

**A previously cryptic diguanylate cyclase can
be active and allows *Bdellovibrio
bacteriovorus* to become a selective
predator on human pathogens**

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Abstract

Bdellovibrio bacteriovorus is a small deltaproteobacterium which invades, grows and replicates rapidly inside the periplasm of other Gram-negative bacteria including human pathogens in a Host-dependent (HD) manner. Cyclic-di-GMP is a second bacterial messenger that controls complex processes in bacteria. Pioneering previous work deduced that *Bdellovibrio bacteriovorus* have four diguanylyl cyclases (Dgcs) responsible for c-di-GMP synthesis and certain diguanylyl cyclases have discrete roles in *Bdellovibrio* lifestyles. In *Bdellovibrio bacteriovorus* HD100, predation ability is controlled by Bd0742 (DgcB) and its deletion led to *Bdellovibrio* growing as a Host-Independent (HI) strain that had lost predation ability. During prolonged co-incubation of this mutant with *E. coli* S17-1, Δ bd0742 suppressor strains arose that could lyse the prey cells.

My project built on this work by co-culturing and screening the predation ability of the HI *B. bacteriovorus* Δ bd0742 mutant strain on a wide range of Gram-negative bacteria including human pathogens. Genomic changes were identified in Δ bd0742 suppressor strains, arising on *E. coli* prey, detecting in which gene the suppressor mutation lies, and the effect of these suppressor point mutations on levels of c-di-GMP production was also assayed. Co-culture of HI *B. bacteriovorus* Δ bd0742 mutant strain in liquid with different human pathogens and non-pathogenic prey cells showed that this strain was originally unable to lyse all different types of prey cells but that suppressors could arise on some types of prey: *E. coli* S17-1 and *A. baumannii* but not on others including *Salmonella typhimurium*, *Serratia marcescens* and *Pseudomonas putida*. Studying the genome sequences of the all Δ bd0742 suppressor strains revealed that there were point mutations in bd3766 (*dgcD*) which encodes another, previously cryptic, diguanylyl cyclase gene. Furthermore, in some strains there was separate point mutation in bd0134 (*aska*), and in all strains mutations in bd0467 transposase

homology gene which were later found to be due to natural variation. The point mutations in *bd3766* occur in bases coding for the coiled coil region and transmembrane domain leading to the proposal that these caused constitutive dimerization of Bd3766 and enhanced Bd3766 activity in the absence of any other regulator protein activity. This was backed up by *E.coli* swimming assays which indicated a higher level of c-di-GMP inhibiting swimming when the *bd3766* suppressor gene alleles were expressed in *E.coli* on swim plates versus wild type *bd3766*.

Gene transfer and predation ability tests of Δ *bd0742* mutant strains showed that the point mutation in *bd3766* was the real cause of suppression of the “non-predatory in liquid culture” phenotype of the HI *B. bacteriovorus* Δ *bd0742* mutant strain. Additional to that, despite the suppressor point mutations in Bd3766 producing more c-di-GMP than wild type Bd3766 but, they still could not restore plaque formation on plates, only in liquid predation, on *E. coli* S17-1 and *A. baumannii* prey. Finally testing function of some point mutations, found in suppressor mutant genomes, in the DAP synthesis pathway gene, (*bd0134*), led to the finding that over expression of *bd0134* restored plaque formation on agar plates to the suppressor strains.

My thesis project (leading from testing *bd0134* suppressor studies) also investigated predatory roles of some further genes involved in cell wall synthesis such as m-DAP/Lysine synthesis pathway genes. This study also found that CpoB, a protein that has an important role in cell wall synthesis regulation, during division, in non-predatory bacteria; is also expressed upon prey during the predatory lifecycle. Concordant with this, deletion studies of *cpoB* suggested that is essential for both HD and HI lifestyles.

عن أبي هريرة رضي الله عنه أن رسول الله صلى الله عليه وسلم قال : ((ما أنزل الله داءً ، إلا أنزل له شفاءً))

The Messenger of Allah Mohammed (peace and blessings be upon him) said:

((Allah did not send down any disease without he also sent its cure))

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List of Abbreviations

~	Approximately
3'	3 prime (hydroxyl) end of DNA/RNA molecule
5'	5 prime (phosphate) end of DNA/RNA molecule
Amp	Ampicillin
AP	Attack Phase
BLAST	basic local alignment search tool
bp	base pairs
Carb	Carbenicillin
c-di-GMP	Cyclic di-GMP
CFU	Colony forming units
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylene diamine tetraacetic acid
EMBOSS	European Molecular Biology Open Software Suite
FOV	field of view
g	gram
<i>g</i>	gravitational acceleration force
hr	hour
HD	Host-dependent
HEPES	N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
HI	Host-independent
IPTG	isopropyl β -D-1-thiogalactopyranosidase
kb	Kilobases
kDa	Kilodalton
kn	Kanamycin
L	litre
LPS	lipopolysaccharide
M	Molar
mCherry	monomeric cherry fluorescent protein
mins	minutes
mRNA	messenger Ribonucleic acid
DNA	deoxyribonucleic acid
NEB	New England Biolabs
ORF	Open Reading Frame
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PFU	Plaque forming units
pH	negative log ¹⁰ of the hydrogen ion activity
ProOpDB	prokaryotic operon database
PSORTdb	subcellular localisation prediction tool
PV	Pixel value
RNA	ribonucleic acid
rRNA	ribosomal Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDW	sterile distilled water
secs	seconds
SignalP	signal peptide prediction tool
m-DAP	meso-diaminopimelic acid
DAP	meso-diaminopimelic acid
WT	Wild type – <i>Bdellovibrio bacteriovorus</i> HD100
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
PG	Peptidoglycan
Δ	Delta, signifying gene deletion

PG	Peptidoglycan
T.P	Time point
DUF	domain of unknown function
OD	optical density
OD600	optical density at 600nm
°C	degrees centigrade
rpm	revolutions per minute
PFAM	protein families database
TFP	teal fluorescent protein
UV	Ultraviolet
mTFP	monomeric teal fluorescent protein
OM	outer membrane
%	percentage
°C	degrees centigrade
α	alpha
β	beta
CFU	colony forming units

1 Chapter One: Introduction

1.1 Overview of c-di-GMP network

1.1.1 c-di-GMP

Cyclic di-guanosine monophosphate (c-di-GMP) is an important bacterial second messenger responsible for regulating essential complex process in bacteria (i.e. the virulence gene expression biofilm formation, motility, pathogenicity and persistency of many bacteria) (Chen et al. 2016). The genes for synthesis and degradation of c-di-GMP are found widely in bacteria but they are depleted in Archaea (Galperin et al. 2001) and also found in eukaryotes but signalling there is more complicated than the bacterial system (Figure 1-1) (Schaap 2013).

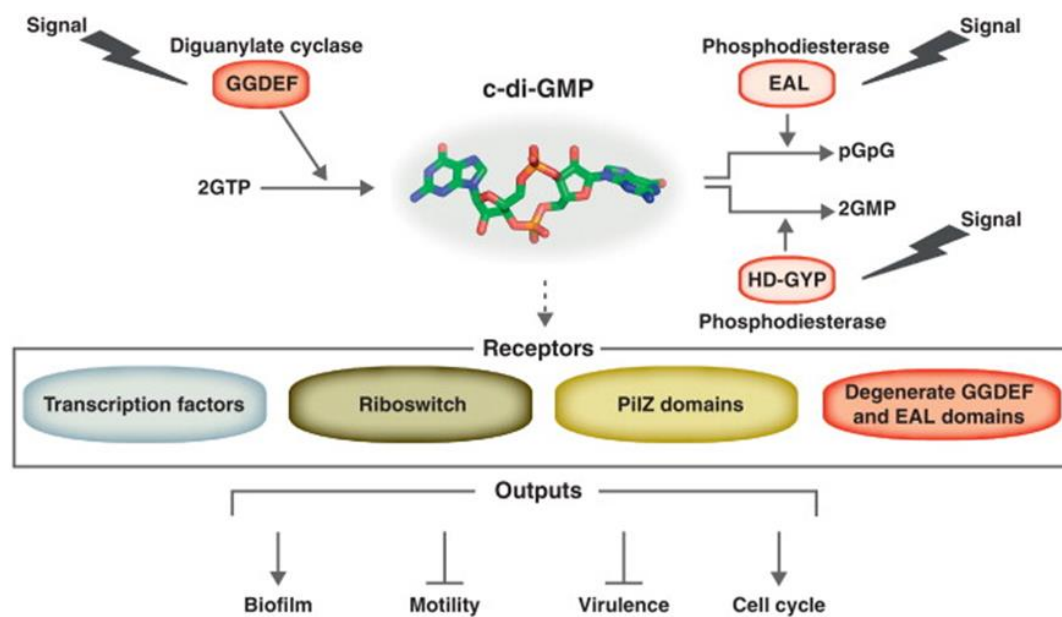


Figure 1. 1: The schematic diagram of basic mechanism of c-di-GMP signalling.
Image reproduced from Sondermann et al. (2012).

The synthesis and degradation of c-di-GMP is controlled by di-guanylate cyclase activity (DGC) and phosphodiesterase activity (PDE) respectively (Tal et al. 1998). c-di-GMP synthesis happens through combination of two GTP molecules by the GGDEF proteins with di-guanylate cyclase (DGC) activity, while the degradation

of it take place by action of the HD-GYP or EAL proteins with phosphodiesterase (PDE) activity which breaks down c-di-GMP into 5' phosphoguanylyl (3'-5') guanosine (pGpG) then it is hydrolysed to two GMP molecules (Römling and Amikam 2006, Hengge 2016) Figure (1-2).

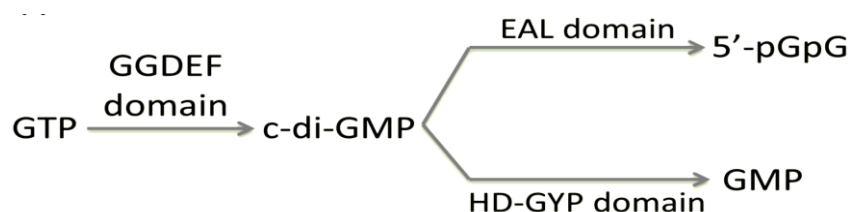


Figure 1.2: The diagram of the domains that involved in synthesis and degradation of the c-di-GMP molecules. Image taken from reproduced from (Wigren et al. 2014).

1.1.2 GGDEF domains

These are widely spread in bacteria proteome and a lot of bacteria have 12–22 different GGDEF domains copies expressed from their genomes. The GGDEF domain acquired its name due to the conserved amino acid residues GG[D/E][D/E]F of their sequence pattern motif (Glycine, Glycine, Aspartic acid or Glutamic acid, and Phenylalanine) and also were originally named domain of unknown function1" (DUF1) (Pei and Grishin 2001). Notably, GGDEF domains are divided into three classes and these are i) lone enzymatically functional domains; ii) enzymatically functional domains linked to an EAL domain (hybrids); and iii) enzymatically non-functional domains. This division depends on the structure of each protein with signature motifs if conserved (Römling 2005, Kulasakara et al. 2006).

Pei and Grishin 2001 predicted that GGDEF domain had diguanylate cyclase activity from the protein sequence alignment and secondary structure comparison with the eukaryotic adenylate cyclases despite the low sequence similarity between them.

The DGC activity of GGDEF domain was firstly demonstrated when studying PleD domain (response regulator) from *Caulobacter crescentus* (Paul et al. 2004).

c-di-GMP biosynthesis results from condensation of two GTP molecules catalysed by diguanylate cyclases (DGC) in the GGDEF domain in the presence of Mg²⁺ or Mn²⁺ ions as a cofactor in order to form phosphoester bond. GGDEF domain has GG (D/E) EF motif that is considered a conserved active site or A-site that involved in GTP binding and the activity of DGC depends on it. Elimination of diguanylate cyclase activity can be obtained by mutation in any residues of GG (D/E) E motif (Chan et al. 2004, Paul et al. 2004, Ryjenkov et al. 2005).

Each residue of the GG (D/E) EF domain has a specific role in catalysis. The first two residues (Gly) are required for GTP binding and the fourth residue (Glu) is responsible for metal ion coordination, while the third residue (Asp/Glu) is crucial for catalysis and it also participates in metal coordination (Pei and Grishin 2001, Chan et al. 2004, Wassmann et al. 2007).

It is rare to find the GGDEF domain standing alone and mostly the GGDEF domains require activating for c-di-GMP synthesis, a process that works due to accessory domains. This regulates the catalysis process through drawing the two GGDEF domains together in order to form a competent homodimer to stimulate the synthesis of c-di-GMP (this will become relevant in chapter 7 on Bd3766). Such response regulator domains are seen in PleD from *Caulobacter crescentus* and WspR from *Pseudomonas aeruginosa* (Chan et al. 2004, Wassmann et al. 2007, De et al. 2009, Schirmer and Jenal 2009). The PleD protein has three domains: a receiver domain (REC), a degenerate receiver domain (REC*) and GGDEF domain, whereas WspR protein has one REC domain, Stalk domain and GGDEF domain.

The activation of the GGDEF domains in PleD protein occurs through REC-REC domain interaction, after phosphorylation of one of REC domain at beginning, that result in formation of antiparallel dimerization of two GG(D/E)EF domains, while activation of the GGDEF domain in WspR protein occurs through connection of REC domain to GG(D/E)EF domain via Stalk domain (Chan et al. 2004, Wassmann et al. 2007, De et al. 2009).

Most GGDEF domains, but not all, contain the RxxD motif (5 amino acids upstream of the GG(D/E)EF motif and located in opposite site to A site) that is named the I-site which represents allosteric feedback inhibition control for c-di-GMP synthesis by slowing down and inhibiting further synthesis of c-di-GMP through binding of c-di-GMP to I site which causes conformational changes in the A site of the GGDEF domain, results in synthesis inhibition (Chan et al. 2004, Christen et al. 2006). In both the PleD and WspR regulators, inhibition of DGC activity happened through binding of c-di-GMP molecules to the allosteric I sites (Schirmer and Jenal 2009).

Dahlstrom et al. (2016) discovered that the I-site of the GGDEF domain can participate in protein-protein interactions with a cyclic di-GMP receptor so specificity of c-di-GMP signalling to that receptor will persist even in fluctuating production of overall global cyclic di-GMP level.

1.1.3 EAL domain

EAL domain Proteins are abundantly found in the proteomes of most bacteria. The EAL domain is about 250 amino acids. It was also known as a domain of unknown function 2 or DUF2. The EAL domain obtained its name from the conserved amino acid residues of a motif sequence (**Glutamic acid, Alanine and Leucine**) (Galperin et al. 2001, Galperin 2004). The EAL domain has the phosphodiesterase activity (PDE). c-di-GMP is degraded by EAL into 5' phosphoguanylyl (3'-5') guanosine (pGpG), then pGpG is hydrolysed to two GMP molecules by action of oligoribonuclease (orn) which is a nanoribonucleases (nanoRNA) which responsible for degradation of small pieces of mRNA (two to five nucleotides) that include pGpG to mononucleotides (Schmidt et al. 2005, Cohen et al. 2015).

It is worth mentioning that EAL domains are divided into three classes and these are catalytically active, potentially catalytically active, and catalytically inactive EAL domains. This division depends on the structure of protein with signature motifs that are conserved (Rao et al. 2009, Romling et al. 2013).

Tischler and Camilli (2004) and Simm et al. (2004) confirmed that the overexpression of EAL domain proteins inhibit biofilm phenotypes and presence of an EAL domain was sufficient for hydrolysis of c-di-GMP specifically. They studied proteins YhjH and VieA from *Salmonella enterica* and *Vibrio cholerae* that contained EAL domains giving the evidence that EAL domain is involved in c-di-GMP specific PDE activity and these studies showed that the consequences of overexpression of these proteins result in reduction of the intracellular level of c-di-GMP in *S. enterica* and *V. cholerae*

The PDE activity of EAL domain is reliant on the presence of divalent cations Mg^{2+} or Mn^{2+} and inhibited efficiently by presence of Ca^{2+} and Zn^{2+} (Tal et al. 1998, Tchigvintsev et al. 2010).

In most microorganisms EAL domains are accompanied by accessory domains that are either separated or fused to signalling or signal output domains (PAS, GAF, MASE, CHASE, REC, and HTH) (Hengge 2009, Schirmer and Jenal 2009). Tchigvintsev et al. (2010) proposed that the PDEs of EAL domains hydrolyse the c-di-GMP using a two-metal-ion catalytic mechanism. In EAL domain motifs, the glutamate (E) residue plays a crucial role in the coordination of one of the metal that is conserved in active enzymes (Barends et al. 2009, Tchigvintsev et al. 2010).

1.1.4 HD-GYP domain

The HD-GYP domain is distributed in bacteria but it is not ubiquitous in bacteria. It has a phosphodiesterase (PDE) activity that breaks down c-di-GMP into two molecules of GMP. HD-GYP domain is a member of HD superfamily of that have metal-dependent phosphohydrolases (PDE) activity. The name of HD-GYP domain is denoted by the presence highly conserved residues of amino acid motifs HD and GYP (Galperin 2005, Chou and Galperin 2016). HD-GYP domain proteins consist of N-terminal regulatory domain and C-terminal HD-GYP domain (Miner and Kurtz 2016).

The first preliminary work to understand HD-GYP proteins was carried out on RpfG PDE of *Xanthomonas campestris* (Ryan et al. 2006). The few studies were done concerned the HD-GYP domain proteins from *P. aeruginosa*, *V. cholerae* and *B. pertussis* and revealed that HD-GYP domain proteins work as a determinators for virulence, motility and biofilm formation and regulate the alteration in c-di-GMP levels significantly (Hammer and Bassler; Ryan et al., 2009; Sultan et al., 2011; Zhang et al., 2013).

Past studies showed that it is difficult to obtain crystal structures of HD-GYP proteins to understand the characteristic of active site and substrate/product binding of this protein until 2011 when Lovering and co-workers solved the structure of catalytically inactive HD-GYP protein from *B. bacteriovorus* Bd1817 and they revealed the HD-GYP domain as a binuclear iron centre. Using this structure and studying c-di-GMP hydrolysis inactive and mutant HD-GYP gives insight for how conserved residues play roles in metal binding. The structure and function of HD-GYP domain was clearly understood when Bellini et al. (2014) resolved the crystal structure of enzymatically active HD-GYP domain protein from *Persephonella marina* (PmGH) alone or when it makes a complex with c-di-GMP or the final reaction product (GMP). Bellini et al. (2014) clarified that the three iron ions of the PmGH domain were arranged as a geometric triangle. Additional to that, Bellini et al. (2014) discovered that the residues conserved E185, H189, H221, D222, H250, H276, H277 and D305 of the HD-GYP domain participate in ligand binding with metal binding co-ordination sphere and all these residues are conserved in HD-GYP family, a site-directed mutagenesis study showed that substitutions of alanine residues from the three metal centre of PmGH domain either sharply reduced or abolished the catalytic activity meaning that the three metal iron ions play vital role in catalytic activity of this domain and Bellini also improved the understanding of c-di-GMP PDE activity of PmGH domain.

Furthermore, this study showed that HD-GYP family can be subdivided into bi- and tri-nuclear catalytic iron centres containing domains (Bellini et al. 2014).

A recent study by Miner and Kurtz (2016) highlighted of the possibility of presences of both di- and tri-metal active sites that accommodated in some bacteria like in HD-GYP domain TM0186, from *Thermotoga maritima* and he proved the presence of reduced Fe in HD-GYP domain TM0186 can catalyse conversion of c-di-GMP to pGpG, while the conversion of pGpG to GMP happened when conversion of c-di-GMP to pGpG was completed and required excess Fe²⁺ or Mn²⁺.

1.1.5 PilZ domain

The PilZ domain was firstly predicted as a receptor for c-di-GMP by Amikam and Galperin (2006) when they used bioinformatics sequences analysis to find out the encoded proteins that follow the phyletic distribution of GGDEF and EAL domain genes. PilZ domain obtains its name from PilZ protein of *P. aeruginosa* that involved in pilus formation. PilZ domain acts as a flexible adaptor domain for combining c-di-GMP with various proteins that regulate important processes in bacteria like flagellar rotation inhibition or exopolysaccharide synthesis (Amikam and Galperin 2006, Boehm et al. 2010, Whitney and Howell 2013)

PilZ domain protein are widely distributed in bacterial phyla and it is the first c-di-GMP binding domain that was distinguished (Amikam and Galperin 2006). Even though, PilZ domain family is widely spread in bacteria it was not in all, including some human pathogens like *Rickettsia* species that do not encode PilZ domains but encoded c-di-GMP synthetases and hydrolases (Galperin 2005, Amikam and Galperin 2006).

The distinguishing feature of PilZ domains from other c-di-GMP binding proteins are that it has two separate motifs. These motifs are; RXXXR motif with conserved arginine residues that surround one of guanine base of c-di-GMP while the other motif DXSXXG surrounding the other guanine bases (Chou and Galperin 2016).

When c-di-GMP molecules bind with PilZ domain, which is structurally rigid, the c-di-GMP molecules do not force the PilZ domain to undergo a major conformational change (Chou and Galperin 2016). Also, Chou and Galperin (2016) pointed out that the process of binding c-di-GMP with PilZ domains affects the unstructured N-terminal of the domain that wraps around the c-di-GMP ligand with assistance of the arginine residues of the RXXXR motif, while the DXSXXG motif acts as a flexible loop that interacts with the second guanine base from above the c-di-GMP (Chou and Galperin 2016). It is worth mentioning that different PilZ domains possess the ability to bind c-di-GMP molecules in either monomeric or dimeric condition (Chou and Galperin 2016).

Remarkably, PilZ domain proteins are quite diverse and currently classified into three different types depending on primary structures: type I, which is a full length domain containing both RXXXR and (D/N)XSXXG motifs and which directly binds with c-di-GMP. Type II, which retains only the (D/N)XSXXG motif but not the N-terminal RXXXR motif, which made it unable to bind to the c-di-GMP. Type III, is truncated that unable to bind cyclic di-GMP (Chou and Galperin 2016). The PilZ II domain which is unable to bind c-di-GMP has the ability to regulate the twitching motility that required presence of type IV pili (Chin et al. 2012).

As with the other c-di-GMP domain proteins, about half of the PilZ domain proteins stand alone with short extensions at the N-terminal of the protein, whereas the other half of PilZ domains are connected with other protein domains like the BcsA domain of glycosyltransferases II protein, REC domain of response regulators, MCP domain of methyl-accepting proteins, and DNA binding protein domains (Ryjenkov et al. 2006, Christen et al. 2007, Freedman et al. 2010).

1.2 Content of *B. bacteriovorus* c-di-GMP proteins

Up to now, the system of the c-di-GMP signalling in *Bdellovibrio* is still not completely understood. *Bdellovibrio* is a predatory bacterium that invades and grows inside other gram-negative bacteria. The network of c-di-GMP signalling in *B. bacteriovorus* HD100 consists of five GGDEF domains, one EAL domain, six HD-GYP domains and 19 PilZ domains, implying many processes are c-di-GMP controlled in *Bdellovibrio*. The studying the network of c-di-GMP in *B. bacteriovorus* is still under research, and only the GGDEF genes were studied in depth and discovered the phenotypes that result from their deletion on both life cycles of *Bdellovibrio*.

1.2.1 GGDEF domains in *Bdellovibrio*

The GGDEF domains in *Bdellovibrio* were previously investigated by Hobley et al. (2012) and they identified five GGDEF domain proteins (four of them are active, and one is inactive). The GGDEF domain proteins in *Bdellovibrio* are: Bd0376 (DgcA), Bd0742 (DgcB), Bd1434 (DgcC), Bd3766 (DgcD) and Bd3125 (CdgA). The DGC activity for all GGDEF domains was confirmed except for Bd3125 that was predicted to function as a c-di-GMP receptor. All the GGDEF proteins have another domain at the N-terminal except Bd1434 that only has annotated a GGDEF domain alone but has a lot of unannotated N-terminus. All these active GGDEF domains in *Bdellovibrio* will be explained in detail with their phenotypes in the Chapters 3 and 7.

1.2.2 EAL domain in *Bdellovibrio*

The *Bdellovibrio* has only a sole EAL domain protein which is Bd1971 in which EAL-type PDE activity and binding ability to c-di-GMP molecules was confirmed (Hobley et al. 2012, Rotem et al. 2015). Bd1971 is a 400 amino acid protein that consists of two domains: Cyclic nucleotide-binding domain (cNMP) (runs from 1-

108 amino acids) and EAL domain (runs from 143-400 amino acids) (<http://smart.embl-heidelberg.de/>) Figure (1-3).

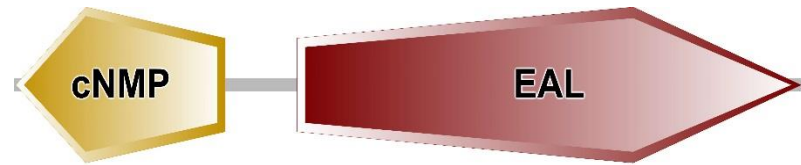


Figure 1. 3: The domains of B1971 protein. The protein consisting of (from N-terminus to the C-terminus) cNMP domain and the EAL domain (<http://smart.embl-heidelberg.de/>).

The expression profile of *bd1971* was examined with RT-PCR, and found to be expressed throughout the predatory cycle of *Bdellovibrio*. The location of Bd1971-mCherry was assessed by a graduate PhD student Sarah Basford and she found that Bd1971-mCherry was mostly located throughout *Bdellovibrio* cells during both predatory and host-independent lifestyles. Deletion of *bd1971* was carried out previously using Kn-insertion deletion method, the deletion strain obtained by the Masters students Rashidah Ahmad and Rowena Fung (AHMAD 2007, FUNG 2008).

The deletion of Bd1971 was obtained as a Host-Independent culture and this strain can return to host-dependent when prey was provided. The efficiency rate of this strain in clearing prey was similar to wild-type strain *B. bacteriovorus* HD100. The distinguishing phenotype for this strain was that the attack phase cells of the $\Delta bd1971$ mutant were longer, thinner and more curved than the wild-type strain (Basford 2014). The other phenotype of $\Delta bd1971$ mutant strain was that the cells aggregated in a clump like rafts in liquid cultures but the extent of this did vary. The level of c-di-GMP in this strain was measured by graduate PhD student Sarah Basford, and she found that there was elevation in the level of c-di-GMP (Basford 2014). Furthermore, Sarah Basford found that there was an interaction between Bd1971 and Bd0367 proteins by bacterial two-hybrid (B2H) assay and this result was confirmed by Ruth Nottingham, another PhD student

who worked more on these proteins, by doing both bacterial two-hybrid assay and β -galactosidase activity assay (Basford 2014, Nottingham 2016). The deletion of *bd1971* affected gliding motility and made the attack phase of the mutant strain glide slower than the attack phase wild-type and this was due to the high level of c-di-GMP causing likely exopolysaccharide (EPS) upregulation that led to sticky cells (Nottingham 2016).

1.2.3 HD-GYP domains in *Bdellovibrio*

The genome of *B. bacteriovorus* HD100 has six genes encoding for HD-GYP domain proteins. These proteins are Bd1822, Bd1762, Bd1817, Bd2325, Bd2421 and Bd3880. From all these six HD-GYP domains proteins, only Bd2421 and Bd2325 have the full conserved HD and GYP motifs amino acid residues, while the remaining four proteins showed some conserved residues but did not contain the full HD-GYP amino acid residues sequence.

Up to date, only three genes were extensively studied in *Bdellovibrio*. These are Bd1817, Bd2325 and Bd2421 (White 2012). The only protein with a HD-GYP family domain in *Bdellovibrio* that has a determined crystal structure is Bd1817 (Lovering et al. 2011). The crystal structure of Bd1817 protein opened the door to understand the nature of the general mechanism of HD-GYP domains with active and c-di-GMP binding site residues for the HD-GYP domain, and more crystal structures could then be studied on HD-GYP domain family proteins from other bacteria (Lovering et al. 2011). Both Bd2325, Bd2421 were studied extensively by the master student Michael White during his MRes project (White 2012).

1.2.3.1 Bd2421

Bd2421 is a protein that consists of the only HD-GYP domain where no other sensory domain can be detected. Michael White MRes student found that *bd2421* was expressed that continuously during the predatory life cycle. The location of this protein illuminated by mCherry tag in attack phase cells was bipolar but

diffuse throughout the cytoplasm when *Bdellovibrio* grew inside the bdelloplast then returning to bipolar when exiting from prey. The location of this protein in HI growing cells was diffuse throughout the cell. The deletion strain of *bd2421* gene was obtained by Michael White and he found that there were no obvious growth phenotype resulting from deletion of this gene and the strain was able to finish the predatory cycle with a similar rate to the *B. bacteriovorus* HD100 (White 2012). The only differences between the deletion strain and *B. bacteriovorus* HD100 were that it has a higher level of c-di-GMP and this indicated that this gene has a role in lowering the level c-di-GMP in *Bdellovibrio*. The phosphodiesterase activity and binding ability of this protein to c-di-GMP was confirmed (White 2012, Rotem et al. 2015).

1.2.3.2 Bd2325

Bd2325 is a protein that found in the *B. bacteriovorus* HD100 that consists of a HD-GYP domain only with an N-terminal domain of unknown function. This gene was also studied by MRes student Michael White. Gene *bd2325* was found to be continuously expressed during the predatory *Bdellovibrio* life cycle. The location of this protein in the predatory life cycle was assessed by mCherry tag and its location in attack phase is bipolar and when growing inside the prey, the location of this protein changed to be diffuse throughout the cell then returning to the poles at the end of the predatory cycle. The location of this protein in HI growing cells was found to be diffuse through the cytoplasm. The deletion strain of *bd2325* was obtained only as a host-independent strain as it was unable to be isolated as a host-dependent strain. The deletion of *bd2325* resulted in a freshly isolated mutant strain that had main phenotypic features growing originally as very long HI cells which apparently had division defects but were still able prey upon *E. coli* and still able to glide on solid surfaces. Further culturing of $\Delta bd2325$ mutant strain gave shorter HI cells suggesting that a secondary mutation might have happened (as yet unstudied) that helped the very long HI cells in the culture of the freshly

isolated mutant strain in division and produced shorter HI cells. Freshly generated *Δbd2325 mutant* strain cultures are needed to check for comparison and will be sent for genome sequencing. This meant that Bd2325 protein may have a role in cell septation processes and cell division. PDE activity of Bd2325 protein was confirmed by detection of an elevated level of c-di-GMP in the deletion strain which meant that Bd2325 PDE has a role in lowering the level of c-di-GMP in *Bdellovibrio* (White 2012).

1.2.3.3 Other HD-GYP domains in *Bdellovibrio*

All the other HD-GYP domain genes in *Bdellovibrio* Bd1822, Bd1762, Bd1817 and Bd3880 are till now not fully studied, but preliminary works were done previously, including the RT-PCR transcriptional profiling and fluorescent protein tagging for each gene, but not deletion of these genes. All the work on these genes has details listed in Table (1-1).

Table 1-1: the summary of work performed on other HD-GYP domains in *Bdellovibrio*. This table includes the Conserved motif, expression profile by RT-PCR, if fluorescence is detectable in HD/HI lifestyle of *Bdellovibrio*, phenotype results from deletion of these genes and Predicted/confirmed function of these gene products.

	Conserve motif	Expression profile by RT-PCR in the predatory lifecycle	Detectable fluorescence of C terminal mCherry tag in HD/HI lifestyle of <i>Bdellovibrio</i>	Deletion phenotype	Predicted/confirmed Function
Bd1762	TD-GFP	Constitutive	Yes/Yes	Not tested	The ability of c-di-GMP binding predicted but no PDE proof by (Hobley et al. 2012).
Bd1817	HD-G-P	Expressed throughout the lifecycle with highest levels at 3-4 hours	No/Yes faint	Deletion obtained, and this deletion does not produce any observable phenotype.	The ability of c-di-GMP binding but no PDE proof predicted by (Lovering et al. 2011, Hobley et al. 2012).
Bd1822	HN-GFP	Constitutive at low levels	Yes/Yes	Not tested	The ability of c-di-GMP binding but no PDE proof predicted by (Hobley et al. 2012).
Bd3880	AD-GFP	Not expressed	No/No	Not tested / Pseudogene?	The ability of c-di-GMP binding but no PDE proof predicted by (Hobley et al. 2012).

1.2.4 PilZ domain proteins in *Bdellovibrio*

The genome of *Bdellovibrio* was previously predicted to encode 15 PilZ domain genes (Hobley et al. 2012). Another four extra PilZ domain genes were identified in a recent study by Rotem et al. (2015). This means that *Bdellovibrio* have 19 PilZ domain proteins, and these are Bd0064, Bd0378, Bd0642, Bd1007, Bd1482, Bd1996, Bd2059, Bd2524, Bd2545, Bd2717, Bd2880, Bd3100, Bd3138, Bd3232, Bd3527, Bd0760, Bd1616, Bd1466 and Bd2147 .

The ability of 11 of the 19 PilZ domain proteins to bind c-di-GMP molecule was confirmed (Rotem et al. 2015). The work on PilZ domain proteins are still ongoing and still not complete. Most works on PilZ domains are still preliminary including fluorescent tagging, the expression profile of PilZ domains and Bacterial two Hybrid (B2H). Until nowadays, the only PilZ domain that has been studied in depth by deletion is Bd3100. However, Bd0064 could be mCherry or mTeal tagged without apparent phenotypic defect and is constitutively fluorescent in predatory cycle so this Bd0064-mCherry is used in Chapter 8 as a fluorescent control.

1.2.4.1 Bd3100

Bd3100 is a PilZ domain protein found in *Bdellovibrio* that consists of two DUF4339 domains and a PilZ domain (from N-terminus to C-terminus). This gene was studied mostly by an undergraduate student, Aidan Taylor with some work by a PhD student, Ruth Nottingham.

Gene *bd3100* was expressed continuously during the predatory lifecycle. The location of Bd3100-mCherry is mostly diffuse through the cells in the attack phase, and Bd3100-mCherry was also expressed in HI growth mode (Taylor 2013).

The interesting feature in *B. bacteriovorus* Bd3100-mCherry strain is surface incubation affected the attack phase cell fluorescence intensity and that this increased with the time of incubation. Also, the surface incubation led to alteration in the distribution of the of Bd3100-mCherry fluorescence inside the

attack phase cells and it became more monopolar than before the incubation when it was mostly diffuse throughout the cell, suggesting protein movement for Bd3100 (Taylor 2013, Nottingham 2016).

The deletion of the *bd3100* resulted in an increase in the frequency of reversals of gliding motility compared with wild-type HD100 of *Bdellovibrio* (Taylor 2013, Nottingham 2016). Bd3100 is the only PilZ protein found that directly interacts with Bd0367, which is one of the DGC protein of *Bdellovibrio* by using B2H and β -galactosidase assays (Milner 2013, Nottingham 2016).

1.3 The effect of GGDEF mutations on levels of c-di-GMP and bacterial virulence factors in other bacteria

In this section, I will explain the effect of the mutation in GGDEF domains on the virulence factors of other bacteria- non-predatory bacteria that have less or more GGDEF domain- genes than the *Bdellovibrio* and how these deletions are either effect positively or negatively on bacterial virulence.

1.3.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram-negative bacterium that is a member of *Enterobacteriaceae* family. It is an opportunistic human pathogen and causative agent of systemic infections in immunocompromised patients and nosocomial associated infections such as catheter-associated urinary tract infection, bloodstream infection, ventilator-associated pneumonia, and surgical site infection (Kulasakara et al. 2006, Hidron et al. 2015). *P. aeruginosa* have several virulence factors like exoenzyme S, and lipopolysaccharides. Additionally, *P. aeruginosa* has the ability to switch its lifestyle from a planktonic growth to a surface-associated life-style (biofilm) when environmental conditions change. Biofilm-associated infections result from the ability of *P. aeruginosa* to form biofilms on the surfaces of medical devices and implants. Therefore, when biofilms formed, bacteria will be more resistant to treatment by antibiotics and will resist

immune system actions (Nickel et al. 1985, Mah et al. 2003, Parsek and Singh 2003, Tseng et al. 2013).

P. aeruginosa has a huge number of the c-di-GMP metabolism proteins and they are classified as 16 GGDEF proteins alone, 5 EAL proteins, 16 dual-domain protein GGDEF-EAL with 3 HD-GYP only proteins (Ha et al. 2014). A recent study on c-di-GMP metabolism protein done by Ha et al. (2014) showed that in *P. aeruginosa*, as in many other bacteria, high levels of c-di-GMP has a correlation with sessile lifestyle, or a biofilm while a decrease in this c-di-GMP level associated with motility and/or planktonic growth. Ha et al. (2014) studied the effect of deletion of c-di-GMP metabolism protein on the major virulence factors activity in *P. aeruginosa* including biofilm formation, exopolysaccharide (EPS) production, swimming motility, swarming motility, and twitching motility. Deletion of each GGDEF domain genes resulted in mutants that still have the ability to form biofilm similar to wild-type ability except six mutants that two of them form impaired biofilm compared with wild type, while the other four mutants form hyperbiofilm about 110 to 140% higher than wildtype levels.

The other virulence factor where production is affected by the level of c-di-GMP is exopolysaccharides (EPS), and *P. aeruginosa* produces two types; Pel and alginate. In previous studies, Pel exopolysaccharide was shown to be essential for biofilm formation in vivo (Friedman and Kolter 2004). Also, the production level of Pel EPS positively correlated with the intracellular level of c-di-GMP (Morgan et al. 2006, Kuchma et al. 2007).

Ha et al. (2014) tested the effect of deleting each of the *GGDEF* domain genes from *P. aeruginosa* on production of the Pel exopolysaccharide using Congo red in growing agar. Congo red was unable to bind to any mutants with defects preventing Pel EPS production, so the colonies grew as a smooth white colonies (Kuchma et al. 2007, Colvin et al. 2012). The results showed that all mutants that result from deletions of GGDEF domains showed the various abilities to binding

Gongo red from weak to very strong except one $\Delta roeA$ mutant that failed to bind this dye even after extended incubation time with the dye.

Swimming motility is a type of flagellar- dependent movement that requires the flagella rotation to occur. This type of motility occurs when individual bacterial cells move in liquid environments (Kearns 2010).

The swimming motility of *P. aeruginosa* is affected by the levels of c-di-GMP. Deletion of 16 GGDEF domain genes from *P. aeruginosa* resulted in 4 mutants with an increase in swimming motility and four mutants with a decrease in swimming motility, and the rest of the mutants having the same wild-type rate (Ha et al. 2014).

Swarming motility is a type of multicellular movement over a solid surface that is energised by flagella rotation and this type of movement occurs in some bacteria. Most bacteria that display the swarming motility have peritrichous flagella on their cell surface. This movement happens on media solidified with 0.3% agar (Alberti and Harshey 1990, Harshey and Matsuyama 1994, Merino et al. 2006, Kearns 2010).

Among the 16 GGDEF mutants, only five mutants swarmed less than wild-type levels, while five mutant were hyperswarmer strains, showing 110 to 150% swarm size higher than of wild-type levels.

Twitching motility is the type of surface motility that is flagellar- independent that happened through extension and retraction of type IV pili (extending from one pole of the bacterial cell) on a moist surface (Mattick 2002, Kearns 2010).

With regarded to twitching motility, Ha et al. (2014) found that all GGDEF mutants revealed a reduction in twitching motility except for one mutant that showed hypertwitching phenotype about 120% of that of the wild-type.

Recently, another study on c-di-GMP signalling confirmed that c-di-GMP influences the ability of *P. aeruginosa*-mediated biofilms to grow during catheter-associated urinary tract infection. This study investigated the outcome of deletion of GGDEF

domain genes on biofilm formation, and this study concluded that three GGDEF mutants were reduced for *P. aeruginosa* colonisation rates in bladder preventing spreading of infection to kidneys (Cole and Lee 2015).

1.3.2 *Staphylococcus epidermidis*

Staphylococcus epidermidis is a gram- positive bacterium that is considered an opportunistic human pathogen normally found on human skin and mucous membranes. It is responsible for causing nosocomial infections and its pathogenicity results from its ability to form biofilms on medical devices and implants. Bacteria in a biofilm are more resistant to antibiotics than planktonic growth counterparts leading to persistent infections and antibiotic resistance (Gotz 2002, Otto 2009). Sequencing the *Staphylococcal* genome has shown that a single gene encodes one GGDEF domain protein and this gene designated as a *gdpS* and one gene encoding either EAL or HD-GYP domain proteins with no c-di-GMP receptor recognised (Holland et al. 2008). However, it was found that this gene regulates c-di-AMP production that is analogous to c-di-GMP (Corrigan et al. 2013, Schuster et al. 2016).

One of the important virulence factors in *Staphylococcus* species is biofilm formation particularly in nosocomial infection related medical device (O'Gara 2007). One of the major components of *S. epidermidis* biofilm is an extracellular polymeric substance (EPS) that consist of a polysaccharide-intercellular adhesin (PIA). PIA is composed of a β -1,6-linked N-acetylglucosamine polymer. The synthesis, export and modification of PIA are under control of enzymes that encoded by the *icaADBC* gene cluster (Heilmann et al. 1996, Gerke et al. 1998, Rohde et al. 2010).

Previous studies in many gram- negative bacteria revealed that the level of c-di-GMP had an essential role biofilm formation. The high level of intracellular c-di-GMP correlated with stimulation of EPS production and increases biofilm

formation, while flagellar motility is induced in a low level of c-di-GMP (Hickman and Harwood 2008, Hengge 2009).

Due to *S. epidermidis* having a sole GGDEF domain it was likely that this gene product plays an essential role in infection of this bacterium. The deletion of *gdpS* resulted in a mutant strain with impaired biofilm formation under both static and dynamic flow conditions, and this indicated that *gdpS* positively regulated the biofilm formation. Moreover, the complementation of GGDEF deletion domain strain with *gdpS* repaired biofilm defect and no longer be affected by deletion of GGDEF (Zhu et al. 2017).

Zhu et al. (2017) pointed out that the mutation in start codon of *gdpS* did not abolish the ability of *gdpS* for enhancing biofilm formation. This meant that *gdpS* has the ability to regulate the biofilm formation independently from its protein-coding function. Zhu et al. (2017) proved that *gdpS* participate in biofilm formation through elevation of the expression of *icaADBC* that responsible for synthesis of polysaccharide-intercellular adhesion (PIA) that is component of extracellular polymeric substance in *S. epidermidis* biofilm.

1.3.3 *Xanthomonas oryzae* pv. *oryzae*

Xanthomonas oryzae pv. *oryzae* (Xoo) is a gram-negative bacterium that belongs to *Xanthomonadaceae* (Swings et al. 1990). It is responsible for bacterial leaf blight disease in rice (Yang et al. 2016). Xoo infection is correlated with production of several virulence factors that play a role in infection like exopolysaccharide (EPS), extracellular enzymes, adhesins and type III secretion system (T3SS) (Das et al. 2009, White and Yang 2009). The genome of the Xoo strain encodes 26 gene products that are involved in c-di-GMP signalling pathway including GGDEF, EAL, GGDEF/EAL or HD-GYP domain proteins (Salzberg et al. 2008, Yang et al. 2012). From all 26 genes, 11 of them are GGDEF-domain proteins, and none of them except one has characterised till now. The only GGDEF domain protein that studied in Xoo from all 11 genes was PXO_00649 that named as GdpX1, which is

the only gene product that contains the most conserved GGDEF domain residues and encode a putative DGC (Xue Dingrong 2015, Yang et al. 2016). Yang et al. (2016) deleted *gdpX1* from Xoo strain which produced a strain that when inoculated on rice leaf cause more severe infection than wild type and the lesion last longer and complementation of this deletion restored the similar wild type disease phenotype. Additionally, overexpression of GdpX1 in Xoo strain decreased the virulence of the strain significantly. All these findings demonstrated that *gdpX1* regulated virulence factors in Xoo strain negatively (Yang et al. 2016). One of the important virulence factors in Xoo infection is EPS production that plays an essential role in the pathogenesis of Xoo strain (Kim et al. 2009).

Yang et al. (2016) also tested the effect of *gdpX1* deletion on EPS production, and they found that Δ *gdpX1* mutant strain colonies were more mucoid and production of EPS increased by about 40% than the wild-type strain. The complementation of this deletion returned the EPS production to near wild-type. In contrast, the overexpression of *gdpX1* affected on EPS production decreasing it by about 30% than that in the wild-type strain (Yang et al. 2016).

In addition to that, deletion of *gdpX1* positively affected *gumB*, *gumD*, *gumK*, *gumM* genes that are involved in EPS synthesis through increasing the transcription of these genes, whereas overexpression of *gdpX1* negatively impacted on the transcription of *gum* genes (Yang et al. 2016). The deletion of *gdpX1* also showed influences on swimming motility, with the mutant strains that forming larger swimming zone diameter compared with wild-type and also increase the expression of flagellin genes. The complemented strain recovered the wild type swimming zone diameter. Moreover, *gdpX1* overexpression reduced the swimming diameter and reduced the expression of flagellin genes (Yang et al. 2016). Yang et al. (2016) discovered by electron microscopy that both Xoo strain (wild-type strain) and Δ *gdpX1* strain had flagella that did not show significant visible differences between them, whereas *gdpX1* overexpressing strain had no flagella. They also concluded that there were no differences in biofilm formation

between $\Delta gdpX1$ strain and wild-type strain, but in *gdpX1* overexpressing strain, there was increase the biofilm formation three-fold more than the wild strain. This finding indicated that *gdpX1* overexpression stimulated biofilm formation, likely not via increased EPS expression, but via sessile status of cells.

1.3.4 *Salmonella*

Salmonella is a genus of gram-negative bacteria that have a rod shape that belongs to the *Enterobacteriaceae* family (Su and Chiu 2007) that is considered a pathogen for both humans and diverse animals.

Solano et al. (2009) studied the c-di-GMP signalling in *Salmonella* when they deleted all the twelve GGDEF domains genes and studied the phenotypes that result from deletion of all twelve genes from the same strain. The deletion of all 12 GGDEF domain in *Salmonella* resulted in a strain that lost the ability to form a biofilm that was unable to produce the EPS, cellulose and fimbriae correlating with negatively decrease the strain motility (Solano et al. 2009).

Comparing with wild type, the 12 GGDEF deletion strain had an avirulent phenotype and was unable to infect mice when this strain was orally administered (10^9 CFU of bacteria) and there was a significant decrease in their ability for adherence and invasion of the intestinal epithelium (Solano et al. 2009). All these findings indicated that the GGDEF domain genes are not vital for *Salmonella* viability but might be important for its survival in the environment and for production of virulence factors.

Solano et al. (2009) discovered by using Microarray and RT-PCR that the inability of 12 GGDEF deletion strain to produce a biofilm and to be fluorescent on Calcofluor plates, colour and morphology of colonies on Congo red plate have a direct connection with lower mRNA level of CsgD. CsgD responsible for controls cellulose and fimbriae production (Zogaj et al. 2001). Additional to that, Solano et al. (2009) detected that there was correlation between decreasing motility of 12 GGDEF deletion strain and increased mRNA levels of flagellar body genes that

contrary to expectation. Also, the deletion of 12 GGDEF domain genes produce strain with severe impairment in exportation of flagellin out of the bacterial cell. One of the important steps that Solano et al. (2009) performed to detect the effect of each *GGDEF* gene in *Salmonella* was to partially complemented 12 *GGDEF deletion* strain with each single *GGDEF* gene and they discovered that the 12 *GGDEF deletion +stm4551* strain from all strains tested was able to recover at least partially most phenotypes that resulted from deletion of all 12 *GGDEF* genes that include virulence, motility, fimbriae synthesis, and long-term survival. Furthermore, Solano et al. (2009) revealed that the 12*GGDEF deletion +stm4551* strain was restored its ability to infect mice through its capacity to adhere and invade the intestinal epithelium with multiplying within spleens and livers cells. These surprising data suggest that the activity of single GGDEF-domain protein could regulate several biological processes in *Salmonella* and the restoration of the phenotypes that were produced from deletion of all *GGDEF* genes happened through a mechanism not solely dependent on the level of c-di-GMP synthesis. Le Guyon et al. (2015) identified the contribution of three di-guanylate cyclases (STM2672, STM4551 and STM1987) in regulating the motility of $\Delta yhjH$ mutant (*EAL* gene deletion strain) through elevating the levels of c-di-GMP which inhibited the motility. This motility inhibition occurred via the c-di-GMP receptors YcgR (PilZ domain protein that regulate the bacterial motility through direct interaction with flagellar motor) for STM2672, STM1987 for the cellulose synthase BcsA (PilZ domain protein that considered as a c-di-GMP receptor that regulate cellulose synthesis for biofilm formation through c-di-GMP binding) and STM4551 for both receptors.

1.4 The effect of the Level of c-di-GMP on the bacterial motility

In this section, it will be explained how the level of c-di-GMP can have effect on flagellar motility of bacteria as this mechanism in *E. coli* was used to see if Δ *bd0742* suppressor mutant strains produce more or less c-di-GMP (See chapter 7 section 7.4.2). Data from several studies have revealed that flagellar motility is promoted by low levels of c-di-GMP while biofilm formation is stimulated by high c-di-GMP levels (Simm et al. 2004, Caiazza et al. 2007). The level of c-di-GMP can regulate bacterial motility through affecting the flagellar biosynthesis or rotational/ propeller function or both (Wolfe and Visick 2008).

Bacteria have different types of motility some of them are flagellar-dependent (swimming motility and swarming motility), and some pili- dependent and do not require flagella to occur (Twitching motility) (Mattick 2002, Kearns 2010). There is another type of bacterial motility that is not dependent on flagella or pili which is gliding motility which is smooth bacterial movement on solid without help of external appendages (Nan and Zusman 2011).

In general, bacterial flagella are defined as a rotary helical thread like appendage that extend from a protein complex in the cell body and is required for movement and considered as a sensory organelle as it is able to respond to sensor reporting the presence of chemicals and temperature changes in the environment via accessory chemotaxis signalling proteins (Bardy et al. 2003, Wang et al. 2005).

In gram-negative bacteria, the flagellum consists of following parts (from outside to inside cell): Cap, Filament, Junction, Hook, Basal body (Rod, L-lipopolysaccharide ring, P-peptidoglycan ring and MS-membrane/supramembrane ring), C-ring, Export apparatus (Figure 1-4) (Xue et al. 2015).

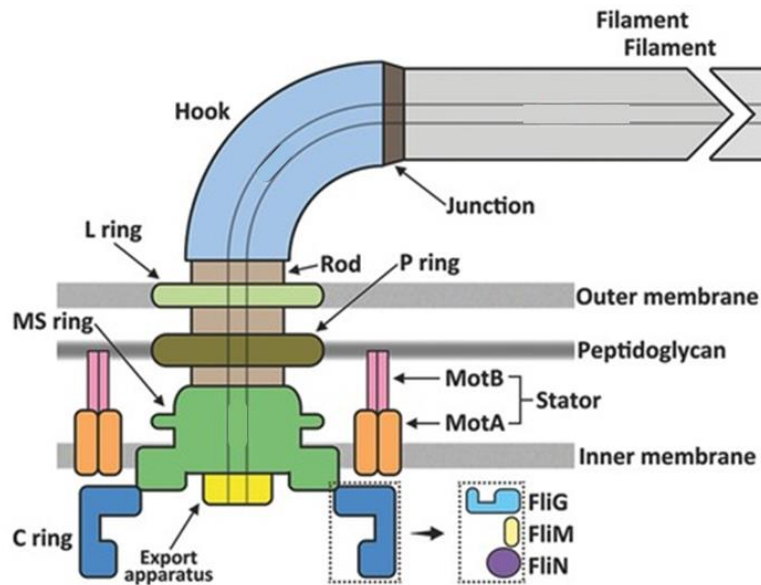


Figure 1. 4 The diagram illustrated the composition of the bacterial flagella. Image adopted from (Xue et al. 2015).

The flagellar motor is composed of a rotating part (the rotor) and the stator which is the non-rotating part that membrane-embedded part. The rotor is consist of about 25 copies of FliG protein, 34 copies of FliM protein, and more than 100 copies of FliN protein and all of them together forming an assembly structure that is referred to as a C-ring or switch complex (Yamaguchi et al. 1986, Francis et al. 1992). The position of the C-ring or switch complex is at the bottom of the basal body, and its function controls the direction of flagella rotation clockwise/counterclockwise (CW/CCW). The stator is composed of the membrane proteins four copies of MotA and two copies of MotB. The function of the Mot proteins is to allow ions across the membrane and link the ion flow to the flagellar rotation (Kojima and Blair 2004, Morimoto and Minamino 2014). The rotation of flagella relies on the electrostatic interactions that occur between specific sites in the stator protein MotA and the rotor protein FliG (Zhou et al. 1998). Interacting with the switch complex that controls the flagellar rotation direction, signaling

protein phospho-CheY (CheY~P) has that ability to change flagellar rotation direction to the CW state by interacting with FliM (Welch et al. 1993)

YcgR is one of the PilZ domain proteins that is considered to function as a c-di-GMP effector. It consists of an N-terminal domain and a PilZ domain at the C-terminal. Proteobacteria highly conserve this C-terminal PilZ domain arrangement (Amikam and Galperin 2006). The ability of YcgR to bind to the c-di-GMP specifically and tightly was detected by Ryjenkov et al. (2006) and they proved that the YcgR protein controls bacterial motility in Enterobacteria. After 2006, a lot of studies on different species of bacteria were conducted and confirmed the ability of PilZ domain protein to control flagellar motility and other motilities additional to biofilm and exopolysaccharide formation (Merighi et al. 2007, Pratt et al. 2007, Pitzer et al. 2011).

A number of studies showed that YcgR protein in *E. coli* and *Salmonella* can be considered as a "backstop brake" for flagella and it plays a role in reducing and changing the rotational direction of flagella. The ability of YcgR protein to impact on the rotation of flagella occurs by direct interactions between YcgR and components of the flagellar motor of switch that are located in the basal body (Boehm et al. 2010, Fang and Gomelsky 2010, Paul et al. 2010). The mechanism of how the YcgR interacted with flagellar rotor to reduce the speed and change rotation direction was determined extensively by Paul et al. (2010). Paul et al. (2010) found that in both *Salmonella* and *E. coli*, high level of c-di-GMP level inhibits the motility. They also discovered that increased levels of c-di-GMP led to YcgR reducing flagellar motor speed and inhibiting CW rotation that results in inhibition of chemotaxis. Additional to that, Paul et al. (2010) proved by the evidence that YcgR strongly interacts with FliG and FliM that are found in rotor structure of flagellar motor and this takes place when c-diGMP is binding to YcgR. So YcgR activity is dependent on the level of c-di-GMP and this developed to an

assay (Fang and Gomelsky 2010) and this assay was used later in Chapter 7 (see section 7.4.2).

1.5 *B. bacteriovorus* HD100

Bdellovibrio bacteriovorus is small short rod-shaped, (0.2 to 0.5 μm wide and 0.5 to 2.5 μm long), Gram negative bacterium which is a member of the *Deltaproteobacteria* class that includes *Myxococcus*, *Desulfovibrio*. In 1963, *B. bacteriovorus* was firstly discovery by Stolp and Starr (Stolp and Starr 1963).

B. bacteriovorus is a highly motile bacterium in liquid by a single polar flagellum (Abram et al. 1974, Rendulic et al. 2004, Sockett 2009). *Bdellovibrio* has been found in, and isolated from, various environments, both terrestrial, aquatic, and recently it was discovered in the intestine of healthy humans in abundant numbers especially in the duodenum (Droop and Jannasch 1980, Iebba et al. 2013). It is a predatory bacterium that has the ability to invade and kill gram-negative bacteria. There are a large number of published studies highlighting the effectiveness of *Bdellovibrio* and its use as a bio-control agent, as a result of their bacteriolytic nature, especially in treatment of shellfish, bird and plant infections, fish (Chu and Zhu 2010, Atterbury et al. 2011, Saxon et al. 2014, Willis et al. 2016). Additional to that, recent studies have highlighted the ability of *Bdellovibrio* to kill Gram-negative human pathogens that are antibiotic-resistant clinical isolates that are a big human health issue like *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Baker et al. 2017, Sun et al. 2017). Another remarkable discovery in the killing of normally lethal *Shigella* infection bacteria by *Bdellovibrio* in vivo was carried out by Willis and co-worker. This work demonstrated the ability of injected *Bdellovibrio* in an animal model to treat an otherwise lethal infection caused by an antibiotic-resistant strain of the human pathogen *Shigella flexneri* by acting together with the host immune system (Willis et al. 2016). Therefore, due to the ability of *Bdellovibrio* to prey on a broad range of Gram-negative bacteria including ones that are pathogenic to humans, it is

considered as a living antibiotic (Tyson and Elizabeth Sockett 2017, Tyson and Sockett 2017).

B. bacteriovorus grow and divide inside the prey cell periplasm, so they do not replicate outside their host cell (Sockett and Lambert 2004). *Bdellovibrio* was previously considered as an endoparasite of other gram negative bacteria (Starr and Baigent 1966). However, from studying how rapidly it kills and digests prey contents, it is seen these days as a predator rather than a parasite. *Bdellovibrio* does not attack cells of human, animal and plant (Iebba et al. 2013, Romanowski et al. 2016, Willis et al. 2016, Shatzkes et al. 2017).

1.6 The life cycle of *B. bacteriovorus* HD100

Bdellovibrio has two replicative life cycles: Host-dependent and Host-independent depending on the presence or absence of the prey cells (Sockett 2009).

1.6.1 Host-Dependent (Predatory) life cycle of *B. bacteriovorus* HD100

The predatory life cycle of *Bdellovibrio* is complex and unusual. *B. bacteriovorus* has a single fast-rotating monopolar flagellum sheathed with outer membrane so they are actively motile. This flagellum helps them to encounter the prey cells and move toward region with many prey, but it is not crucial for invading the prey cell to release of replicated predators (Lambert et al. 2006, Iida et al. 2009). The predatory life cycle of *B. bacteriovorus* takes about 240 min from time of finding the prey cell (Lambert et al. 2010). The predatory life cycle is generally divided into two phases: free-living attack phase and intra- periplasmic growth phase in which the predator- prey association is known as a bdelloplast (Figure 1-5) (Varon and Shilo 1969).

The predatory cycle starts in liquid culture when the freely swimming attack phase *Bdellovibrio* seeks the prey. The attack phase *Bdellovibrio* swims at high speed that reach up to $160\mu\text{m sec}^{-1}$, and collide with the prey cell (or also at this stage Gram-positive cells). Upon encountering region rich in prey cells, the attack phase

Bdellovibrio uses either its single polar flagellum to swim in liquid media or use gliding engines for slow gliding motility on solid media which also brings predator to prey (Hobley et al. 2006, Lambert et al. 2006, Iida et al. 2009, Lambert et al. 2011).

The attachment stage starts after the predator-prey collision occurs, at this point when the *Bdellovibrio* come in to contact with the prey cell using Type IV pili. In this stage, the attachment is either reversible, which means that *Bdellovibrio* able to detach and move away from prey cells if the prey cell is occupied by other *Bdellovibrio*, (and if S-layer present above the cell envelope of the prey cell), or irreversible, when the attack phase *Bdellovibrio* find suitable the prey and start the host invasion (Koval and Hynes 1991, Hobley et al. 2006, Lambert et al. 2006, Evans et al. 2007).

When *Bdellovibrio* attaches properly to the prey cell, the next stage of the cycle occurs which is invasion; *Bdellovibrio* start to make a penetration pore in the outer-membrane and cell wall of the prey cell to enter the prey cell periplasm to begin the intra- periplasmic growth phase of *Bdellovibrio* (Stolp and Starr 1963, Burnham et al. 1968). The formation of this pore occurs when *Bdellovibrio* locally secretes hydrolytic enzymes, these are currently not fully characterised but they locally break down the constituents of the prey cell outer envelope and wall (Hasegawa et al. 2003, Lambert et al. 2006).

A very recent study by Sockett laboratory working with Kuru et al. (2017) proved that the modification in the prey cell wall by *Bdellovibrio* starts during entry of the *Bdellovibrio* to the prey cell. They shown that by labelling the prey cell and *Bdellovibrio* using Fluorescent D-amino acids (FDAA) with different colours and doing a pulse with blue HADA FDAA which was incorporated into dynamically changing cross-link of the peptidoglycan in a very efficient way. Kuru et al. (2017) observed that there were annular ring structures of HADA on prey peptidoglycan

(PG) that labelled at the point of prey-predator contact when the *Bdellovibrio* was attached on outside or through entering the prey cell or inside it. Invasion is accompanied by morphological changes in the prey cell that Thomas Lerner previously showed involves *Bdellovibrio* DacB enzymes (DD-endopeptidase) (Lerner et al. 2012). The cells become more rounded to a structure called a 'bdelloplast'. When the *Bdellovibrio* completely enters the prey cell, *Bdellovibrio* seals the penetration pore, so it will be able to continue the predatory cycle (This will explain in Chapter 8 what Sockett laboratory and this study found recently about how *Bdellovibrio* seal the penetration pore after the invasion as the work on this subject is still ongoing in Sockett laboratory).

B. bacteriovorus requires the presence of type IV pili (T4P) to commence their invasion which is found at the opposite pole to the flagellum (Capeness et al. 2013, Milner et al. 2014). The shortening/retraction of pili is thought to pull *Bdellovibrio* through the pore that it has made in the prey cell. Before *Bdellovibrio* enters to the prey cell, it usually (but not always) sheds its flagellum from the tail pole (Rendulic et al. 2004, Lambert et al. 2006). The formation of the bdelloplast needs alteration the peptidoglycan cross- linkage of the prey cell to make it more flexible and allow rounding up into spherical bdelloplasts. This occurs through the action of *Bdellovibrio* D,D-transpeptidase Bd816, Bd3459 with L,D-transpeptidase Bd0886 and Bd1176 strengthening the rounded bdelloplast wall and making it more resistance to bursting while *Bdellovibrio* grows inside the prey (Lerner et al. 2012, Kuru et al. 2017).

Then predator growth is initiated forming an elongated, snake-like *Bdellovibrio* cell that is several times longer than a single *Bdellovibrio* unit cell size. (Fenton et al. 2010, Lerner et al. 2012). The bdelloplast is an osmotically stable structure for this process to happen inside (Lambert et al. 2006, Sockett 2009). Formation of the bdelloplast is considered as an initial method for protecting *Bdellovibrio* from unfavourable conditions and enhancing its survival as well as allowing nutritional

uptake and replication (Markelova 2002, Hobley et al. 2006). For this nutrition, degradation of the prey cell components occurs, allowing *Bdellovibrio* to use them as precursors for nutrients and energy (Lambert et al. 2006). After that, *Bdellovibrio* begins to synthesise DNA, RNA, protein, lipid and polysaccharide (Burns and Slater 1982, Tudor et al. 1990). As *B. bacteriovorus* have the apparent ability to synthesise only eleven amino acids, the synthesis of *Bdellovibrio* proteins depends on consuming products that result from degradation of prey cell components (Rendulic et al. 2004). The *Bdellovibrio* septation stage starts after the formation of the filamentous *Bdellovibrio* cell inside the bdelloplast. Subsequently, *Bdellovibrio* detect (by currently unknown mechanisms Lukasz Makowski), the depletion of available nutrients in the prey cell and undergo septation to produce an odd or even number of progeny cells (4-7 progeny produced if *E. coli* S17-1 if used as prey) (Barabote et al. 2007, Sockett 2009, Fenton et al. 2010).

The last step in the cycle is when the progeny of *Bdellovibrio* secrete hydrolytic enzymes that lyse the prey cell and they exit, either using their flagella (in liquid culture) or their gliding motility on a solid surface. The new attack phase starts as the progeny search for new prey cells and repeat the cycle (Sockett and Lambert 2004, Barabote, Rendulic et al. 2007).

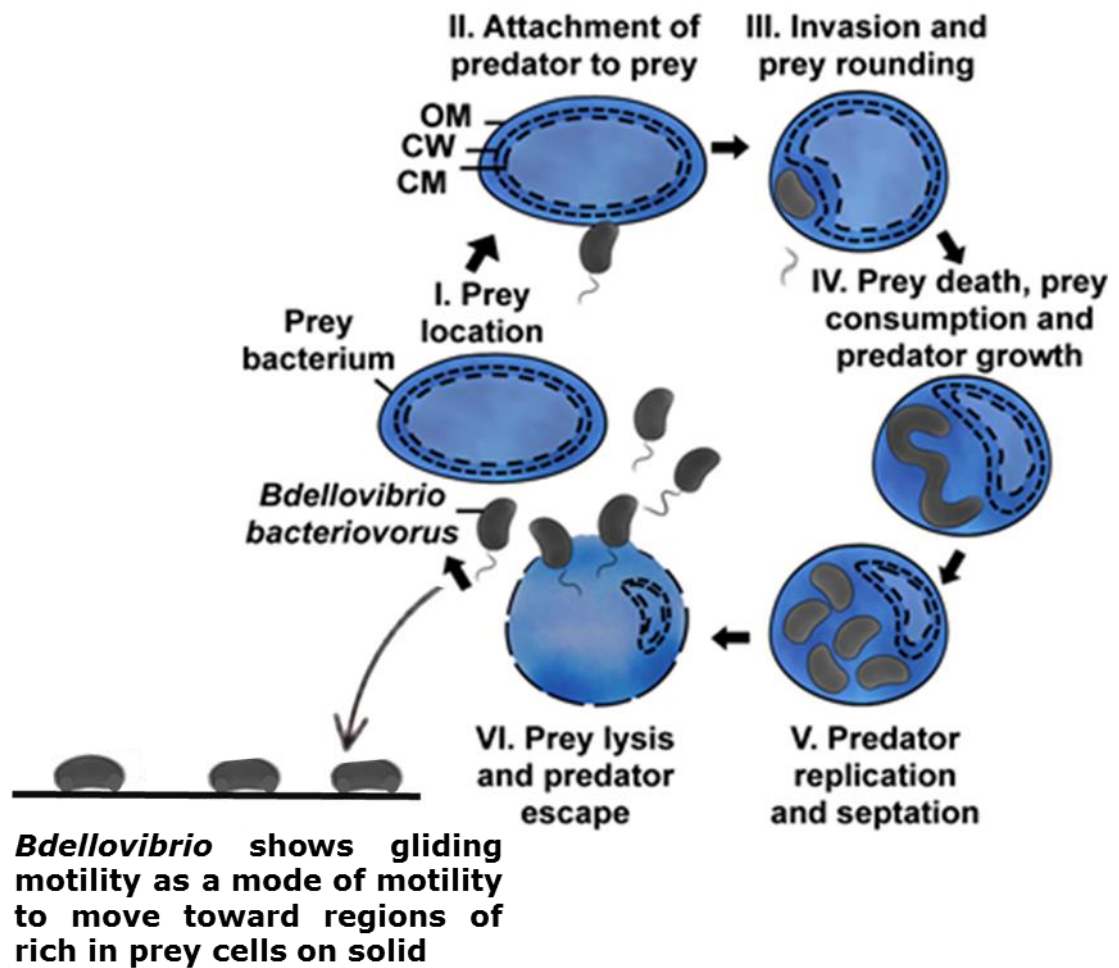


Figure 1. 5: The lifecycles of host- dependent replication of *B. bacteriovorus*. This image adapted from (Willis et al. 2016). The cycle consists of six stages that start when *Bdellovibrio* finds the prey and attach to start invasion that is followed with morphological changes to resize and round the prey cell wall to allow the intracellular growth of the *Bdellovibrio* without bursting the prey cell. After that, *Bdellovibrio* replication and septation occurs followed by prey lysis and new *Bdellovibrio* progeny exit to repeat the same cycle.

1.6.2 Host- Independent *Bdellovibrio* growth

In the absence of the prey cells, *B. bacteriovorus* can survive and grow slowly as Host- independent (HI) cells on amino-acid rich media (Figure 1-6). HI cultures can be derived from a small percentage Host-dependent *B. bacteriovorus* (HD) (Seidler and Starr 1969, Cotter and Thomashow 1992). A single HI colony can

be obtained from 5×10^8 attack phase of *B. bacteriovorus* when plated on rich media (Stolp and Starr 1963)

HI *Bdellovibrio* cells have to grow outside bdelloplast protection and show a broad diversity in cells size (2- 30 μm), shape (short, filamentous, attack phase cells, etc.), the formation of pigmentation and size of plaques made on prey lawns (Figure 1-6-B)(Barel and Jurkevitch 2001). Providing rich media such as PY, enhances HI *Bdellovibrio* growth. HI growth is reported to be enhanced by adding also host cell extract or divalent cations to the media (Barel and Jurkevitch 2001), and HI cultures also can show a reduced level of motility in host free media (Seidler and Starr 1969) with flagella still present, albeit at non-polar locations on longer cells than in attack phase (Seidler and Starr 1968, Seidler and Starr 1969) so motility is not rapid. HI *B. bacteriovorus* colonies have a slow growth rate that takes 7-10 days to grow on plate media that are provided by peptone- or yeast-extract at 29°C (Sackett 2009).

The ability *Bdellovibrio* to switch from HD to HI of results from single mutation, often, (but not always), in the small open reading frame of the host interaction locus (*hit locus*) including gene *bd0108* which controls the Type IV pili and this acquired mutation found in about 89% of HI *B. bacteriovorus* isolates cells (Wurtzel et al. 2010, Roschanski et al. 2011, Capeness et al. 2013). The prey entry requires the presence of Type IV pili and deletion of *bd0108* results in significant a reduction of pili extrusion and this indicated that production and extrusion/ retraction of type IV pili was regulated by Bd0108 (Roschanski et al. 2011, Capeness et al. 2013). HI *B. bacteriovorus* which grow axenically on rich media still retain the ability to grow intraperplasmically on prey but, produce smaller and more turbid plaques than those produced by wild-type HD *B. bacteriovorus* on *E. coli* lawns (Cotter and Thomashow 1992). HI cells have been shown to have the ability to form biofilms and the predatory ability of HI cells that form biofilm can be restored by releasing HI cells from the biofilm and attack the

prey cells that provided externally (Medina and Kadouri 2009). Until now not too much is known about the proteins that play a role in biofilm formation in HI cells that needed to search a lot in mixed prey predator films. In the recent past, a study carried out by Lambert and Sockett (2013) they found that nuclease genes (*bd1244*, *bd1934*) of the *B. bacteriovorus* play a role biofilm formation and deletion of these genes result in mutant strains have less efficiency in biofilm production compared with wild type control.

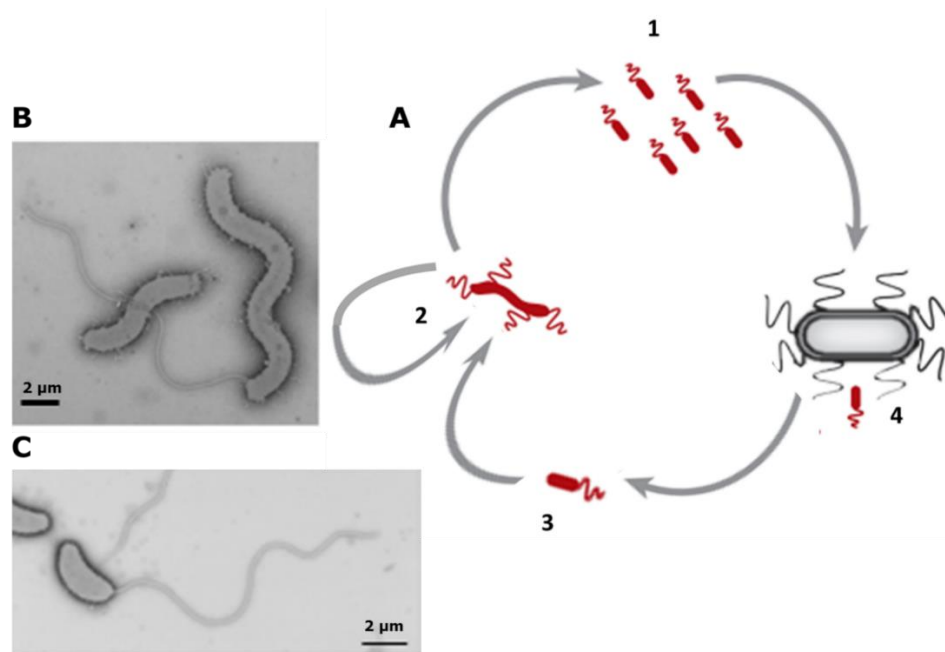


Figure 1. 6: The host-independent (HI) growth cycle of *Bdellovibrio*. A: Stages of HI growth cycle of *Bdellovibrio* include, Stage 1: When the high-density population of host-dependent attack phase *Bdellovibrio* (1×10^7 cells) with the presence of a rich source of nutrients can switch on to HI growth cycle. Stage 2: The HI growing cells display various shapes and size (filamentous, serpentine, attack phase or selfbdelloplast etc.). Stage 3: Replication of HI cells is happened by 'budding'. Stage 4: With providing the prey, HI cells of *Bdellovibrio* can go back to the HD lifecycle B: Electron microscopy image showing the HI cells (filamentous and short attack phase like), C: Electron microscopy images showing HD attack phase cell. Figure is taken from (Sockett 2009) and Electron microscope images are taken from (Hobley et al. 2012).

1.7 Bacterial cell envelope

The bacterial cell envelope is clearly important in predatory bacterial interaction. It is a structure of complex multilayered that surrounds bacterial internal structures that are known to give shape and protect bacteria cell from undesirable environmental conditions. At the same time, the bacteria cell envelope allow selective passage for nutrients to enter to the cell from the outside and discharge waste products from the inside cell to outside (Silhavy et al. 2010). One of the major components in the bacterial cell envelope is peptidoglycan. Depending on the structure of cell envelope, the most bacteria can be divided into two major groups: Gram- positive and Gram-negative (Silhavy et al. 2010). This division depends on the bacterial cell wall reaction with Gram method that detected thickness of peptidoglycan retaining the first Gram stain (John G. Holt 1994). The bacterial envelope in Gram-negative bacteria consists of the outer membrane (OM), a thin layer of peptidoglycan, and the cytoplasmic or inner phospholipid membrane (IM) (Silhavy et al. 2010, Todar 2013). The outer membrane consists of two leaflets: an inner leaflet that composed of phospholipids and an outer leaflet compose of lipopolysaccharide that is also called lipopolysaccharide (LPS) as seen in Figure (1-7).

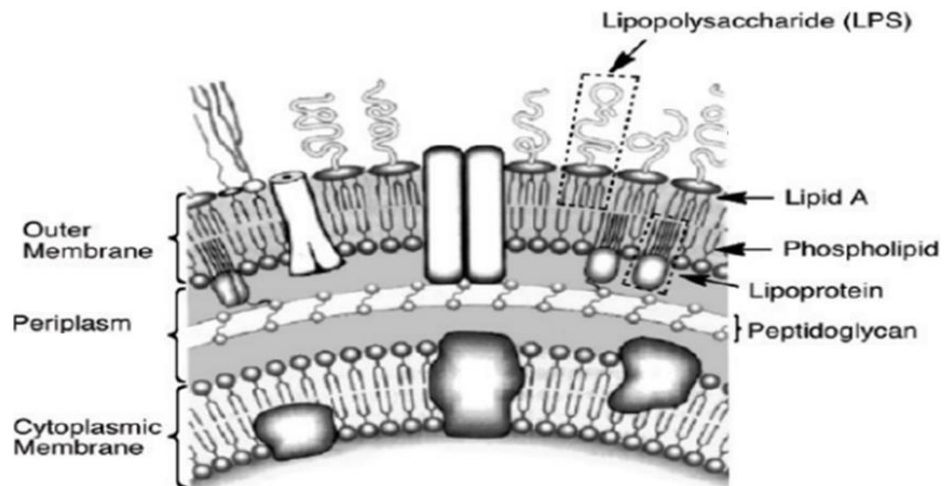


Figure 1. 7: The Gram-negative cell envelope. The envelope consists of an outer membrane, peptidoglycan and the cytoplasmic membrane that was taken from (Fujimoto and Fukase 2011).

In contrast, Gram-positive bacteria have no an outer membrane and are surrounded by very thick layers of peptidoglycan (15-80 nanometers) comparing with the single layer in the Gram-negative bacteria. The other differences that the gram-positive cell wall have, that not are found in Gram-negative, are containing teichoic acids (anionic polymers) that penetrate through the peptidoglycan layers (Figure 1-8) (Silhavy et al. 2010, Todar 2013).

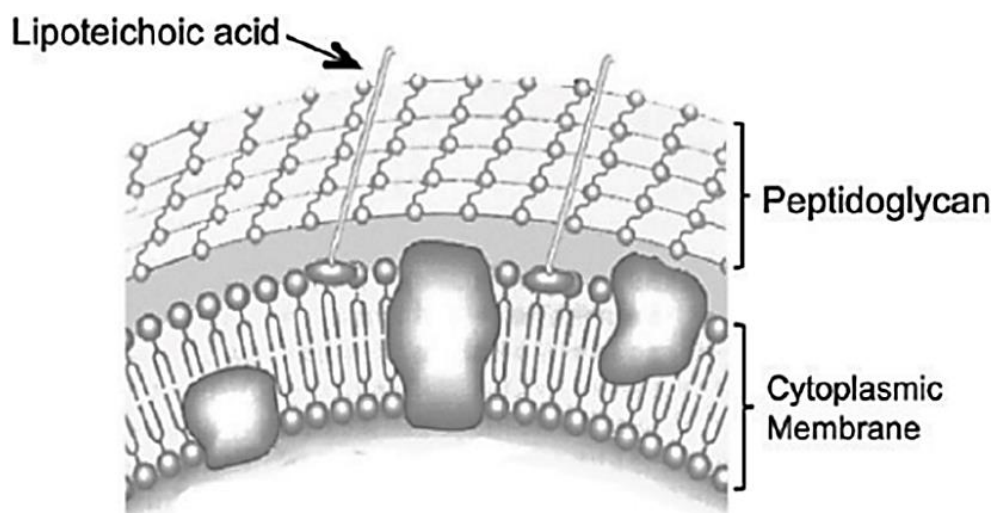


Figure 1. 8: The Gram-positive cell envelope. The envelope consists of the Lipoteichoic acid, thick layers of peptidoglycan and cytoplasmic membrane that was taken from (Fujimoto and Fukase 2011).

There are some Gram- positive bacteria with exception to what mention in Gram-positive envelope structures and these bacteria are *Corynebacterium* and *Mycobacterium* that cell envelope complex architectures than normal Gram-positive cell envelope. *Corynebacterium* cell envelope consist of (from inside to outside) plasma membrane, Peptidoglycan, Arabinogalactan, with outer layer of Mycolic Acids and Trehalosyl Mycolates (Daffé 2005, Burkovski 2013). The cell envelope of mycobacterium consist of (from inside to outside) plasma membrane, Peptidoglycan, Arabinogalactan, Mycolic Acids and capsular layer that made of protein and polysaccharide (Kieser and Rubin 2014, Alderwick et al. 2015).

1.8 Periplasm of Gram- negative bacteria

During invasion, *Bdellovibrio* inhabit the region between Gram-negative inner and outer membrane which is the periplasm. Periplasm is a gel like region that located between the outer membrane and the inner cytoplasmic membrane in Gram-negative bacteria. The periplasm consist of water and proteins and layer of peptidoglycan that gives Gram-negative bacteria strength and shape (Todar 2013, Paustian 2017). The periplasm is considered as an oxidative environment that participates in much protein folding and export and it is contain a lot of enzymes and components that essential for cell viability and sensing the environment and some of them are listed in table (1-2) (Todar 2013, Kojer and Riemer 2014). The thickness of periplasm of the *E. coli* varies from 10 to 50 nm (Graham et al. 1991). The periplasm is the place where *B. bacteriovorus* HD100 after invasion enter and start its intracellular growth phase stage inside the prey cell therefore the periplasm is considered as a home for *Bdellovibrio* (Sockett and Lambert 2004). For *Bdellovibrio* to prepare the periplasm to be its home it is required to decrosslink the prey peptidoglycan layer that found in the periplasm and this happened through DD-endopeptidase enzymes (Bd0816 and Bd3459) that responsible for rounded the prey cells (Lerner et al. 2012).

Table 1-2: showed some representative enzymes that found in *E. coli* periplasm.
The table was taken from (Todar 2013)

Enzyme Type	Examples
Binding proteins	For amino acids (e.g. histadine, arginine)
	For ions (e.g. phosphate, sulfate)
	For sugars (e.g. glucose, maltose)
	For vitamins (e.g. thiamine, vitamin B12)
Biosynthetic enzymes	For murein assembly (e.g. transglycosylases, carboxypeptidases, transpeptidases)
	For fimbrial subunit secretion and assembly (e.g. chaperonins)
Degradative enzymes	Phosphatases, proteases
Detoxifying enzymes	Beta-lactamases (penicillinase)
	Aminoglycoside-phosphorylating enzymes

This study will focus on Gram-negative bacteria especially *E. coli* that are considered the best model used to study the cell wall of Gram-negative and as *Bdellovibrio* is a Gram-negative bacteria, and all prey that used in this study are also Gram-negative bacteria.

1.9 Peptidoglycan structure and content

Peptidoglycan (PG) is a key component of the bacterial cell wall which surrounds the inner membrane of most bacteria. It is found to maintain the bacterial cell shape and prevents cells from bursting due to internal pressure from cytoplasm or external pressure (Vollmer et al. 2008, Popham 2013, Egan et al. 2014). It also has to be modified to participate in cell growth and division (Typas et al. 2012). Peptidoglycan is found in nearly all bacteria with the exception of *Mycoplasmas*,

Chlamydia and *Rickettsia* (Tamura et al. 1995, Razin et al. 1998, Razin and Hayflick 2010). At 2014, Liechti and co-worker provided firm evidence that *Chlamydia* is possessing functional PG (only at the septum) by using the fluorescent D-amino acids (FDAAs) finishing the debate about the presence of a cell wall in that group.

Additional to that, there is what are termed L-form bacteria which are fragile, osmotically sensitive, pleomorphic bacteria that are wall-deficient and have the ability to go back to a walled state. The L-form results from the mutation in enzymes that are responsible for peptidoglycan precursor synthesis or from the action of antibiotics that work on the cell wall lytic enzymes (Joseleau-Petit et al. 2007, Kawai et al. 2014).

1.9.1 Peptidoglycan structure

The peptidoglycan (PG) polymer consists of glycan strands which are cross-link with short side chain peptides. Each glycan strand is composed of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) residues arranged in an alternating way and both residues linked by β 1,4 glycosidic bonds. The short peptides that are cross-linked with glycan joined to an N-acetylmuramic acid (NAM) residue, after substitution of the D-lactoyl group of each NAM molecule by a peptide stem. In newly synthesised peptidoglycan, the composition of peptide stem which some times varies in some bacteria but is mostly composed of L-alanine-D-glutamate-*meso*-diaminopimelicacid-D-alanine-D-alanine (L-ala-D-glu-mDAP-D-ala-D-ala) while, in mature peptidoglycan, the terminal D-alanine is cleaved resulting in the tetrapeptide stem (see Figure 1-9) (Barreteau et al. 2008, Hayhurst et al. 2008). Meso-diaminopimelicacid (mDAP) is an amino acid whose synthesis will be studied further in this thesis (Chapter 5 and 6).

The glycan chain cross-linking happens via binding the carboxyl group of D-alanine at position 4 from one peptide stem with the amino group of the m-DAP

from other peptide stem at position 3 (see Figure 1-9). This type of cross link is called 3-mDAP-4-Alanine (Barreteau et al. 2008). This cross-linking event takes place with the help of D,D transpeptidase enzymes in *E. coli* (see Figure 1-10 A) (Magnet et al. 2008).

The existence of peptide stems that are used for cross-linking with D- amino acids give the glycan strands resistance to peptidases that cleave the cross-links that lead to lysis the cell wall (Cava et al. 2011).

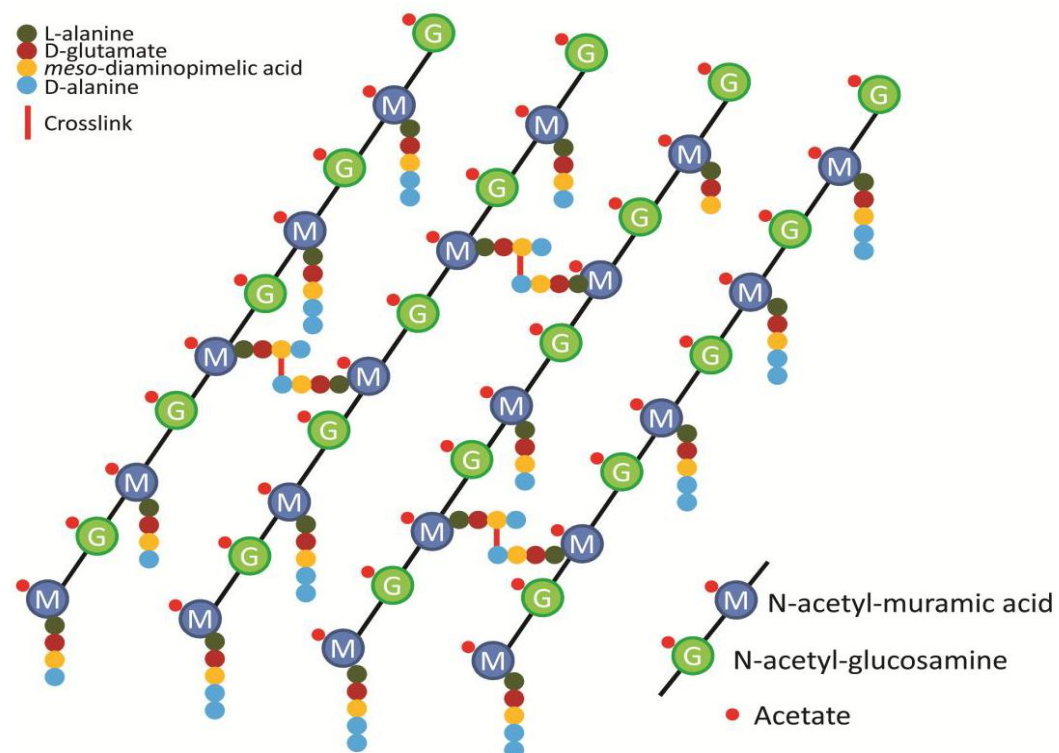


Figure 1. 9: The basic chemical structure of peptidoglycan in Gram-negative bacteria. Single glycan strand consists of N-acetylglucosamine and N-acetylmuramic acid residues arranged in an alternating way and both residues linked by β 1,4 glycosidic bonds. The pentapeptide stems participate in crosslinking the glycan strands giving tetrapeptide and tripeptide cross-links (Lerner 2012).

In *E. coli* and many other species, there is another type of crosslinking type between 3-mDAP-3-mDAP cross-links that occur through binding of the L-carboxyl group of m-DAP in one glycan strand with the D-amino group of m-DAP in a neighbouring strand (Magnet et al. 2008, Peltier et al. 2011). This cross-linking happens through action of L,D transpeptidase enzymes (see Figure 1-10 B) (Magnet et al. 2008).

The 3-mDAP-3-mDAP cross-links have been found to play a role in increasing the rigidity of the in *E. coli* cell wall and adaption to specific stress conditions additional to participating in bacterial survival in non-replication periods (Goffin and Ghuysen 2002). The 3-mDAP-3-mDAP cross-links increase in some bacteria leading to resistance to penicillin-type antibiotics in *Enterococcus faecium*, while in *E. coli*, 3-mDAP-3-mDAP cross-links increase when there are temperature elevations, osmotic stress and in nutrient limitation (Glauner et al. 1988, Mainardi et al. 2000). In *E. coli* 3-mDAP-4-Alanine cross linking occurs during the exponential phase, while 3-mDAP-3-mDAP cross linking occurs during the stationary phase, nutrient depletion and stress (Wietzerbin, et al. 1974, Templin, et al. 1999).

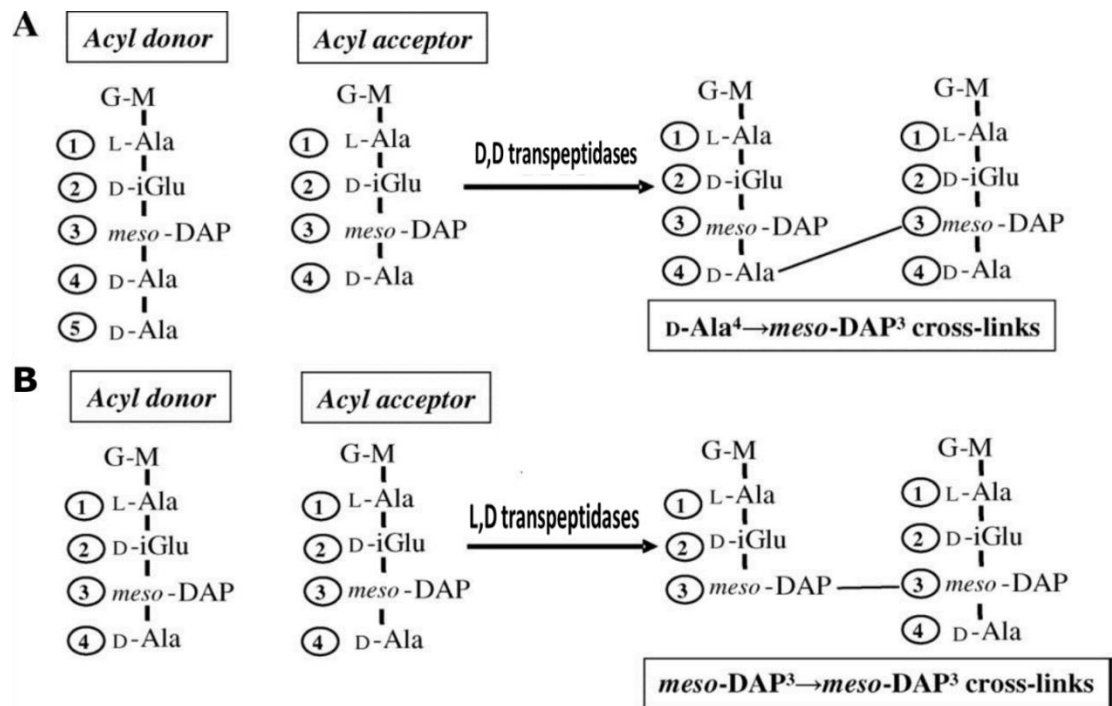


Figure 1. 10: The schematic diagram of Gram- negative cell wall cross- links. The cell wall cross- links are catalysed by D,D transpeptidases which responsible for 3-mDAP-4-Alanine cross-link and L, D transpeptidases which responsible for 3-m-DAP-3-m-DAP cross-link from *E. coli* studies reproduced from (Magnet et al. 2008).

The “sacculus” is the term that denoted for the macromolecules that consist of the glycan strands with its cross-linked peptide stems that that wrap the cytoplasmic membrane which that play a role in protecting the bacterial cell from lysis by internal osmotic pressure (Vollmer and Bertsche 2008). For a long time, the debate about these structures and how the peptidoglycan is arranged in the sacculus continues until coming of the new technology, Electron cryotomography and atomic force microscopy solved the basic architecture of the sacculus for *E. coli*, *Streptococcus pneumoniae*, *Enterococcus faecalis* (Gan et al. 2008, Wheeler et al. 2011). The length of glycan strands can be obtained from determining the proportion of mucopeptides with 1,6-anhydro-N-acetyl-muramic acid (1,6-anhydroMurNAc) that are found at the end of glycan strand digestion of the sacculus (Glauner et al., 1988). The length of the glycan strands in *E. coli* varies and depends on the type of strain and growth stage. The average glycan strand

in *E. coli* is 18 and 60 disaccharides (DS) units (Vollmer et al. 2008). The PG is predominantly made from approximately 70–75% of glycan strand, with length 1–30 DS units and short glycan strand with length 7–12 DS units, while the rest made from 25–30% of glycan strand with length up to 80 DS units (Harz et al. 1990).

It is worth to mention that Gram-negative purified sacculi have different thicknesses, for example, purified *Pseudomonas aeruginosa* sacculi are about half the thickness of *E. coli* sacculi (Yao et al. 1999). Even though later in my thesis, I used several Gram-negative bacteria that should all have similar envelope architecture PG but may be differ in thickness

1.10 Why meso-diaminopimelic acid (m-DAP) is important for Gram-negative bacteria

Meso-diaminopimelic acid (m-DAP) is considered as a one of the important metabolites that participates in lysine biosynthesis and also in the peptidoglycan synthesis where it plays a crucial role in glycan strand cross-linking that gives the PG strength and bacterial cells resistance to lysis (Pavelka and Jacobs 1996). DAP is found in all gram-negative and some gram- positive bacteria like Bacilli (Todar 2013).

Furthermore, m-DAP is found in the spores and mucopeptides of gram- positive bacteria and *Mycobacterium tuberculosis* (Pavelka and Jacobs 1996, McCoy, et al. 2006). The lack of the m-DAP/ Lysine synthesis pathway in mammalian cells but its existence in bacteria, makes this pathway is a good target for antimicrobial therapy development (Usha, et al. 2012). DAP is synthesised as an (intermediate) metabolite of the aspartate metabolic pathway which involves leucine, methionine, threonine and lysine synthesis (Pavelka and Jacobs 1996, McCoy, et al. 2006).

In general, Gram- negative bacteria require the m-DAP for 3-mDAP-4-Alanine cross-linking and 3-m-DAP-3-m-DAP cross-linking to strength cell wall. Furthermore, in Gram- negative bacteria the m-DAP partakes in connecting the

peptidoglycan sacculi with the outer membrane through Braun's lipoprotein (Figure 1-11) (Magnet et al. 2007). Braun's lipoprotein is a protein 6 kDa in size and it is composed of 78 amino acids from these amino acid, 20 amino acid make up the signal peptide at the amino terminus (Dramsai et al. 2008, Konovalova and Silhavy 2015).

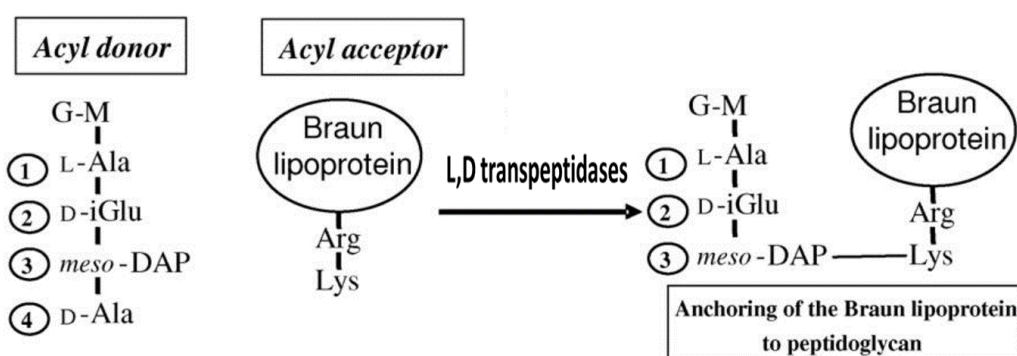


Figure 1. 11: The schematic diagram of Gram- negative cell wall showing the connection of peptidoglycan with outer membrane. The connection occur through Braun lipoprotein that catalysed by L, D transpeptidases from *E. coli* studies reproduced from (Magnet et al. 2008).

This connection take place by binding L- carboxyl group of m-DAP from peptidoglycan with the side chain amide group of lysine from Braun lipoprotein (Magnet et al. 2007). This connection results from the action of L,D transpeptidases which breaks the bond between DAP and D-ala in tetrapeptide and connects it to Lysine (Figure 1-11). This may explain why gram-negative bacteria need DAP "more" than D-ala due to its multiuse in three different cross-links.

In the case of *B. bacteriovorus HD100*, the requirement of m-DAP definitely is more because *B. bacteriovorus HD100* needs m-DAP for its cell wall cross-links and for connection to its outer membrane for its own wall and also likely for prey wall modification to make it more strong so it can grow inside the prey cell.

1.11 Peptidoglycan synthesis

Peptidoglycan is a key component of the bacterial cell wall that give bacteria protection and its shape and the synthesis of PG occurs in complex process and required participate of a lot of enzymes. Synthesis of PG consist of three steps.

1. Synthesis of the precursor molecule of the PG in the cytoplasm: the precursor molecule of the glycan are synthesised in the cytoplasm which are uridine-diphosphate-N-acetyl-glucosamine (UDP-NAG) and uridine-diphosphate-N-acetyl-muramic acid (UDP-NAM) by action of MurA and MurB that committed step in bacterial cell wall biosynthesis. During that time, the peptide side chain is synthesised by attaching L-amino acids which are L-ala, D-glu, meso-DAP, D-ala-D-ala to the lactyl group of UDP-NAM by action of MurC, MurD, MurE, MurF ligases respectively (see Figure 1- 13). D- alanine is synthesised by Ala racemases (DadX) and the dipeptide D-ala-D-ala happened through action of D-ala-D-ala ligase (DdlA) (Barreteau et al. 2008, Bugg et al. 2011, Typas et al. 2011).
2. Transport of the PG precursor molecules across the cytoplasmic side of inner membrane: the soluble UDP-NAM –pentapeptide joining with undecaprenyl phosphate (lipid carrier molecule) by translocase of MraY in order to form Lipid I in the inner face of cytoplasmic membrane (undecaprenyl pyrophosphoryl-NAM-pentapeptide). After that, NAG (from UDP-NAG) is attach to lipid I (undecaprenyl pyrophosphoryl-NAM-pentapeptide) by glycosyltransferase of MurG to form Lipid II (Bouhss et al. 2008, Bugg et al. 2011). The Lipid II molecule flipped across the inner membrane into the periplasm by action of lipid II flipase (FtsW-RodA or MurJ) (see Figure 1- 13) (there was a big controversy about who is playing the role of Lipid II flipase as Mohammadi et al. (2011) provided the biochemical evidences that FtsW is Lipid II flipase while, Sham (2014) gives the genetic evidences that MurJ is the Lipid II flipase) (Mohammadi et al. 2011, Sham et al. 2014).

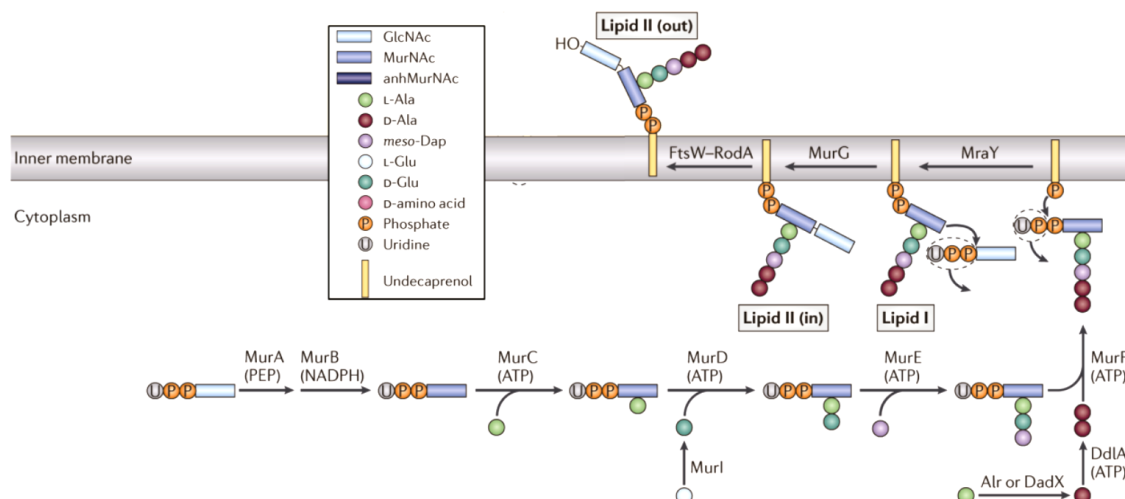


Figure 1. 12: The cytoplasmic steps of the peptidoglycan synthesis. First step include the synthesis of precursor cell UDP-MurNAc via MurA and MurB. The side peptide amino acids L-ala, D-glut, *meso*-DAP and D-ala- D-ala are sequentially added by action of MurC, MurD and MurE, and MurF. Second step include UDP-NAM-pentapeptide joining with undecaprenyl phosphate and form lipid I by action of MraY and Lipid I converted to Lipid II by attaching of UDP-NAG with action of MurG to form Lipid II. Then LipidII flipped across inner cytoplasm membrane into periplasm. The figure was adapted from (Typas et al. 2011).

3. Polymerization of Lipid II by PG synthases (Penicillin binding proteins): the PG synthases can be classified to either glycosyltransferase (GTases), transpeptidases (TPases) or have both glycosyltransferase and transpeptidases (GTases-TPases) reactions. This explained more in details in Chapter 8 in this thesis. The LipdII is polymerised by reaction of glycosyltransferase with release of undecaprenyl pyrophosphate, while the crosslinked occur through the reaction by transpeptidases (TPases) (Vollmer and Bertsche 2008) (Typas et al. 2011) (Figure 1- 14).

There are two types of transpeptidase enzymes that play role in peptidoglycan cross-linking. These are D, D transpeptidases and L, D transpeptidases (Figure 3). D,D transpeptidase enzymes are members of pencillin-binding protein family (PBPs) and function by forming D- Ala⁴- DAP³ cross-linkage (4-3) in peptidoglycan (Glauner et al. 1988). L, D transpeptidases have two major functions, they are

able to form DAP-DAP (3-3) cross-linkages and also connecting peptidoglycan with Braun lipoprotein (Magnet et al. 2007, Magnet et al. 2008).

In addition all L, D transpeptidases enzymes are responsible for cleaving peptide bonds between m-DAP³-D-Ala⁴ (Figure 3). In an *E. coli*, deletion of all genes encoding L, D transpeptidases produced cells starved for DAP, had deleterious effect on the growth and integrity of *E. coli* which evidence that L, D transpeptidases are responsible for anchoring Braun lipoprotein to the PG and for 3-3 cross-linking of the PG cell wall (Sanders and Pavelka 2013).

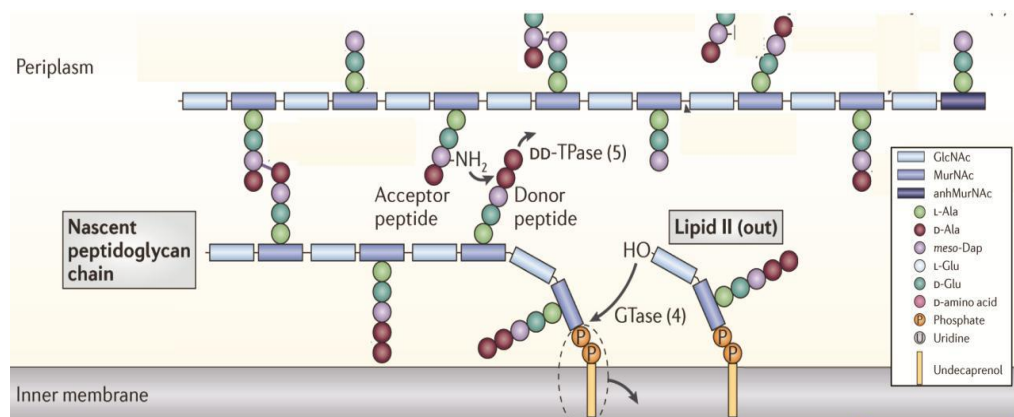


Figure 1. 13: Third step in PG synthesis. Lipid II is polymerised by action of glycosyltransferase that led to release of undecaprenyl pyrophosphate and the crosslinked occur through transpeptidases (TPases). The figure was adapted from (Typas et al. 2011).

1.12 From the past till today what new discoveries are there about *Bdellovibrio* and the prey wall modification?

The predatory life cycle of *Bdellovibrio* is complex and requires several modifications in the wall of *Bdellovibrio* and in the prey cell walls that are controlled by various genes encoded in the *Bdellovibrio* genome. The modifications start from the attachment step of the predatory cycle to prepare (predator or/and prey) wall for entry of *Bdellovibrio* to the niche and then physical invasion into the periplasm of Gram-negative bacteria cells to form bdelloplasts

which remain strong and protect the progeny of *Bdellovibrio* while they grow inside it. This bdelloplast modification needs numbers of physiological and chemical activities (Parish 1979). To date, all the proteins that play a role in the morphological changes that accompanied the predatory life cycle are not clearly identified. In this section, all up to date proteins that play role in either self- wall modification of *Bdellovibrio* or prey wall modification genes that participate on going of the predatory lifecycle in a normal way, will be mentioned.

The first attempt to understand how *Bdellovibrio* entry to the prey cell started at 1978 when Thomashow and Rittenberg (1978), when they noticed that *Bdellovibrio* produced specific enzymes that were associated with the invasion and establishment of bdelloplast. Invasion of prey cell by *Bdellovibrio* needed passage through of the outer membrane and the peptidoglycan layer and this was first thought to happen through mechanical drilling (Starr and Baigent 1966). This theory was refuted by Lambert et al. (2006) when they proved that strain of *Bdellovibrio* with a non-motile flagellar mutation was still able to invade the prey cell and so *Bdellovibrio* flagellum is for motility but not required for predation. Preliminary work on how the pore was made during invasion, Fackrell and Robinson (1973) and Huang and Starr (1973) pointed out that pore must result from enzymatic digestion of the prey peptidoglycan by *Bdellovibrio* enzymes when they found muramic acid with activity of one lysozyme and one peptidase in lysate of multicycles of *Bdellovibrio* prey on *E. coli*.

A new recent study by (Kuru et al. 2017) that used pulsed fluorescent D-amino acids and 3D Structured Illumination Microscopy (3D SIM), they revealed that the modification of the prey cell wall starts from the attachment time when they found annular ring structure of FDAAs at point where the *Bdellovibrio* comes into contact with prey cell to make the entry pore. Moreover, a related result was obtained by Sam Greenwood and myself from fluorescently mCherry tag and dual colour mCherry and mTFP tags for *CpoB* (*Bd0635*) of *Bdellovibrio* that detected

spot like patches at the site of attachments of the *Bdellovibrio* to prey cell (see Chapter 8).

Thomashow and Rittenberg (1978) found that there was 10-15% of prey PG cell solubilised in the first 20 mins after mixing the *Bdellovibrio* with prey and there were two enzymes active during this period which have glycanase and peptidase activities. Furthermore, early workers reported that the majority of the free DAP molecules released from the prey cell during invasion localized in the peptidoglycan of the prey bdelloplast wall and not in the *Bdellovibrio* cell wall (Ruby and Rittenberg 1984). Thomashow and Rittenberg (1978) also detected that 30% of solubilized *E. coli* diaminopimelic acid residues were found as free diaminopimelic acid after 90 mins of mixing *Bdellovibrio* with *E. coli* prey in the culture. Kuru et al. (2017) found most extensive prey cell wall modification appeared at 30-45 mins after predator-prey mixing and it comes from the activity of L,D transpeptidase Bd1176 and Bd0886 of *Bdellovibrio* through increase the incorporation of tetrapeptide FDAAs into the bdelloplast wall which gives the strength to the bdelloplast to avoid the bursting of it during the intracellular growth of *Bdellovibrio*. Deletion of both of *bd1176* and *bd0886* L,D-transpeptidase genes resulted in decrease in incorporation of FDAAs into the bdelloplast and the bdelloplasts that resulted from the deletion strain were less rounded than that of wild type strain. The FDAAs could be substituting for m-DAP in corporation using free DAP as reported by Thomashow and Rittenberg (1978)

Transition in prey cell shape is needed during the invasion to make prey cell accommodating for intracellular growth of *Bdellovibrio* forming the rounded structure called bdelloplast. Removal of diaminopimelic acid (DAP) and other peptide amino-acids (such as D-ala) from prey peptidoglycan was reported in early papers to occur via a peptidase enzyme and indeed many be acting not this one (Thomashow and Rittenberg 1978). These amino acids would become free in

the periplasm of the prey bdelloplast. Work since then Lerner et al. (2012) and Lerner (2012) has shown that several peptidoglycan peptidases work on different prey wall components.

Lerner and co-worker (2012) found that Bd0816 and Bd3459 play role in rounding the prey cell and both of them are DD-endopeptidases that hydrolyse the 3m-DAP-4-D-aLa cross-links in the prey cell wall that lead to rounding of the prey cell. Additional to that, these enzymes give signals for other attack phase predation that the prey cell is invaded by *Bdellovibrio* so should not be attached by other attack phase to improve efficiencies invasion process. Deletion of either or both *bd0816* and *bd3459* resulted in production of bdelloplasts that were less rounded than produced by wild type strain with an increase in the rate of double prey-entry (Lerner et al. 2012). Lerner and co-worker (2012) discovered that Bd3459 is a PBP with both DD-carboxypeptidase/ endopeptidase activity acting on existing prey PG cross link due to its ability to cleave trimers crosslinked in PG and additionally to trim the terminal D-alanine from the pentapeptide stems.

Older research showed that the incorporated DAP in the wall of bdelloplast starts to solubilize during the lysis of bdelloplast (just before *Bdellovibrio* progeny release). This indicates that DAP attachment to bdelloplast wall is controlled by *Bdellovibrio* enzyme(s) to make the bdelloplast stable in some way until later lysis. Thus the activity of the invaded prey cell was not influencing the process of m-DAP attachment to wall of bdelloplast, the *Bdellovibrio* are actively doing it with their enzymes as part of predation (Ruby and Rittenberg 1984, Araki and Ruby 1988).

The discovery that the m-DAP incorporation process was insensitive to metabolic inhibition was reported to be evidence that the *Bdellovibrio* invasive cell may be regulating the activity of DAP incorporation in the bdelloplast by secreting extracellular enzyme(s) in early stages of attack phase of *Bdellovibrio* and

bdelloplast formation (Araki and Ruby 1988). After all what we know up to the minute about the modification that occurs in the predatory life cycle of *Bdellovibrio*, the question arises: "How is *Bdellovibrio* able to protect itself from all these enzymes?". Self-protection is a very crucial aspect in the predatory life cycle of *Bdellovibrio* to protect itself from action of its PG modification enzymes that modify the prey wall during invasion to avoid prey cell from breaking and accommodating the intracellular growing *Bdellovibrio* within prey cell until reaching the lysis step and exit from prey shell.

Dr. Andy Lovering working with Sockett laboratory answered this question for the product of one of genes that plays a role in *Bdellovibrio* self-protection which is Bd3460. Dr. Andy Lovering noted that *bd3460* gene was adjacent to PG endopeptidase gene *bd3459* and had a predicted Ankyrin structure. Ankyrins are typically immunity proteins. Bd3460 is an ankyrin repeat protein that identified as a self-protection of *Bdellovibrio*. This protein have ability to made complexes with Bd3459 and Bd0816 which are DD-endopeptidases. This complexation resulted in inhibition of endopeptidase activity and stopped the cell wall decrosslinking. The deletion of *bd3460* resulted in a *B. bacteriovorus* strain that was able to attach to prey cell in normal manner of wild type but, upon invasion, this strain started to decrosslink its self-peptidoglycan and rounded up itself with lose of predatory ability and death (Figure 1-12) (Lambert et al. 2015). This meant that *bd3460* gene product acts as an antidote for endopeptidase enzymes that modify prey cell wall prey wall which is secreted by *Bdellovibrio* into its own periplasm to protect its self-peptidoglycan from decrosslinking

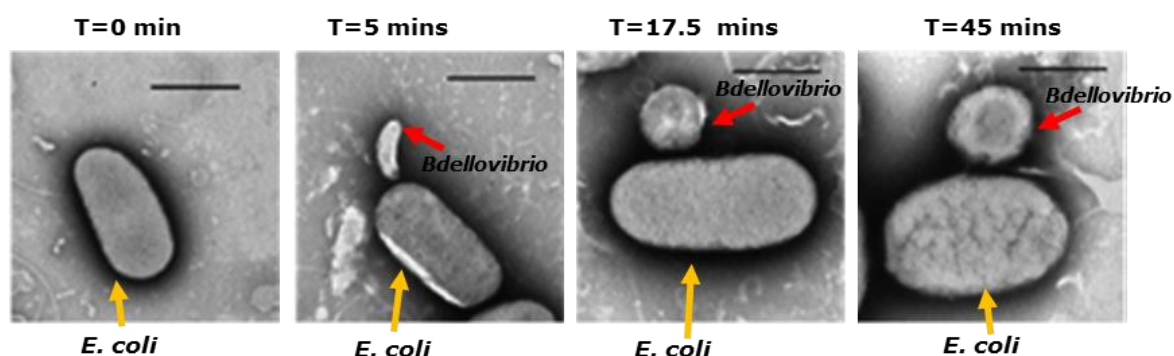


Figure 1. 14: Electron micrographs showing the attachment ability of *B. bacteriovorus* *bdΔ3460* strain in different stages. *bdΔ3460* strain have the ability to find the prey cell and attach to prey cell but then, up on invasion, *Bdellovibrio* adopt a round morphology. The images were taken from (Lambert et al. 2015).

The highest expression of DAP-incorporating enzyme activity was shown in the first 60 mins of predation during attack and was proposed to solidify the wall of the bdelloplast (Ruby and Rittenberg 1984). All these early reports indicate that *Bdellovibrio* have the ability to regulate the synthesis of enzyme(s) in the bdelloplast which is external to themselves. DAP attachment is considered in these papers as an example of the ability of *Bdellovibrio* to modify biosynthetically the prey cell wall (Ruby and Rittenberg 1984). It will become clear that a m-DAP synthesis gene of *Bdellovibrio* was one of the potential suppressing genes studied in this thesis.

1.13 Aspartokinase III enzyme and DAP synthesis

Aspartokinase enzyme is a key enzyme in the aspartate pathway that directly participates in the synthesis of lysine, threonine, methionine and isoleucine. One of the essential metabolic products of the aspartate pathway is *meso*-Diaminopimelic acid (m-DAP), so this pathway (Figure 1-12) play a role in m-DAP/Lysine synthesis pathway that might be used by *Bdellovibrio*, to add to the prey wall (or even to its own wall). Lysine is a crucial amino acid for protein synthesis, and lysine originates from a *meso*-Diaminopimelic acid precursor

(Figure 1-12). Moreover, both *meso*-Diaminopimelic and lysine also have an important role in the synthesis of peptidoglycan in all bacteria by cross linking chains of glycan to give rigidity and strength for the cell wall of bacteria (van Heijenoort 2001, Viola 2001). The questions being asked here is about synthesising DAP for self and bdelloplast peptidoglycan.

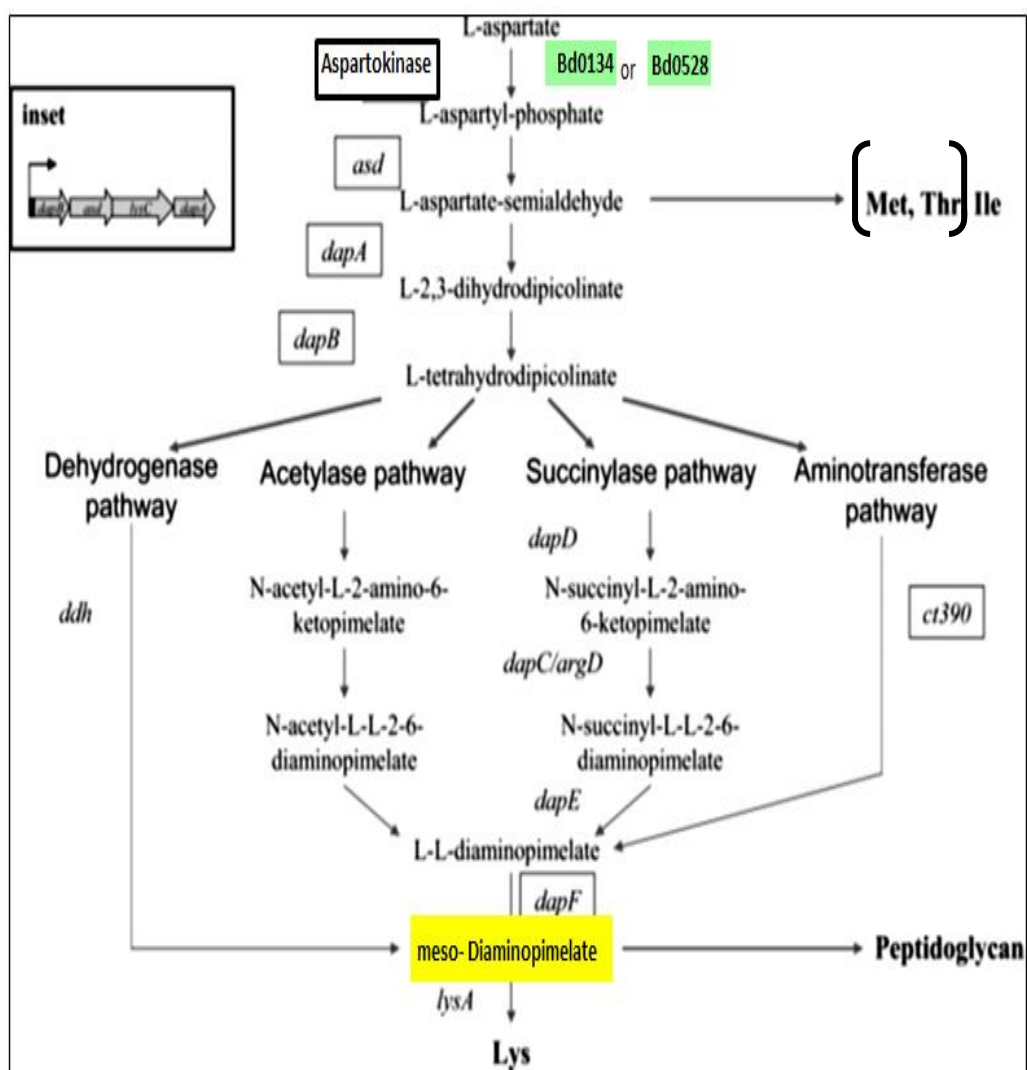


Figure 1. 15: The diagrammatic scheme of the pathway of lysine synthesis in plants and bacteria. The pathway taken from (McCoy et al. 2006) with my addition of the *Bdellovibrio* enzymes and deletion of the Met and Thr pathways which are not present in *Bdellovibrio* (Rendulic et al 2004).

Aspartokinase (ASK) is one of the enzymes that required for the cell wall synthesis in bacteria and plants, and as a family divided into three enzymes types: ASKI,

ASKII and ASKIII. Both ASKI and ASKII enzymes are bifunctional enzymes that have aspartokinase and homoserine dehydrogenase activities (ASKI-HDHI and ASKII-HDHII) and they are encoded by the *thrA* gene and the *metL* gene respectively (Katinka et al. 1980, Zakin et al. 1983).

ASKIII enzyme has aspartokinase activity only that does not show homoserine dehydrogenase activity, and it encoded by the *lysC* gene. It is a dimeric enzyme with 4.8 KD subunit that contains two N and C-terminal domains (Cassan et al. 1986, Ramon-Maiques et al. 2002). The catalytic activity of ASKIII leads to the combination of L-aspartate with ATP and converting to 4-phospho-L-aspartate (Lo et al. 2009). The N-terminal domain shows catalytic activity, and near its end, there is a highly conserved motif which has three variants. These are KFGG, KYGG and KIGG, while the C- terminal domain has an inhibitory effect on lysine synthesis by this enzyme (Cassan et al. 1986, Patte 1996).

All ASK enzymes have two adjacent domain copies ACT1 and ACT2 that merged with the catalytic domain of AK enzyme. Each ACT domain contains numbers of conserved domain (cd) signatures in the database that help in the allosteric specificity prediction. ACT domains are difficult to find by straightforward sequence homology (Liberles et al. 2005, Grant 2006, Marchler-Bauer et al. 2007).

ASK enzyme subhomology is divided into ASK α and ASK β and both contain an ASK domain followed by two ACT domains ACT-1 and ACT-2. The greater numbers of cd signatures are found in the ASK β subsection. The ASK α subhomology group are the ancient/ ancestral grouping which includes all Archaea, Eukarya and many bacterial ASKs also belong to the ASK α group (Curien et al. 2008, Lo et al. 2009).

1.14 Aspartokinase enzymes in *B. bacteriovorus*

The genome analysis of *B. bacteriovorus* HD100 revealed that *B. bacteriovorus* HD100 has gene for potentially possess two Aspartokinase enzymes. These are ASK α (Bd134) and ASK β (Bd0528) (Rendulic et al. 2004, Lo et al. 2009). So in

this section, the general features of them are explained and in Chapter 5, the function of them in the both lifestyles of *Bdellovibrio* is clarified.

1.14.1 Lysine sensitive Aspartokinase Bd0134 ASK α of *Bdellovibrio*

One type of ASK enzyme in *Bdellovibrio bacteriovorus* HD 100 is ASK α Bd0134 that contains an aspartokinase domain and catalytic/substrate-binding residue (Figure 1-13). The type of ASK ACT is categorized as having the conserved domain cd04258. It has two adjacent ACT domains at the C- terminus ACT-1/ ACT-2. ACT-1 contains cd04932 and ACT-2 contains cd04917. Furthermore, ASK enzyme has indel region, which is approximately 50 amino acid residues. The site of this indel region's proximity of 70 residues downstream of the N- terminal and it is placed between catalytic residues as seen in Figure (1-13) (Lo et al. 2009).

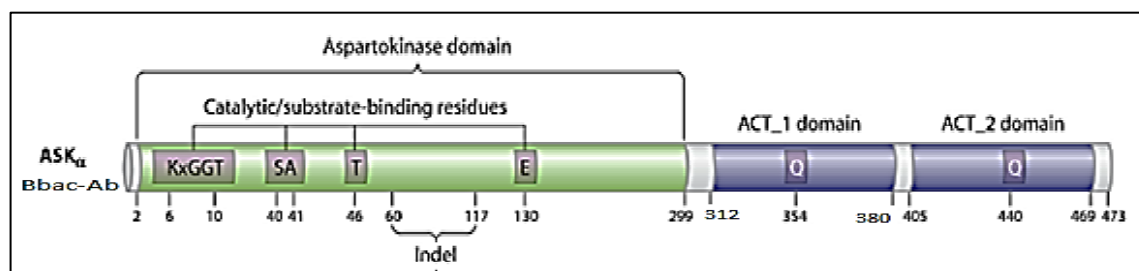


Figure 1. 16: The schematic diagram showed the components of *B. bacteriovorus* ASK α enzyme.Adapted from (Lo et al. 2009)

ASK α of *Bdellovibrio bacteriovorus* has a KXGGT motif in which (K) comprises the catalytic residue and (GGT) comprise the three substrate-binding residues for ATP. In addition, it has a Q...Q motif which contains a Q354 for glutamine residue near the midpoint of the ACT-1 domain and Q440 a glutamine residue near the midpoint of the ACT-2 domain which shows allosteric regulation for catalytically important threonine. The ACT-1 Q354 residue is located in the high-affinity region and it is vital for inhibiting threonine of ASK, whereas the ACT-2 Q440 residue is found in the low-affinity region. The presence of a Q...Q motif signature between

ACT-1 and ACT-2 region means that there is feedback inhibition by threonine on ASK activity (Xie, Keyhani et al. 2003, Lo, Bonner et al. 2009). ASK α of *B. bacteriovorus* is encoded by *lysC* gene (Aspartate kinase III) and more details about this found in chapter 5 (Lo et al. 2009). As mentioned before *Bdellovibrio* has to modify self- peptidoglycan with m-DAP during cell growth, and may act on prey bdelloplast peptidoglycan. Most bacteria have one aspartokinase gene, but *Bdellovibrio* has two aspartokinase genes (*bd0134*, *bd0528*) (Figure 1-15).

1.14.2 ASK α evolution in *B. bacteriovorus* lateral genealogy of Bd0134

Bdellovibrio acquired ASK α gene from a lower *Gammaproteobacteria* donor that belongs to cohesion group -21 (CG-21) named by Lo and co-workers (Lo et al. 2009). In lower *Gammaproteobacteria*, the 17 genomes sequenced of *Enterobacterales* encode a lysine-specialized ASK that that they are thought to have received via lateral gene transfer (LGT) from a CG-11 source in a *Bacteriodes* family (Figure 1-17). The recipient lineage then diverged to give CG-21 members. In lower *Gammaproteobacteria*, ASK α (*lysC*) is a monofunctional ASK (for the lysine DAP pathway) that belongs to CG-21 (Lo et al. 2009).

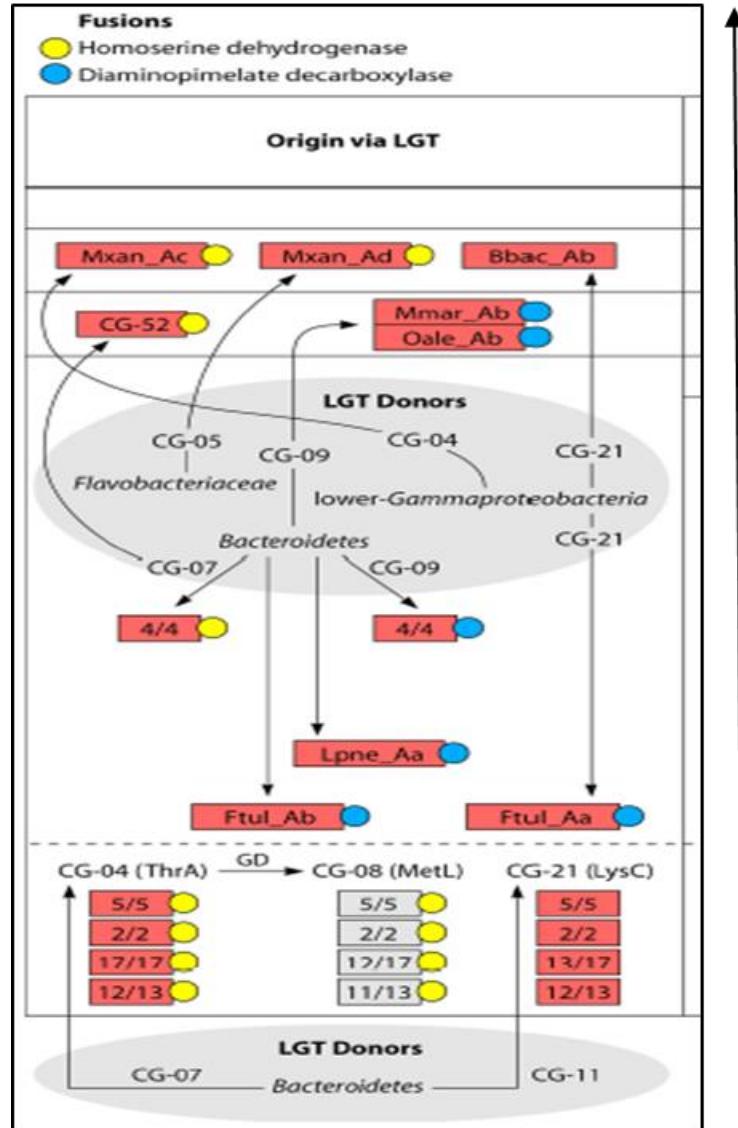


Figure 1. 17: The schematic events of lateral gene transfer within the vertical genealogy for *bd0134 ASK α* (*Bbac Ab*) evolution of *B. bacteriovorus*. Reproduced from (Lo et al. 2009).

1.14.3 Alternative Aspartate kinase Bd0528 of *B. bacteriovorus*

One, other type of ASK enzyme, encoded by a predicted ancestral gene, in *B. bacteriovorus* is ASK β (Bd0528) that contains an aspartokinase domain and catalytic/ substrate binding residues. ASK β is encoded by *lysC* (*bd0528*) gene. This Bd0528 enzyme is predicted by KEGG (Genomes 2013) to catalyze the combination of ATP with L-aspartate. Lo and coworkers define it as coming from a different cohesion group CG-27 (Lo et al. 2009).

Bd0528 (ASK β) is a possible alternative first enzyme to Bd0134 in the pathway (Figure 1-12). ASK β domain has two ACT domains (Figure 1-16), ACT-1 contains cd04261 and ACT-2 contains cd04891. ASK β does not contain indel region and it is split by about 25 residues (T47 and E74). ASK β of *B. bacteriovorus* has a KYGG motif (Figure 1-15) (Lo et al. 2009).

The ACT-1 (cd04891), which is at the C-terminus, contains a homologous position for glutamine residues that were previously reported to be inhibited by threonine or threonine plus lysine (*Thr plus Lys*) this is the Q motif (Q298). The ACT-2 domain (cd04261) found in the N-terminus—does not possess the Q residues due to a very small deletion (Lo et al. 2009). ASK β is typified by presence of cd04261 for a functional aspartokinase domain (Datta and Prakash 1966).

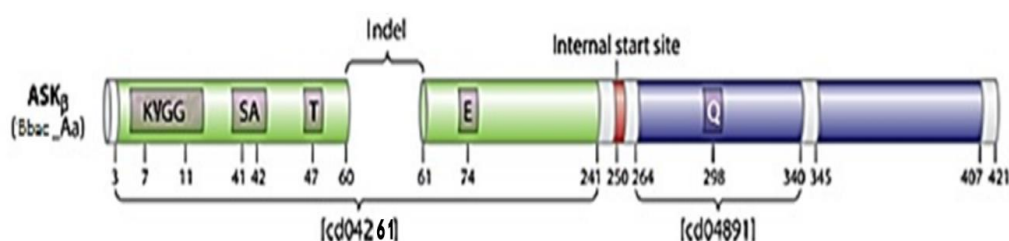


Figure 1. 18: The schematic diagram showed the components of *B. bacteriovorus* ASK β enzyme. Adapted from (Lo, et al. 2009).

1.14.4 Vertical genealogy of bd0528 for ASK β in *B. bacteriovorus*

Gene *bd0528* (ASK β) of *B. bacteriovorus* is in an ancestral gene grouping CG-27 and this belongs to a genealogy studied by Lo, Bonner and coworkers (Lo et al. 2009). They showed that *deltaproteobacteria* (such as *Bdellovibrio*) has ancestral ASK orthologs found common to sixteen genomes, that divided into three cohesion groups and two orphans. These are CG-27, CG-37, CG-36, Mxan- Ab and Sfum-Aa. This relatedness suggests that all genes for this type of enzyme evolved from an ancestral ancestor, and that possibly Bd0528 may be a “core enzyme” required for “self” DAP synthesis for housekeeping of the “own” cell walls

of *B. bacteriovorus*. No recognizable cd hit of the CDD found in twin ACT domain regions of *Bacillus* like CG-27 ASK enzymes are found in the Bd0528 family enzymes so Bd0528 would not encode a secondary C terminal only extra enzyme due to an secondary translational start, but would encode a potential functional ASK alternative enzyme to Bd0134. (Lo et al. 2009). So part of my project is testing which one of both aspartokinase (Bd0134 and Bd0528) that *Bdellovibrio* have is core enzyme and if both of them have a predatory role or not.

1.15 Overall research aims of this study

The overall aims of my PhD research were to understand the hidden cross talk that allowed genetic suppression between diguanylate cyclase genes allowing *Bdellovibrio* to become a selective predator on human pathogens and who this hidden cross talk related with some DAP/Lysine pathway genes that is involved in cell wall synthesis.

Specifically my thesis concerned with six themes:

Chapter 3: screening the possibility of suppression occurrences in genome of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain and what genome sequence tell about suppressor strains:

- To investigate the ability of occurrence of suppression mutation in genome of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain upon incubation with different human pathogens and if the suppression also occur on prey lawns plates with all types cell of prey.
- Test if deletion of *bd0742* affected the attachment ability of HI *B. bacteriovorus* $\Delta bd0742$ mutant stain to prey cells and discuss what the genome sequencing of suppressor strains discovered.

Chapter 4: Evaluation the impact of point mutation in *bd0467* discovered by genome sequencing data of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain

- Test the effect of the point mutation in *bd0467* gene through addressing the function and the effect of deletion of this gene on predation ability and plaque formation of *B. bacteriovorus* HD100.

Chapter 5: Evaluation of the impact of a point mutation in *bd0134* that was discovered by genome sequencing data in some $\Delta bd0742$ suppressor strains and its relationship with cell wall synthesis

Aspartokinase is a Key enzymes in DAP/lysine pathway that involved in cell wall synthesis but *B. bacteriovorus* has two genes encoding the Aspartokinase instead of one (*bd0134* and *bd0528*). The genome sequencing data of some $\Delta bd0742$ suppressor strains showed that there was point mutation in *bd0134*, therefore the main aims in this chapter are:

- To examine the expression, location and function of *bd0134* and *bd0528* in both *Bdellovibrio* lifestyle and the effect of deletion of these genes on the predatory life cycle of *B. bacteriovorus* HD100.
- To evaluate the effect of the point mutation in *bd0134* on the predation ability of *B. bacteriovorus* HD100 and plaques formation on prey lawn plate.
- To test the effect of addition of extra copy of the WT*bd0134* on restoring plaque formation ability of HI *B. bacteriovorus* $\Delta bd0742$ deletion mutant and $\Delta bd0742$ suppressor strains.

Chapter 6: Investigating some of the DAP/Lysine synthesis pathway genes and find out their relationship with PG modification during *Bdellovibrio* invasion.

- To investigate the function of further m-DAP Bd0047 (DapB) and Bd0129 (DapE) genes in the *B. bacteriovorus* lifecycle.
- To assess whether Bd0047 and Bd0129 genes are involved in predation *Bdellovibrio*

- To test the effect of deletion of Bd0129 on the predatory lifecycle of *Bdellovibrio* if the deletion is possible in the presence and absences of external source of DAP.
- To discuss what the genome sequences discovered in genome of HI *B. bacteriovorus* *bd0129-mcherry* strain.

Chapter 7: screening the effect of suppression on predation ability of *B. bacteriovorus* Δ bd0742 suppressor strains

The strain of HI *B. bacteriovorus* Δ bd0742 mutant strain that have the suppression in genome in *bd3766* were named as a Δ bd0742 suppressor strains

- To investigate the effects of point mutations in *bd3766* on ability Δ bd0742 suppressor strain to prey on different human pathogens
- To assess if the point mutations in *bd3766* of Δ bd0742 suppressor strains led to restoration of the plaque formation ability on prey lawn plate as well as in liquid culture for some types of prey, investigate whether these point mutations in *bd3766* produced more or less c-di-GMP.
- To assess if the point mutations in the Δ bd0742 suppressor strains able to restored the phenotypes that resulted from deletion of *bd1434* and *bd0367*.

Chapter 8: PG modification during *Bdellovibrio* invasion a role for CpoB in sealing of a prey entry pore.

- To investigate the function of *cpoB_{Bd}* (*bd0635*) gene in the *B. bacteriovorus* genome.
- To assess whether if *cpoB_{Bd}* (*bd0635*) are involved in *Bdellovibrio* entry pore sealing.
- To test the effect of deletion of *cpoB_{Bd}* (*bd0635*) on the predatory lifecycle of *Bdellovibrio* if the deletion is possible.

2 Materials and Methods

2.1 Strains, plasmids and primers used

The strains of *E. coli* that used to generated the plasmid constructs is *Escherichia coli* DH5α which after that transformed into *Escherichia coli* S17-1 that used for *B. bacteriovorus* HD100 conjugation.

2.2 The bacterial strains that used in this study

Strains	Description	Confirmation	Reference / Source
<i>Escherichia coli</i>			
DH5α	F ⁻ <i>endA1 hsdR17</i> (r _k ⁻ mk ⁻) <i>supE44 thi-1 recA1 gyrA</i> (Nal ^r) <i>relA1 Δ</i> (lacIZYA-argF) U169 <i>deoR</i> (80dlacΔ(lacZ)M15); used as a cloning strain	N/A	(Hanahan 1983)
S17-1	<i>thi,pro,hsdR⁻,hsdM⁺,recA</i> ; integrated plasmid RP4-Tc::Mu-Kn::Tn7; used as donor for conjugating plasmids into <i>B. bacteriovorus</i>	N/A	(Simon R. 1983)
S17-1 (pZMR100)	S17-1 strain containing pZMR100 plasmid used to confer Kan ^R ; used as Kan ^R prey for <i>Bdellovibrio</i> .	N/A	(Rogers et al. 1986)
K-12 MG1655	F ⁻ <i>lambda- ilvG⁻ rfb-50 rph-1</i> (K12 derivative); Used for swimming motility test	N/A	(Bachmann 1972)
<i>B. bacteriovorus</i> strains			
HD100	Wild-Type strain, genome sequenced.	N/A	(Stolp and Starr 1963)
HID13	Host-independent derivative of HD100	N/A	(Lambert et al. 2010)
HID2	Host-independent derivative of HD100	N/A	(Lambert et al. 2010)
HID22	Host-independent derivative of HD100	N/A	(Lambert et al. 2010)
HID26	Host-independent derivative of HD100	N/A	(Lambert et al. 2010)

HD100 <i>bd0047</i> -mCherry	HD100 strain carrying integrated plasmid pK18:: <i>bd0047</i> -mCherry at the <i>bd0528</i> locus.	Gen PCR	This study
HD100 <i>bd0129</i> -mCherry	HD100 strain carrying integrated plasmid pK18:: <i>bd0129</i> -mCherry at the <i>bd0528</i> locus.	Gen PCR	This study
HD100 <i>bd0134</i> -mCherry	HD100 strain carrying integrated plasmid pK18:: <i>bd0134</i> -mCherry at the <i>bd0528</i> locus.	Gen PCR	This study
HD100 <i>bd0528</i> -mCherry	HD100 strain carrying integrated plasmid pK18:: <i>bd0528</i> -mCherry at the <i>bd0528</i> locus.	Gen PCR	This study
HD100 <i>bd0467</i> -mCherry	HD100 strain carrying integrated plasmid pK18:: <i>bd0467</i> -mCherry at the <i>bd0528</i> locus.	Gen PCR	This study
HD100 <i>bd0635</i> -mCherry	HD100 strain carrying integrated plasmid pK18:: <i>bd0635</i> -mCherry at the <i>bd0528</i> locus.	Gen PCR	Rob Till
HD100 <i>bd0064</i> -GFP (<i>bd0635</i> -mCherry)	HD100 <i>bd0064</i> -GFP double cross over strain carrying integrated plasmid pK18:: <i>bd0635</i> -mCherry at the <i>bd0635</i> locus	Gen PCR	This study
HD100 <i>bd0047</i> -mCherry HI	Host-independent derivative of HD100 <i>bd0134</i> -mCherry	N/A	This study
HD100 <i>bd0129</i> -mCherry HI	Host-independent derivative of HD100 <i>bd0134</i> -mCherry	N/A	This study
HD100 <i>bd0528</i> -mCherry HI	Host-independent derivative of HD100 <i>bd0528</i> -mCherry	N/A	This study
HD100 <i>bd0134</i> -mCherry HI	Host-independent derivative of HD100 <i>bd0134</i> -mCherry	N/A	This study
HD100 <i>bd0467</i> -mCherry HI	Host-independent derivative of HD100 <i>bd0467</i> -mCherry	N/A	This study
HD100 <i>bd0635</i> -mCherry HI	Host-independent derivative of HD100 <i>bd0635</i> -mCherry	N/A	This study
HD100 Δ <i>bd0134</i>	HD100 with <i>bd0134</i> markerless Deletion	Gen PCR, RT-PCR, Seq	This study
HD100 Δ <i>bd0134</i> HI	Host-independent derivative of HD100 with <i>bd0134</i> markerless Deletion	N/A	This study

HD100 <i>bd0134</i> Δ <i>bd0134</i>	HD100 Δ <i>bd0134</i> strain carrying integrated plasmid pK18:: <i>bd0134</i> .	Gen PCR, Seq	This study
HD100 <i>bd134-T355I</i>	HD100 with <i>bd0bd134-T355I</i> double cross over	Gen PCR	This study
HD100 Δ <i>bd0467</i> HI	Host-independent derivative of HD100 with <i>bd0467</i> markerless Deletion	N/A	This study
HD100 Δ <i>bd0742</i> HI	Host-independent derivative of HD100 with <i>bd0742</i> markerless deletion carrying integrated plasmid pK18:: <i>bd0134</i> .	WGen seq	(Hobley et al. 2012)
HD100 Δ <i>bd0742</i> HI (<i>WTbd0134</i>)	Host-independent derivative of HD100 with <i>bd0742</i> markerless deletion strain carrying integrated plasmid pK18:: <i>bd0134</i>	Gen PCR	This study
<i>742 suppressor HD1</i>	Host-dependent derivative of HI <i>bd0742</i> markerless deletion contain point mutations in T355I, R135G and LL439L of <i>bd0134</i> , <i>bd0467</i> and <i>bd3766</i> respectively.	WGen seq	Simon King
<i>742 suppressor HD2</i>	Host-dependent derivative of HI <i>bd0742</i> markerless deletion contain point mutations in T355I, R135G and LL439L of <i>bd0134</i> , <i>bd0467</i> and <i>bd3766</i> respectively.	WGen seq	Simon King
<i>742 suppressor HD3</i>	Host-dependent derivative of HI <i>bd0742</i> markerless deletion contain point mutation in R135G of <i>bd0467</i> and point mutations in F394C and LL439L of and <i>bd3766</i> .	WGen seq	David Milner
<i>742 suppressor HD4</i>	Host-dependent derivative of HI <i>bd0742</i> markerless deletion contain point mutation in R135G of <i>bd0467</i> and point mutations in F394C and LL439L of and <i>bd3766</i> .	WGen seq	David Milner

742 suppresor HD5	Host-dependent derivative of HI <i>bd0742</i> markerless deletion contain point mutation in R135G of <i>bd0467</i> and point mutations in F394C and LL439L of and <i>bd3766</i> .	WGen seq	David Milner
742 suppresor HD6	Host-dependent derivative of HI <i>bd0742</i> markerless deletion contain point mutations in R135G and Q429L in <i>bd0467</i> and <i>bd3766</i> .	WGen seq	This study
742 suppresor HD7	Host-dependent derivative of HI <i>bd0742</i> markerless deletion contain point mutations in R135G and Q429L in <i>bd0467</i> and <i>bd3766</i> .	WGen seq	This study
742 (WT <i>bd3766-mcherry</i>)	Host-independent derivative of HD100 with <i>bd0742</i> markerless deletion strain carrying integrated plasmid pK18:: <i>WTbd3766-mcherry</i> .	Gen PCR	This study
742 (<i>bd3766-F394C-mcherry</i>)	Host-independent derivative of HD100 with <i>bd0742</i> markerless deletion strain carrying integrated plasmid pK18:: <i>bd3766-F394C-mcherry</i> .	Gen PCR	This study
742 (<i>bd3766-Q429L-mcherry</i>)	Host-independent derivative of HD100 with <i>bd0742</i> markerless deletion strain carrying integrated plasmid pK18:: <i>bd3766-Q429L-mcherry</i> .	Gen PCR	This study
742 (<i>bd3766-LL439L-mcherry</i>)	Host-independent derivative of HD100 with <i>bd0742</i> markerless deletion strain carrying integrated plasmid pK18:: <i>bd3766-LL439L-mcherry</i> .	Gen PCR	This study
Human pathogen			
<i>Acinetobacter baumannii</i> TCDC-AB0715	Clinical isolate (plate F1865) of <i>A. baumannii</i> identified from 16S rDNA sequencing used as prey.	Gift from Dr W. Ashraf and Dr. R Bayston, University of Nottingham	

<i>Pseudomonas putida</i> UWC1	Saprophytic soil organism; PGPR	(McClure et al. 1989)
<i>Serratia marcescens</i> ATCC-274	Wild type strain	(Gargallo et al. 1987)
<i>S. typhimurum</i> SJW1103	Wild type strain	(Yamaguchi et al. 1984)

Table 2-1: showed the bacterial strains that used in this study.

Abbreviations: HD100 = *B.bacteriovorus* HD100; Gen PCR = PCR was carried out using genomic DNA isolated from the strain to observe the expected band shift resulting from a gene deletion; RT-PCR = RT-PCR was carried using RNA isolated from the strain to confirm gene deletion by absence of the transcript of gene; Seq = genomic sequencing across integrated construct; WGen seq= the strain was sent for whole genome sequencing; Kn-selection= strain was selected for growth with kanamycin at a concentration of 50 µg ml⁻¹ in the growth medium.

2.3 The plasmids and plasmid construct used in this study

Plasmid	Description	Confirmation	Source
pK18mobsacB	Suicide vector (in <i>Bdellovibrio</i>) used from conjugation and recombination into the <i>B. bacteriovorus</i> genome, Kn ^R	N/A	(Schafer et al. 1994)
pAKF04-mcherry	Cloning vector, Amp ^R	N/A	(Fenton et al. 2010)
pAKF56-mcherry	Cloning vector, Amp ^R	N/A	(Fenton et al. 2010)
pAKF04::b b4024	The <i>b04024</i> ORF was amplified from <i>E. coli</i> genomic DNA using primers B4024tag-F and B4024tag-R and the PCR product then ligated into pAKF04 cut with EcoRI and SacI. This placed the B4024 ORF (minus stop codon) upstream of the mTFP ORF in the plasmid.	DD	This study
pAKF56::b0031	The <i>b00031</i> ORF was amplified from <i>E. coli</i> genomic DNA using primers B0031tag-F and Bd00031tag-R and the PCR product then ligated into pAKF56 cut with EcoRI and KpnI. This placed the B0031 ORF (minus stop codon) upstream of the mCherry ORF in the plasmid.	N/A	This study
pAKF56::b2472	The <i>b2472</i> ORF was amplified from <i>E. coli</i> genomic DNA using primers Bd02472tag-F and Bd02472 tag-R and the PCR product then ligated into pAKF56 cut with SacI and KpnI. This placed the B2472 ORF (minus stop codon) upstream of the mCherry ORF in the plasmid.	N/A	This study
pAKF56::bd0047	The <i>bd0047</i> ORF was amplified from <i>B. bacteriovorus</i> HD100 genomic DNA using primers Bd0047tag-F and Bd0047tag-R and the PCR product then ligated into pAKF56 cut with EcoRI and KpnI. This placed the Bd0047 ORF (minus	N/A	This study

	stop codon) upstream of the mCherry ORF in the plasmid.		
pAKF56::bd0129	The <i>bd0047</i> ORF was amplified from <i>B. bacteriovorus</i> HD100 genomic DNA using primers Bd0129tag-F and Bd0129tag-R and the PCR product then ligated into pAKF56 cut with EcoRI and KpnI. This placed the Bd0129 ORF (minus stop codon) upstream of the mCherry ORF in the plasmid.	N/A	This study
pAKF56::bd0134	The <i>bd0047</i> ORF was amplified from <i>B. bacteriovorus</i> HD100 genomic DNA using primers Bd0134tag-F and Bd0134tag-R and the PCR product then ligated into pAKF56 cut with EcoRI and KpnI. This placed the Bd0134 ORF (minus stop codon) upstream of the mCherry ORF in the plasmid.	DD	This study
pAKF56::bd0528	The <i>bd0047</i> ORF was amplified from <i>B. bacteriovorus</i> HD100 genomic DNA using primers Bd0134tag-F and Bd0134tag-R and the PCR product then ligated into pAKF56 cut with EcoRI and KpnI. This placed the Bd0047 ORF (minus stop codon) upstream of the mCherry ORF in the plasmid.	DD	This study
pAKF56::bd0635	The <i>bd0635</i> ORF was amplified from <i>B. bacteriovorus</i> HD100 genomic DNA using primers Bd0635tag-F and Bd0635tag-R and the PCR product then ligated into pAKF56 cut with EcoRI and KpnI. This placed the Bd0635 ORF (minus stop codon) upstream of the mCherry ORF in the plasmid.	DD	Constructed by Sam Greenwood
pK18::bd0047-mCherry	The Bd0047-mCherry fragment was cut out of the pAKF56::bd0047 plasmid using EcoRI and XbaI and ligated into pK18 <i>mobsacB</i> using the same enzymes.	DD	This study
pK18::bd0129-mCherry	The Bd00129-mCherry fragment was cut out of the pAKF56::bd0129 plasmid using EcoRI and XbaI and ligated into pK18 <i>mobsacB</i> using the same enzymes.	DD	This study
pK18::bd0134-mCherry	The Bd0134-mCherry fragment was cut out of the pAKF56::bd0134 plasmid using EcoRI and XbaI and ligated into pK18 <i>mobsacB</i> using the same enzymes.	DD	This study
pK18::bd0528-mCherry	The Bd0528-mCherry fragment was cut out of the pAKF56::bd0528 plasmid using EcoRI and XbaI and ligated into pK18 <i>mobsacB</i> using the same enzymes.	DD	This study
pK18::bd0635-mCherry	The Bd0635-mCherry fragment was cut out of the pAKF56::bd0635 plasmid using EcoRI and XbaI and ligated into pK18 <i>mobsacB</i> using the same enzymes.	DD	Rob Till
pK18::bd0467-mCherry	The Bd0467 ORF was amplified from <i>B. bacteriovorus</i> HD100 genomic DNA using primers Bd0467gib-F and Bd0467gib-R and mCherry ORF was amplified from pKFA56 plasmid using	Seq	This study

	primers mcherrygib-F and mcherry gib-R. Both of these PCRs product were ligated with pK18mobsacB plasmid cut with EcoRI, HindIII using isothermal Gibson enzymes to join overlapping regions.		
pUC19:: <i>Δbd0134</i>	The deletion construct was generated using primers bd0134-F and <i>Δbd0134_R</i> to create the 5' fragment, and <i>Δbd0134-F</i> and bd0134_R to create the 3' fragment. These two fragments were joined using binding PCR. This PCR product was cut with BamHI and HindIII and ligated into pUC19.	DD	This study
pK18:: <i>Δbd0134</i>	The pUC19:: <i>Δbd0134</i> plasmid was cut with BamHI and HindIII and the knockout construct fragment was ligated into pK18mobsacB which had been cut with the same enzymes.	DD	This study
pK18:: <i>Δbd0528</i>	The deletion construct was generated using primers bd0528-F and <i>Δbd0528_R</i> to create the 5' fragment, and <i>Δbd0528-F</i> and bd0528_R to create the 3' fragment. These two fragments were joined using binding PCR. This PCR product was cut with EcoRI and HindIII and ligated into pK18mobsacB.	DD	This study
pK18:: <i>Δbd0129</i>	The deletion construct was generated using primers bd0129-F and <i>Δbd0129_R</i> to create the 5' fragment, and <i>Δbd0129-F</i> and bd0129_R to create the 3' fragment. These two fragments were joined using binding PCR. This PCR product was cut with EcoRI and XbaI and ligated into pK18mobsacB.	DD	This study
pK18:: <i>Δbd0467</i>	The deletion construct was generated using primers bd0467 gib-F and <i>Δbd0467 gib-R</i> for amplify 1kb upstream of <i>bd0467</i> and <i>Δbd0467 gib-F</i> and bd0467 gib-R for amplify 1Kb downstream the gene. Both of these PCRs product were ligated with pK18mobsacB plasmid cut with EcoRI, HindIII using isothermal Gibson enzymes to join overlapping regions.	Seq	This study
pK18:: <i>Δbd0635</i>	The deletion construct was generated using primers bd0635gib-F and <i>Δbd0635_R</i> for amplify 1kb upstream of <i>bd0635</i> and <i>Δbd0635_F</i> and bd0635gib-F for amplify 1Kb downstream the gene. Both of these PCRs product were ligated with pK18mobsacB plasmid cut with EcoRI, HindIII using isothermal Gibson enzymes to join overlapping regions.	Seq	This study
pK18:: <i>bd0134</i>	pK18mobsacB suicide plasmid containing approximately 1 Kb of 5'- and 100 bp of 3'-flanking genomic DNA from around <i>bd0134</i>	DD	This study
pK18:: <i>WTbd3766-mCherry</i>	The WT Bd3766 ORF was amplified from the original <i>B. bacteriovorus Δ bd0742 deletion strain</i> genomic DNA using primers Bd3766gib-F and Bd3766gib-R and mCherry ORF was amplified from pKFA56 plasmid using primers mcherrygib-	Seq	This study

	F and mcherry gib-R. Both of these PCRs product were ligated with pK18 <i>mobsacB</i> plasmid cut with EcoRI, HindIII using isothermal Gibson enzymes to join overlapping regions.		
pK18:: <i>bd3766-F394C-mCherry</i>	The Bd3766-F394C ORF was amplified from 742 suppressor <i>Bd3766-F394C</i> genomic DNA using primers Bd3766gib-F and Bd3766gib-R and mCherry ORF was amplified from pKFA56 plasmid using primers mcherrygib-F and mcherry gib-R. Both of these PCRs product were ligated with pK18 <i>mobsacB</i> plasmid cut with EcoRI, HindIII using isothermal Gibson enzymes to join overlapping regions.	Seq	This study
pK18:: <i>bd3766-F394C-mCherry</i>	The Bd3766-F394C ORF was amplified from 742 suppressor <i>Bd3766-F394C</i> genomic DNA using primers Bd3766gib-F and Bd3766gib-R and mCherry ORF was amplified from pKFA56 plasmid using primers mcherrygib-F and mcherry gib-R. Both of these PCRs product were ligated with pK18 <i>mobsacB</i> plasmid cut with EcoRI, HindIII using isothermal Gibson enzymes to join overlapping regions.	Seq	This study
pK18:: <i>bd3766-Q429L-mCherry</i>	The Bd3766-F394C ORF was amplified from 742 suppressor <i>Bd3766-Q429L</i> genomic DNA using primers Bd3766gib-F and Bd3766gib-R and mCherry ORF was amplified from pKFA56 plasmid using primers mcherrygib-F and mcherry gib-R. Both of these PCRs product were ligated with pK18 <i>mobsacB</i> plasmid cut with EcoRI, HindIII using isothermal Gibson enzymes to join overlapping regions.	Seq	This study
pK18:: <i>bd3766-LL439L-mCherry</i>	The Bd3766-F394C ORF was amplified from 742 suppressor <i>Bd3766-LL439L</i> genomic DNA using primers Bd3766gib-F and Bd3766gib-R and mCherry ORF was amplified from pKFA56 plasmid using primers mcherrygib-F and mcherry gib-R. Both of these PCRs product were ligated with pK18 <i>mobsacB</i> plasmid cut with EcoRI, HindIII using isothermal Gibson enzymes to join overlapping regions.	Seq	This study

Table 2-2: Plasmids and plasmid construct used in this study.

Abbreviations: HD100 = *B. bacteriovorus* HD100; Kn^R = kanamycin resistance; Amp^R = ampicillin resistance; DD = plasmid confirmed by diagnostic digests using restriction endonucleases; Seq = plasmid confirmed by sequencing.

2.4 The primers used in this study

Primer	Description	Sequence (5'-3')
RT-PCR primers		
bd0528 RT-F	Primers to amplify a 110 bp fragment of HD100 <i>bd0528</i> ORF.	GACCCAACATCGTGAAATC
bd0528 RT-R		AGTTCCACCGAGCGATAGTG
bd0134 RT-F	Primers to amplify a 98 bp fragment of HD100 <i>bd0528</i> ORF.	GCTTGTGACCTTTCCACTC
bd0134 RT-R		TCGCGTCGATACTGACTTTG
bd0529 RT-F	Primers to amplify a 88 bp fragment of HD100 <i>bd0528</i> ORF.	AAGTCCACCCACGTTCAATC
bd0529 RT-R		GGAAGTAGGCGATCCACTGA
bd0046 RT-F	Primers to amplify a 96 bp fragment of HD100 <i>bd0528</i> ORF.	ATTTCAAAGGCACGTTCAACC
bd0046 RT-R		CAGCCAGTTGTTGCTTCAGA
bd0047 RT-F	Primers to amplify a 102 bp fragment of HD100 <i>bd0528</i> ORF.	TGTGGTCTTCGAACATGAGC
bd0047 RT-R		GGTGGATTTCCTCAATCTGG
bd0129 RT-F	Primers to amplify a 114 bp fragment of HD100 <i>bd0528</i> ORF.	ACCACTCACGGCAATAAAG
bd0129 RT-R		ACATTGCGCTGTCAAGGTC
bd0127 RT-F	Primers to amplify a 92 bp fragment of HD100 <i>bd0528</i> ORF.	TATTCACCTGATGGGCCACT
bd0127 RT-R		GCTGCAAGTTCCTTGAGTC
danKRT-F	Primers to amplify a 90 bp fragment of HD100 <i>bd0528</i> ORF.	TGAGGACGAGATCAAACGTG
danKRT-R		AAACCAGGTTGTCGAGGTTG
bd0467RT-F	Primers to amplify a 112 bp fragment of HD100 <i>bd0528</i> ORF.	AGCCATGCATGTTGTGTTGT
bd0467RT-R		GTATTTTCGCGCTGACTCT
bd0635RT-F	Primers to amplify a 89 bp fragment of HD100 <i>bd0528</i> ORF.	TGATGAAAACCCGAAAGGTC
bd0635RT-R		TTGCCTCGTCTTTCATACCC
bd0633RT-F	Primers to amplify a 89 bp fragment of HD100 <i>bd0528</i> ORF.	GAGTGGATGAAGGCAATCC
bd0633RT-R		CCCAAAGCCACGTTGTATTC
bd0634RT-R	Primers to amplify a 99 bp fragment of HD100 <i>bd0528</i> ORF.	CCGCATCTATTTGGAAGACC
bd0634RT-R		ATCAAACGAGCCGAATTCTC
bd0636RT-R	Primers to amplify a 93 bp fragment of HD100 <i>bd0528</i> ORF.	ATCCACCTCTGCGTTACTG
bd0636RT-R		ATCCAAAGCCGACACTTCAC
bd0742RT-F	Primers to amplify a 173 bp fragment of HD100 <i>bd0528</i> ORF.	AGTTGTCATCGTTGTGAGCC
bd0742RT-R		AAACAAGGTGGCCGTAACCG
bd0743RT-F	Primers to amplify a 254 bp fragment of HD100 <i>bd0528</i> ORF.	GCTCTTTTCCGAACCTCGTG
bd0743RT-R		TACAGCCAATTGCACATCGT
bd0135RT-F	Primers to amplify a 137 bp fragment of HD100 <i>bd0528</i> ORF.	GTCGATCAGTTCCTGGGTGT
bd0135RT-R		CAAGTTACGCGACCCTCAAT

Primer	Description	Sequence (5'-3')
bd0130RT-R	Primers to amplify a 90 bp fragment of HD100 bd0528 ORF.	CGAGGATTTCAAAGCGTCTC
bd0130RT-R		AAATTTCTTCGCCGACTTCA
Bd0048RT-R	Primers to amplify a 86 bp fragment of HD100 bd0528 ORF.	ATCTGACGCTGGATCTGGTC
bd0048RT-R		AATCGCCACAATGTCTCCAC
Primers for fluorescent tagging		
b0031-tag-F	Contain six random 5' bases then a EcoRI site that followed by the <i>b0031</i> ORF.	TCACTGGAATTCATGCATGATGCAAACA TCCG
b0031-tag-R	Contains the 3' region of the <i>b0031</i> ORF without stop codon followed by a KpnI site and six random 3' bases for cutting efficiency	cggagcGGTACCCAAATTATTGAGATCAA GTA
B2472-tag-F	Contain six random 5' bases then a EcoRI site that followed by the <i>b2472</i> ORF.	gctcacGAGCTCATGTCGTGCCCGGTTAT TGA
B2472-tag-R	Contains the 3' region of the <i>b2472</i> ORF without stop codon followed by a KpnI site and six random 3' bases for cutting efficiency	cggagcGGTACCGGCGACGAGCTGTTCC ATGA
B4024-F	Contain six random 5' bases then a EcoRI site that followed by the <i>bd4024</i> ORF.	TCACTGGAATTCATGTCTGAAATTGTTGT CTC
B4024-R	Contains the 3' region of the <i>bd4024</i> ORF without stop codon followed by a SacI site and six random 3' bases for cutting efficiency	cggagcGAGCTCCTCAAACAAATTACTAT GCA
bd0047 -tag-F	Contain six random 5' bases then a EcoRI site that followed by the <i>bd0047</i> ORF.	TCACTGGAATTCGTGAAGAAAATCAAAA TCGG
bd0047 -tag-R	Contains the 3' region of the <i>bd0047</i> ORF without stop codon followed by a KpnI site and six random 3' bases for cutting efficiency	cggagcGGTACCTTTTTTGCTTTTCCGAA CA
bd00129 -tag-F	Contain six random 5' bases then a EcoRI site that followed by the <i>bd0129</i> ORF.	TCACTGGAATTCCTGGACTTCATCGAGG CCTG
bd0129 -tag-R	Contains the 3' region of the <i>bd0129</i> ORF without stop codon followed by a KpnI site and six random 3' bases for cutting efficiency	cggagcGGTACCTAAACAAAACCTTTCGA TGA
bd0134 -tag-F	Contain six random 5' bases then a EcoRI site that followed by the <i>bd0134</i> ORF.	TCACTGGAATTCGTGGCAAACTGGTAG TTTC
bd0134 -tag-R	Contains the 3' region of the <i>bd0134</i> ORF without stop codon followed by a KpnI site and six random 3' bases for cutting efficiency	cggagcGGTACCGGCCATTCTTCGATGC CGG
bd0528-tag-F	Contain six random 5' bases then a EcoRI site that followed by the <i>bd0528</i> ORF.	TCACTGGAATTCATGAAACCCCTGATTG TTCA
bd0635 -tag-R	Contains the 3' region of the <i>bd0635</i> ORF without stop codon followed by a KpnI site and six random 3' bases for cutting efficiency	TCACTGGAATTCATGAAGTTGATCATTCT TGT
bd0635-tag-F	Contain six random 5' bases then a EcoRI site that followed by the <i>bd0635</i> ORF.	cggagcGGTACCGTTTGAAGGGTTTAAAA AAG
bd0528 -tag-R	Contains the 3' region of the <i>bd0528</i> ORF without stop codon followed by a KpnI site and six random 3' bases for cutting efficiency	cggagcGGTACCAATCAAAGGATGAAGG CACT
bd0467 gib-F	Used to amplify 624 bp of <i>bd0467</i> from HD100 genomic has an overlap compatible with pk18 cut with EcoRI	cgttgtaaacgacgcccagtgccaGTGAAACC TATCATTCCG

Primer	Description	Sequence (5'-3')
bd0467 gib-R	Used to amplify 624 bp of <i>bd0467</i> from HD100 genomic has an overlap compatible with pk18 cut with HindIII	ccttgctcacatGATTCTTAGAGGATAGGG CTG
bd0467gibmcherry F	Used to amplify 711 bp from pAKF56 that contain <i>mcherry</i> that has an overlap compatible with pk18 cut with EcoRI	ccttgctcacatGATTCTTAGAGGATAGGG CTG
bd0467gibmcherry R	Used to amplify 711 bp from pAKF56 that contain <i>mcherry</i> that has an overlap compatible with pk18 cut with HindIII	ggaaacagctatgaccatgattacgTTACTTGT ACAGCTCGTCCATG
Bd3766 gib-F	Used to amplify 1881 bp of <i>bd0467</i> from HD100 genomic that has an overlap compatible with pk18 cut with EcoRI	cgttgtaaaacgacgcccagtgccaTTGTCCGA TGAATCTGGAAGTATG
bd3766 gib-R	Used to amplify 1881 bp of <i>bd0467</i> from HD100 genomic has an overlap compatible with pk18 cut with HindIII	ccttgctcacatCGACACCAACCGCAAGCT TAC
Bd3766gib-mcherryF	Used to amplify 711 bp from pAKF56 that contain <i>mcherry</i> that has an overlap compatible with pk18 cut with EcoRI	gcggttggtgtcgATGGTGAGCAAGGGCGA G
Bd3766gib-mcherryR	Used to amplify 711 bp from pAKF56 that contain <i>mcherry</i> that has an overlap compatible with pk18 cut with HindIII	ggaaacagctatgaccatgattacgTTACTTGT ACAGCTCGTCCATG
Primers for gene deletion		
Bd0134-F	Six random 5' bases for cutting efficiency then an EcoRI site followed by a 1029 bp upstream of <i>bd0134</i> ORF.	GATCGGGAATTCaagaccgctgatttcttcgg
Bd0134-R	Six random 5' bases for cutting efficiency then a HindIII site followed by reverse complemented sequence 1061 bp downstream of <i>bd0134</i> ORF.	GATCGGAAGCTTtcggtgacgacaacactggc
ΔBd0134-F	Twenty bases chosen after leave 48 bases downstream from the start of the <i>bd0134</i> ORF, followed by an XbaI site then 20 bp sequence was chosen after left 48 bases from the 3' end of the <i>bd0134</i> ORF.	GCAGACTGTATGCTTCGCTCTAGACCGA GGTCATCAAACGCC
ΔBd0134-R	Reverse complement of ΔBd0134-F	GGCGTTTGATGACCTCGGTCTAGAGCG AAGCATACAGTCTGC
Bd0528-F	Six random 5' bases for cutting efficiency then an EcoRI site followed by a 598 bp upstream of <i>bd0528</i> ORF.	GATCGGGGATCCcatcagtagcgtgatgcc
Bd0528-R	Six random 5' bases for cutting efficiency then a HindIII site followed by reverse complemented sequence 932 bp downstream of <i>bd0528</i> ORF.	GATCGGAAGCTTcgatcagcctgtgatcaggc
ΔBd0528-F	Fourteen bases upstream of the <i>bd0528</i> ORF then followed by first two codons, XbaI site, last two codons then followed by 14 bases downstream of stop codon.	aaagttgcttgaATGAAATCTAGAATTGA ttttgatttaagg
ΔBd0528-R	Reverse complement of ΔBd0528-F	ccttaaatcaaaaTCAAATCTAGATTTCAT tccaaagcaacttt
Bd0129-F	Six random 5' bases for cutting efficiency then an EcoRI site followed by a 943 bp upstream of <i>bd0129</i> ORF.	GATCGGGGATCCcgctggatcgcaacctgcg
Bd0129-R	Six random 5' bases for cutting efficiency then a HindIII site followed by reverse complemented sequence 1066 bp downstream of <i>bd0129</i> ORF.	GATCGGGCATGCacataaaattcttcgccc
ΔBd0129-F	Fourteen bases upstream of the <i>bd0129</i> ORF then followed by first two codons, XbaI site then 20 bp sequence was chosen after left 50 bases from the 3' end of the <i>bd0134</i> ORF.	ttgaaggagtctagTTGGACTCTAGACGTCT CTATAGAGGATCTGC

Primer	Description	Sequence (5'-3')
ΔBd0129-R	Reverse complement of Δ <i>bd0129</i> -F	GCAGATCCTCTATAGAGACGTCTAGAGT CCAActagactccttcaa
Bd0467 gib-F	Used to amplify 100 bases upstream of <i>bd0467</i> from HD100 genomic has an overlap compatible with pk18 cut with EcoRI	cgttgtaaaacgacggccagtccaCCAGCTTT TCCATGCGGA
ΔBd0467 gib-R	Used to amplify 1000 bases upstream of <i>bd0467</i> from HD100 genomic has an overlap compatible with pk18 cut with HindIII	aagcaggtaccttGCGATGGTATTTTATTG GACATTTTT
ΔBd0467 gib -F	Used to amplify 1000 bases downstream of <i>bd0467</i> from HD100 genomic has an overlap compatible with pk18 cut with EcoRI	aaaataccatcgcaAGGTACCTGCTTACTTT TTC
Bd0467 gib -R	Used to amplify 1000 bases downstream of <i>bd0467</i> from HD100 genomic has an overlap compatible with pk18 cut with HindIII	ggaaacagctatgaccatgattacgCATCCTCT GTTATGGTGC
Bd635 gib-F	Used to amplify 1000 bases upstream of <i>bd0467</i> from HD100 genomic has an overlap compatible with pk18 cut with EcoRI	cgttgtaaaacgacggccagtccaAAACTGCG CCTGTTCCCC
ΔBd635 gib-R	Used to amplify 1000 bases upstream of <i>bd0467</i> from HD100 genomic has an overlap compatible with pk18 cut with HindIII	cgagcctctctgCAGCCCGTCAGCAAAGTC
ΔBd635 gib-F	Used to amplify 1000 bases downstream of <i>bd0467</i> from HD100 genomic has an overlap compatible with pk18 cut with EcoRI	tgctgacgggctgCAGAAGAGGCTCGTCGT G
Bd635 gib-R	Used to amplify 1000 bases downstream of <i>bd0467</i> from HD100 genomic has an overlap compatible with pk18 cut with HindIII	ggaaacagctatgaccatgattacgATCCAAAG CCGACACTTC
Complementation primer		sequences
Bd0134COMP-F	Six random 5' bases for cutting efficiency, then EcoRI site followed by a 199 bp upstream of the <i>bd0134</i> ORF.	TCACTGGAATTCgctgatttctcggtcttcc
Bd0134COMP-R	Six random 5' bases for cutting efficiency, then a HindIII site followed by a reverse complemented 100 bp downstream of the <i>bd0134</i> ORF.	cggagcGAATTCgaatggacttctggattcc

Table 2-3: List of primers used in this study with descriptions.

2.5 Bacterial growth conditions:

2.5.1 *E. coli* DH5 α , *E. coli* S17-1, *E. coli* MG1655, *A. baumannii*, *P. putida*, *S. marcescens* and *S. typhimurum* growth

These species were streaked from frozen stocks to give single colonies on YT agar (with appropriate antibiotic added if necessary) and grown for 16 hrs at 37 °C. Single colonies were picked into YT broth (with appropriate antibiotic added if necessary) to prepare bacterial culture and incubated for 16 hrs overnight at 37 °C at 200rpm. After the bacterial cultures were grown, these cultures (stationary phase) centrifuged and back-diluted to O.D600 1.0 except the *E. coli* MG1655 back diluted to O.D 5.0 and then used in different experiments.

2.5.2 *B. bacteriovorus* (Host-dependent)

Host-dependent *Bdellovibrio* strains were grown on YPSC overlay plates that consisted of a solid layer of YPSC bottom agar (with appropriate antibiotics added if necessary) and top layer consisted of 5ml of semisolid YPSC top agar mixed with 150 μ l *E. coli* S17-1 prey cells or *E. coli* S17-1 (pZMR100) for kanamycin resistant strains. The plates were incubated at 29 °C for 3-10 days until plaques appeared. To revive *Bdellovibrio* HD strains from frozen stocks, 100 μ l of the frozen stock was added onto such YPSC overlay plates and incubated at 29°C for 3-7 days until a clear region was visible.

For growing *Bdellovibrio* strains in liquid predatory cultures, 2 ml predatory culture was prepared by picking up single plaques or regions of lawn clearing into bijoux tubes containing 2 ml CaHEPES and 150 μ l of prey (*E. coli* S17-1 or *E. coli* S17-1 pZMR100 for kanamycin-resistant strains) and incubated at 29°C with 200 rpm shaking for 24hr-48 hr until the prey cells were cleared from the predatory culture (as checked by light microscopy). Host- dependent *Bdellovibrio* strains culture can be grown in 10 ml predatory cultures (10 ml CaHEPES, 600 μ l prey, 200 μ l previous *Bdellovibrio* culture in 50 ml falcon tubes) or 50 ml predatory cultures (50 ml CaHEPES, 3 ml prey, 1 ml previous

Bdellovibrio culture in 250 ml conical flasks) and incubated at 29°C with 200 rpm shaking. For quantifying viable cells of *Bdellovibrio* strains, 100 µl of a serial dilution was added to 150 µl of prey prior to adding 5 ml of melted YPSC top agar (cooled to ~50°C) which was then mixed and poured on the YPSC bottom agar plates. The plates were incubated at 29°C for 3-10 days until single plaques are formed and can be counted. For RNA isolation for time course experiments, 1 litre predatory culture was grown in a 2 litre conical flask (1 L CaHEPES, 60 ml pre-grown *E. coli* S17-1, 20 ml previous *Bdellovibrio* culture in 2 litre conical flask).

2.5.3 *B. bacteriovorus* growth (host-independent strains)

Host-independent *B. bacteriovorus* strains were revived from frozen stocks by spreading 100µL onto SPY agar (with appropriate antibiotics supplied if necessary) and incubated at 29°C for 3-12 days. When growth appeared, a large inoculum was picked from plate into 500 µl PY broth and incubated at 29°C with 200 rpm shaking for 1-2 days. The culture was then doubled in volume by the addition of PY broth until the volume of culture reached 2ml which can then be diluted into large volumes.

For isolation of RNA from growing HI *Bdellovibrio* strains, strains were back-diluted in PY to OD₆₀₀ 0.1 in a total volume of 50 ml in a 250 ml conical flask and left to grow for approximately 16 hours at 29°C with 200 rpm shaking until the culture reached an OD₆₀₀ 0.6. For HI *Bdellovibrio* strains used in predation assays, the cultures were sub- cultured 24 hours before the assay by the addition of 5 ml HI culture to 5 ml PY.

2.5.4 Frozen stocks of *B. bacteriovorus* HD100 and bacterial strains

For long-term storage of *B. bacteriovorus* (both HD and HI) and other bacterial strains, 150 µl of 80% glycerol (v/v) was added to 700 µl of a freshly grown bacterial culture in screw-cap tube (2 ml) and mixed by inversion. The tubes were snap-frozen in liquid nitrogen and stored at -80°C.

2.6 Antibiotics

All antibiotics that used in this study were purchased from the Sigma-Aldrich company (www.sigmaaldrich.com) and prepared by dissolved the antibiotics powder with sterile AnalaR water or with some solvent according to manufacturer's instructions as shown in the Table (2-4).

Table 2-4: shows the antibiotics used in this study. The table illustrating the antibiotic name with abbreviation (in brackets), stock concentration with solvent used to dissolve each antibiotic and final concentration of each antibiotic used in this study.

Antibiotic / Additive	Stock Concentration (mgml ⁻¹)	Final Concentration (µgml ⁻¹)
Ampicillin sodium (Amp)	50 (AnalaR water)	50
Carbenicillin disodium (Carb)	50 (AnalaR water)	50
Kanamycin sulphate (Kan)	50 (AnalaR water)	25 (<i>E. coli</i>) or 50 (<i>Bdellovibrio</i>)
Isopropyl-β-D-thiogalactopyranoside (IPTG)	200 (AnalaR water)	200
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)	20 (Dimethyl-formamide, DMF)	200
m-DAP	50 (HCL)	100

2.7 Bacterial manipulations

2.7.1 Viable cell enumerations of *Bdellovibrio* and prey cells

Serial dilutions of *B. bacteriovorus* and prey bacteria (*E. coli*, *A. baumannii*, *P. putida*, *S. marcescens* and *S. typhimurum* strains) were prepared and plated out in order to obtain the number of viable cells. Host-dependent *B. bacteriovorus* strains were plated out at dilutions 10^{-4} to 10^{-7} on YPSC agar prey overlay plates (section 2.2.2), host-independent *B. bacteriovorus* strains were plated out (10^{-4} to 10^{-7}) on SPY agar plates and the prey were plated out at dilutions 10^{-4} to 10^{-8} in triplicate on YT agar using the Miles Misra method (section 2.4.2). CFU/PFU per ml values were calculated by counting separate colonies (for the prey or HI *Bdellovibrio*) and plaques (for the HD *Bdellovibrio*) from the plates.

2.7.2 The Quantification of Colony Forming Units (CFU) per ml of bacterial suspensions by the Miles and Misra method

In order to quantify CFU/ml that were present in bacterial suspensions during the 120 hr predation assays, the Miles and Misra method was used (Miles et al. 1938). This method provided an accurate and faster method to quantify CFU/ml that were present in bacterial suspensions and determine the CFU of viable bacteria that were not killed by the *Bdellovibrio* strain. Bacterial culture of prey (*E. coli* S17-1, *A. baumannii*, *P. putida*, *S. marcescens* and *S. typhimurum*) was grown under standard condition and centrifuged for 10 mins at $5525 \times g$ at 29°C and back-diluted to OD₆₀₀ 1.0 in CaHEPES. From these, the OD₆₀₀ 1.0 prey suspensions were serially diluted 1:10 with CaHEPES by adding 100 µl of each prey suspension to 900 µl of CaHEPES. 20 µl of serial dilution was taken from the predation suspension samples every day and pipetted onto YT agar plates with triplicate repeats for each dilution. These dilutions were carried out every day at the same time for 120 hrs. The plates were left to dry and then incubated upside down for 24 hours at 37 °C. The plates of dilutions that showed discrete colonies were counted. For achieving CFU/ml of diluted suspension, CFU/ml of original

suspension was calculated by multiplying the average number of colonies across the triplicate repeat that were prepared from the same dilution, then multiplying by the dilution factor.

2.7.3 Swarming assay

Swarming assay was carried out as described previously by (Ryjenkov et al. 2006) on soft agar swarming plates (0.25% agar, 1% tryptone and 0.5% NaCl). The *E. coli* MG1655 strains containing plasmids with either wild-type *bd3766* *bd3766* with of different version of point mutations (table 2.1 & 2.2) were grown under standard conditions alongside one another in YT broth provided with kanamycin ($50 \mu\text{g ml}^{-1}$) at 37°C at 200 rpm for 16 hrs, to measure their swarming zone. 3 ml from each bacterial culture were centrifuged at $5525 \times g$ at 29°C for 5 mins and the supernatant removed. The cell pellets were re-suspended with distal water and back-diluted to OD_{600} 5. Then 5 μl from these OD_{600} 5 bacterial suspensions were taken and inoculated onto the soft agar swarming plates and dried briefly. After that, the plates were incubated for 4-6 hr at 37°C . The zones formed by the swarming bacteria were measured after 5 h and all swarming zone measurements then were analysed. This assay was performed three times independently on separate days.

2.7.4 Production of chemically competent *E. coli* cells

Chemically competent *E. coli* cells (DH5 α and S17-1) was prepared by growing *E. coli* cells in 10 ml YT at 37°C overnight. The culture was back diluted 1:100 in 600 ml YT and re-incubated to grow to OD_{600} 0.4-0.6 at 37°C with 200 rpm shaking. The culture was then split into 50 ml and centrifuged at $5525 \times g$ at 4°C for 5 mins. The supernatant was removed and the cell pellets re-suspended in 20 ml of 4°C TFB1 (recipes in appendix). The cell suspension was incubated on ice for 5 mins then centrifuged (as before) for 5 mins. The supernatant was removed and each cell pellet re-suspended in 2ml 4°C TFB2 and incubated on ice for at least

one hour. The suspension was then divided into 200 µl aliquots in 1.5 ml microcentrifuge tubes before being snap-frozen in liquid nitrogen and stored at -80°C until used. Transformation efficiency of the competent *E. coli* cells was assessed by transformation with a high copy-number plasmid (typically pUC19) and counting the number of colonies formed on the YT agar supplemented with the appropriate antibiotic (expectation is > 1000 transformants from 50 ng plasmid DNA).

2.7.5 Transforming plasmids DNA into competent *E. coli* strains

To transform plasmid DNA, aliquots of *E. coli* DH5α or S17-1 competent cells were thawed on ice and the DNA from a ligation or miniprep (at the required concentration) was added and mixed by gentle pipetting to prevent cell lysis. The cells were incubated on ice for 30 mins and heat shocked at 42°C for 1 min, then placed on ice for 5 mins. 1 ml of SOC medium was then added and the cells were incubated at 37°C with 200 rpm shaking for 1 hour in order to allow the cells to recover. The cells were then centrifuged at 17000 x *g* for 2 mins, re-suspended in 100 µl YT and plated onto YT agar provided with the appropriate antibiotic selection. The plates were incubated at 37°C for 16 hours. The positive and negative control transformations were also performed (positive control = 1 µl empty plasmid from a miniprep; negative control = 1 µl water).

2.7.6 Isolation of Plasmid from *E. coli* strains

The best method to screen multiple transformants that might contain the target plasmid is the alkaline-SDS lysis method (Birnboim and Doly 1979). This was carried out by taking 300 µl of transformed *E. coli* that was lysed by adding 300 µl of alkaline SDS (1% (w/v) SDS and 0.2 M NaOH). The mixtures then were neutralised by the addition of 300 µl 3 M potassium acetate (pH 5.5). Samples were then centrifuged at 17000 x *g* for 10 mins 4°C and the supernatants were transferred to tubes containing 700 µl chilled isopropanol. The samples were

mixed by inversion and then centrifuged at $17000 \times g$ for 10 mins. The resultant pellets were washed with 500 μ l 70% (v/v) ethanol and recentrifuged for 2 mins. The supernatant was then removed, and DNA pellet allowed to dry before being resuspended in 25 μ l (10 mM Tris-HCl pH 8, 1 mM EDTA) containing 10 μ g ml⁻¹ RNaseA. In order to obtain highly purified plasmid extracts from *E. coli* strains, the GenElute™ plasmid miniprep kit was used according to the manufacturer's instructions.

2.7.7 Isolation of bacterial genomic DNA

Total bacterial genomic DNA was isolated by using a genomic DNA kit (Sigma-Aldrich GenElute™ Bacterial). 10 ml of a *B. bacteriovorus* predatory culture (attack-phase only) or 2 ml of HI *B. bacteriovorus* cultures were harvested by centrifugation in a Sigma 4K15 and centrifuged at 5,100 rpm ($5,525 \times g$) for 20 mins, then following the manufacturer's instructions. For genome sequencing services, MicrobesNG (<https://microbesng.uk>) was used.

2.7.8 Generating host-independent isolates of *B. bacteriovorus* strains

10 ml of a predatory culture of *Bdellovibrio* was filtered through a 0.45 μ m filter and centrifuged at $5525 \times g$ (in a Sigma 4K15 centrifuge) at 29°C for 20 mins. The supernatant was removed, and the pellet was re-suspended in 110 μ l of PY broth and spread on to SPY agar (with the selection, if required) and incubated at 29°C for 7-12 days until colonies appeared.

2.7.9 Plasmid conjugation into host-dependent *B. bacteriovorus*

The *E. coli* strain S17-1 was used as the donor strain to conjugate pK18*mobsacB* into *Bdellovibrio*, which is a plasmid used for single crossover recombination of fluorescent tags or deletion constructs into the *Bdellovibrio* genome. 10 ml of the recipient *B. bacteriovorus* strain host-dependent culture, grown under standard conditions, was centrifuged in a Sigma 4K15 centrifuge at $5525 \times g$ at 29°C for

20 mins. The supernatant was then removed, and the pellet was resuspended in 100 µl of the residual supernatant. The suspension was loaded onto a small piece of sterile transfer membrane (Nytran SuPerCharge 0.45 µm Nylon- Whatman), immobilised on a SPY agar plate and allowed to dry. Under standard conditions, 50 ml YT broth containing the donor *E. coli* strain with the desired plasmid was grown in the presence of kanamycin (50 µg ml⁻¹). 10 ml of this *E. coli* culture was centrifuged at 29°C for 10 mins at 5525 x *g* and the supernatant was discarded and the pellet resuspended with 100 µl YT broth. This suspension was transferred to the membrane and left to dry on top of *B. bacteriovorus* cells and incubated at 29°C for 16 hours.

After incubation, sterile forceps were used to transfer the membrane to a 50 ml falcon tube and the cells were washed off with 1 ml CaHEPES. Aliquots of this suspension (divided to 10 µl, 100 µl, 200 µl and 690 µl) were then added to 5 ml YPSC top agar mixed with 150 µl *E. coli* S17-1 containing pZMR100 prey cells before being poured onto YPSC bottom agar plates (plates contained 50 µg ml⁻¹ kanamycin). Plates were incubated at 29°C for 3-10 days until *B. bacteriovorus* merodiploid plaques appeared.

2.7.10 Plasmid conjugation into host-independent *B. bacteriovorus* strains

Under standard conditions, the recipient host-independent *B. bacteriovorus* strain was grown in a 2 ml culture and 500 µl of this culture was centrifuged (Sigma 4K15) at 17000 x *g* at 29°C for 2 mins. The pellet was resuspended in 100 µl of PY, and the suspension was loaded onto a small piece of sterile transfer membrane (Nytran SuPerCharge 0.45 µm Nylon - Whatman) immobilised on a SPY agar plate and allowed to dry. 5 ml of YT broth containing the donor *E. coli* strain with the desired plasmid construct was grown in the presence of kanamycin (50 µg ml⁻¹) under standard conditions and centrifuged at 29°C for 10 mins at 5525 x *g*. The supernatant was discarded and pellet resuspended with 100 µl PY broth. This

suspension was transferred to the membrane and left to dry on top of *B. bacteriovorus* cells and incubated at 29°C for 16 hours.

After incubation, sterile forceps were used to transfer the membrane to a 50 ml falcon tube and the cells were washed off with 5 ml of PY and the cell suspension was incubated for 30 mins in order to recover at 29°C with 200 rpm shaking. For plasmid conjugations into HI strains, the cell suspension acquired from washing the membrane was filtered through a 0.45 µm filter to remove the residual *E. coli* donor cells and remaining *B. bacteriovorus* cell suspension was centrifuged for 2 mins at 17000 x *g* at 29°C. The pellet was re-suspended in 100 µl of PY broth and spread onto SPY plates (containing 50 µg ml⁻¹ kanamycin).

2.7.11 Preparation of host-dependent *B. bacteriovorus* strain samples for RNA isolation

A 40 ml predatory culture of *B. bacteriovorus* (grown under standard conditions) was added to 10 ml 5% (v/v) phenol in ethanol. The sample was incubated on ice for at least 1 hour, then the sample was centrifuged for 10 min in a Sigma 4K15 benchtop centrifuge at 5525 x *g* at 4°C. The supernatant was discarded and the pellet was stored at -80°C. RNA was extracted as in section 2.7.14.

2.7.12 Preparation of host-independent *B. bacteriovorus* strain samples for RNA isolation

4 ml of *B. bacteriovorus* host-independent culture, grown under standard conditions to reach OD₆₀₀ 0.6, was added to 1 ml 5% (v/v) phenol in ethanol. The sample was incubated on ice for at least 1 hour, then the sample was centrifuged for 10 min in a Sigma 4K15 benchtop centrifuge at 5525 x *g* at 4°C. The supernatant was discarded and the pellet was stored at -80°C. RNA was extracted as in section 2.7.14.

2.7.13 Preparation of samples from a predatory time-course for RNA isolation

RNA isolation from a predatory time-course was carried out as previously described in (Lambert et al. 2006). In brief: 1 L of the required predatory culture of *B. bacteriovorus* (grown under standard conditions) was setup 24 hr prior to RNA isolation. The growth and complete lysis of prey by the *B. bacteriovorus* strain was checked by light microscopy and the culture was centrifuged at 17,000 x *g* in a Sorvall RC-5B Plus centrifuge for 30 mins. The pellet was re-suspended in fresh 100 ml of CaHEPES buffer and incubated for 3 hrs at 29°C with 200 rpm shaking in order to stabilise gene expression. At the same time, a 50 ml culture of *E. coli* S17-1 (grown under standard conditions) was centrifuged at 5,000 x *g* in a Sigma 4K15 benchtop centrifuge for 10 mins at 29°C. The prey cell pellet was re-suspended in 50 ml of fresh CaHEPES and back-diluted to an OD₆₀₀ of 1.0 and incubated at 29°C with 200 rpm shaking for 3 hours in order to stabilise gene expression.

After 3 hours of incubation at 29°C, synchronous predatory cultures were started by adding 40 ml OD₆₀₀ 1.0 *E. coli* and 50 ml *Bdellovibrio* culture to 30 ml CaHEPES, alongside controls of *E. coli*-only (40 ml OD₆₀₀ 1.0 *E. coli* and 80 ml CaHEPES) and *Bdellovibrio*-only (50 ml *Bdellovibrio* and 70 ml CaHEPES).

The number of *E. coli* and *Bdellovibrio* cells from the controls were enumerated by serial dilution to calculate the ratio of *Bdellovibrio* to *E. coli* in order to evaluate the infection efficiency (the multiplicity of infection). At each timepoint, the synchronicity of the culture was assessed by microscopy and 4ml of the culture was taken and added to 1 ml 5% (v/v) phenol in ethanol. The samples were incubated on ice for at least 1 hour then the sample was centrifuged for 10 min in a Sigma 4K15 benchtop centrifuge at 5525 x *g* at 4°C. The supernatant was discarded and the pellet stored at -80°C. RNA was extracted as in section 2.7.14.

2.7.14 RNA extraction:

The frozen cell pellets were defrosted on ice and RNA extraction carried out by using the Promega SV Total RNA Isolation kit which contains a single DNase treatment following manufacturer's instructions. A second DNase treatment to remove any residual DNA after RNA extraction was performed using Ambion DNA-free kit. To be sure that the RNA samples were free from DNA, Dream Taq PCR were run for these samples using primers amplifying a fragment of the *fliC1* gene and PCR product only seen if DNA remained in samples. The quality and yield of RNAs samples were then tested using an Agilent 2100 bioanalyzer to confirm that the 16S and 23S rRNA were present. Dr. Carey Lambert supplied HI RNA that I used in my project.

2.7.15 Measurement of the host-independent *Bdellovibrio* growth rate

To observe the host-independent (HI) *B. bacteriovorus* strains (wild type and mutant) growth rates were performed using OD₆₀₀ measurements. The Host-independent strains were grown in 10 ml of PY broth under standard condition and back diluted to OD₆₀₀ 0.1 in PY broth. 255 µl of back diluted culture was then transferred into the wells of a 96-well microtitre plate. Typically, three independently isolates for each mutant HI strain were taken with 4 replicates for each isolate, and 6 replicates were used for each wild type HI strain control. The plate was then sealed with transparent nylon membrane and incubated at 29°C with 200 rpm shaking in a BMG FluoStar microplate reader for three day with the OD₆₀₀ measurements taken every 30 mins. A growth curve was obtained by plotting the average OD₆₀₀ values of each strain at each time point from three biological repeats.

2.7.16 Growth curve of *E. coli* MG1655

To evaluate if expression of Bd3766 point mutation variants have an effect on *E. coli* MG1655 cells growth in comparison to wild type Bd3766 , strains of *E. coli* MG1655 containing all versions of point mutations in Bd3766/pK18mobsacB were grown beside *E. coli* MG1655 containing Wild type Bd3766/pK18mobsacB for 16 hours in LB/kan50 at 37°C. All cultures were back diluted to an OD₆₀₀ of 0.02 in 100 ml with LB/kanamycin (50 ug ml⁻¹) and incubated at 37°C with 200rpm shaking. Every 30 mins, 1 ml samples were taken and the OD₆₀₀ measured and the growth curve obtained by plotting the average OD₆₀₀ values of each strain at each time point from two biological repeats at least.

2.8 DNA manipulations

2.8.1 Agarose gel electrophoresis

In order to separate and visualise DNA fragments of varying sizes, agarose gel electrophoresis (2% agarose gel used for DNA fragments size 50 bp to 1 kb and 0.8% agarose gel used for DNA fragments size 500 bp to 12 kb) was used. The agarose gel run in tank (Qiagen) contained 1 x TBE buffer provided with 0.1 µg ml⁻¹ ethidium bromide that help in visualising the DNA bands under UV light. The samples of DNA were mixed with 3 x Orange G loading buffer and loaded into the gel wells (recipes in Appendix) alongside appropriate DNA markers (1 kb DNA ladder for 0.8% agarose gels or 100 bp DNA ladder for 2% agarose gels). Typically, the gels were run for 1 hr at 100 volts and images for DNA bands in gel were taken using UV light in a BioRad™ gel Quantity One Software system.

2.8.2 Restriction enzymes digests

Fermentas Fast Digest restriction endonuclease enzymes were used to digest DNA in the provided Fermentas green universal buffer. Digests were carried out in 10 – 50 µl reactions in accordance with the manufacturer's guidelines, with the

glycerol concentration of the restriction enzymes not exceeding 5% (v/v). Digests were carried out for 2 hours at 37°C for each enzyme.

2.8.3 Pfu fill-in reactions

Pfu DNA polymerase (Stratagene) was used to remove 3' overhangs or fill-in 5' overhangs for 30 mins at 72°C and typical reaction mixtures are shown in table (2-5).

50 µl	DNA template
10 µl	5x Pfu buffer
5 µl	10 mM dNTPs
1 µl	Pfu DNA polymerase
34 µl	AnalaR water

Table 2-5: shows the recipe of Polishing/Pfu fill-in reactions.

2.8.4 Dephosphorylation

Alkaline phosphatase was added to restriction digests in order to remove 5' phosphate groups from cut vector DNA to prevent recircularisation of the vector during blunt cloning. 1 µl FastAP Thermosensitive Alkaline Phosphatase was added to Fermentas FastDigest reactions.

2.8.5 Ligations of DNA fragments

Ligations of DNA fragments was carried out using T4 DNA ligase (Fermentas) in 20 µl reactions where, 1 µl of T4 DNA ligase was added to each reaction with the accompanying ligase buffer in order to catalyse the joining of the cut vector with the insert (ratio of vector to insert, 1:3). Ligation reactions were incubated at room temperature for 2 hours or at 16°C for 16 hours.

2.8.6 Gel extraction

Fragments of DNA from PCR reactions (to isolate PCR product from non-specific products) or digested DNA (to purify the desired restriction digested vector from any residual uncut plasmid) were electrophoresed on an agarose gel. The desired fragment was excised from the gel using a sterile scalpel blade whilst being visualised on a UV transilluminator. The DNA fragment was extracted and purified from the gel sliced using a GenElute™ gel extraction kit (Sigma-Aldrich) according to manufacturer's instructions.

2.8.7 Purification of DNA fragments from PCR restriction digest mixture

In order to purify DNA fragments that did not need to be resolved by agarose gel electrophoresis (digest of PCR products produce sticky ends fragments), a GeneElute PCR Cleanup Kit (Sigma-Aldrich) was used according to the manufacturer's instructions.

2.8.8 DNA sequencing

To sequence DNA (plasmid or PCR product), 5 µl of a plasmid miniprep (diluted to 100 ng µl⁻¹) or 5µl of PCR product (diluted to 1 ng µl⁻¹ per 100 bp), and 10 µl of the required primer per reaction (diluted to 3.2 pmol µl⁻¹) were sent for Sanger sequencing at Source BioScience (<https://www.sourcebioscience.com>).

2.9 Amplification of DNA/RNA

2.9.1 Polymerase Chain reaction (PCR)

Two types of polymerase enzymes were used to carry out PCR in this study:

Phusion® High-Fidelity DNA polymerase which was used to amplify DNA for cloning or sequencing (25-100 µl reactions), and **Dream Taq DNA polymerase** used for screening potential *Bdellovibrio* deletion strains by colony PCR (by adding 2 µl of HD or HI culture as template to a 25 ul reaction mixture). Typical

reaction mixtures are shown in table (2-6) and cycling temperatures are shown in table (2-7).

PCR component	Phusion	DreamTaq
Buffer (including MgCl ₂)	5 µl (5x)	2.5 µl (10x)
10 mM dNTPs	1 µl	1 µl
Primers (100 pmol µl ⁻¹)	0.5 µl	0.3 µl
Template genomic DNA	0.5 µl	0.5 µl (or 2 µl <i>Bdellovibrio</i> culture)
DNA Polymerase	0.25 µl	0.2 µl
AnalaR water	17.25 µl	20.2 µl (or 18.7 µl)
TOTAL	25 µl	25 µl

Table 2-6: the typical PCR reaction mixtures for Phusion and DreamTaq DNA polymerase.

Stage	Temperature	Duration	Cycles
Initial denaturation	98°C Phusion 95°C DreamTaq	5 mins	x 1
Denaturation	98°C Phusion 95°C DreamTaq	10 s 30 s	x 30 Phusion x 30-35 DreamTaq
Annealing	50-70°C Phusion 50-65°C DreamTaq	30 s	
Extension	72°C	30 s kb-1 1 min kb-1	
Final extension	72°C	5 mins	x 1
Hold	4°C	∞	x 1

Table 2-7: shows the conditions of PCR cycling for Phusion and DreamTaq PCR programs.

2.9.2 Touchdown PCR

Touchdown PCR was used to fuse separate PCR products with overlapping homology, such as 1 kb upstream and downstream flanking DNA regions of a gene for generating deletion constructs. The same Phusion DNA polymerase mixture (table 2-6) used in this type of PCR reaction, changing a genomic DNA for the PCR products to be joined as the template with the PCR cycle used as shown in table (2-8).

Stage	Temperature	Duration	Cycles
Initial denaturation	98°C	5 mins	x 1
Denaturation	98°C	1 min	x 20
Annealing	64-45°C (ramp)	1 min	
Extension	72°C	1 min	
Denaturation	98°C	1 min	x30
Annealing	55- 70°C	1 min	
extension	72°C	1 min	
Final extension	72°C	10 mins	x 1
Hold	4°C	∞	x 1

Table 2-8: Shows the conditions of PCR cycling for Touch down PCR programs.

2.9.3 Reverse-transcriptase polymerase chain reaction (RT-PCR)

RT-PCRs were carried out using the Qiagen OneStep RT-PCR kit that converts RNA to DNA through the reverse transcriptase and then amplifies the DNA via DNA polymerase. The typical RT-PCR reaction mixtures consist of the following:

Buffer (including 2.5 mM MgCl ₂)	5 µl (5x)
Q solution	5 µl (5x)
10 mM dNTPs	1 µl
Primers (100 pmol µl ⁻¹)	0.15 µl
Template (DNA/RNA)	0.5 µl (50-500 ng)
Polymerase	1 µl
AnalaR water (RNA free water)	12.2 µl

The cycling and temperatures of RT-PCR can be seen in Table (2-9)

Stage	Temperature	Duration	Cycles
Reverse transcriptase	50	30 mins	x 1
Initial denaturation	94°C	15 mins	x 1
Denaturation	94°C	1 min	x 28-30
Annealing	50°C	1 min	
Extension	72°C	1 min	
Final extension	72°C	10 mins	x 1
Hold	4°C	∞	x 1

Table 2-9: shows the conditions of PCR cycling for Reverse-transcriptase polymerase chain reaction (RT-PCR) programme.

2.9.4 Using RT-PCR to visualise the gene expression across the predatory cycle of *Bdellovibrio*.

RNA samples isolated from specific time points of the predatory life cycle of *B. bacteriovorus* (section 2.4.12) were used to identify when genes are expressed during the predatory cycle. Specific primers annealing to genes of interest were designed to amplify DNA fragments of approximately 100 bp from the RNA samples by using RT-PCR, with the number of cycles varying from 25-30 to ensure the PCR product is not saturated when visualising the relative transcription levels on an agarose gel. Furthermore, each set of RT-PCR reactions were run alongside a positive control reaction from HD100 genomic DNA, and two negative control reactions using no-template and RNA isolated from *E. coli* S17-1. The expression of each gene was confirmed by repeating the RT-PCR reaction at least two times from two separate sets of RNA samples.

2.9.5 Using RT-PCR to compare the expression levels on matched RNA amounts in host-independent strains

Previously isolated RNA samples from different HI strains as described in Section 2.7.11 were matched to 10 ng μl^{-1} for each HI strain and also for attack phase of *B. bacteriovorus* HD100 RNA using a NanoDrop spectrophotometer. Specific primers annealing to genes of interest were designed to amplify DNA fragments of approximately 100 bp by using RT-PCR with 30 cycles to ensure the PCR product is not saturated when visualising the relative transcription levels on an agarose gel. Furthermore, each set of RT-PCR reactions were run alongside a positive control reaction from HD100 genomic DNA, and two negative control reactions using no-template and RNA isolated from *E. coli* S17-1.

2.9.6 Using RT-PCR to detect co-transcription of genes

RT-PCR was performed to identify if neighbouring genes share the same promoter and are therefore on a single mRNA transcript. RT-PCR for each gene throughout the predatory lifecycle was first performed to find a suitable time point where both genes had peak expression, and the RNA sample for that time point was then used for checking co-transcription. Three sets of RT-PCR reactions were set up: one of these reactions used primers specific for the upstream gene, the second reaction used primers specific for the downstream gene and the third reaction used the forward primer from the upstream gene with the reverse primer from the downstream gene. With each RT-PCR reaction a positive control reaction from HD100 genomic DNA was setup. Two negative control reactions with no template and RNA isolated from *E. coli* S17-1 were also performed. The number of cycles used was 30 in this type of RT-PCR to amplify DNA and determine whether there is a continuous transcript across both genes.

2.10 Strategies of bacterial cloning and mutant strain isolation

2.10.1 Generation of the fluorescently tagged gene constructs and strains in *B. bacteriovorus*

To assess the cellular localisation of any *Bdellovibrio* proteins of interest, C-terminal fluorescent protein tagging was required. ORF-mCherry or ORF-mTFP constructs were generated by cloning the wild type gene (minus the stop codon) into either pAKF56 or pAKF04 (plasmids containing mCherry or mTFP genes respectively). The fluorescently tagged gene construct was then cloned into the final vector, pK18*mobsacB*. This plasmid containing the tagged gene was then transferred to *E. coli* S17-1 and subsequently conjugated into *B. bacteriovorus* HD100. The resulting strain is merodiploid containing fluorescently-tagged copy of the protein of interest.

2.10.2 Generating markerless gene deletions of *Bdellovibrio*

To gain gene deletion strains in *Bdellovibrio*, 1 kb regions upstream and downstream of the gene to be deleted were amplified and phusion polymerase (NEB) (section 2.9.1) was used to fuse the fragments together. The deletion construct was firstly transformed into *E. coli* DH5a using pUC19 vector then transformed into *E. coli* S17-1 using vector pK18 *mobsacB*. To obtain single cross overs, Kanamycin- resistant merodiploid *Bdellovibrio*, the pK18- deletion construct was conjugated in *B. bacteriovorus* HD100 using *E. coli* S17- pZMR (100) lawns on YPSC overlay plates with antibiotic selection provided by Kanamycin (50 $\mu\text{g}/\text{ml}^{-1}$). Resulting *B. bacteriovorus* merodiploid strains were then grown in 2.5% or 5% sucrose CaHEPES for inducing the second cross over event (pK18*mobsacB* is a sucrose suicide vector that encoding a levansucrase) using *E. coli* S17-1 prey (live or heat-killed 3 min at 105°C). Heat-killed prey was used to prevent *E. coli* from using the sucrose as a source of carbon while *Bdellovibrio* are able to invade them. After at least 3 rounds of growth in sucrose, lysed predatory cultures (by taking 100 μl of it) were plated out using a series of dilution (10^{-4} ,

10⁻⁵ and 10⁻⁶) onto YPSC overlay plates (no antibiotic selection) and incubated at 29 °C until single plaques appeared. The plaques were then picked into 96 wells plate and incubated for 2-3 day then these were subcultured into two 96 wells plates, one with kanamycin and the other without kanamycin. All strains not grown in the presence of kanamycin were screened by Dream Taq PCR (section 2.9.1) for potential gene deletions. The potential deletion strains were then confirmed by genomic DNA extraction and sequencing.

2.10.3 Gibson cloning method for generating markerless deletions and fluorescent protein tagging in *B. bacteriovorus*

Constructs of fluorescently tagged genes or constructs to create *B. bacteriovorus* deletion strains, can be obtained by using another method additional to the method that is mentioned in section (2.10.1 and 2.10.2), by Gibson assembly cloning method. This method works through combining multiple DNA fragments in one reaction via the action of multiple enzymes. Fragments to be joined together are generated with primers containing complementary overlapping regions to adjacent fragments. All primers for the Gibson assembly method designated by using NEBuilder tool <http://nebuilder.neb.com> website.

After that, the DNA fragments that need to go through the Gibson cloning were amplified by using Phusion® High-Fidelity DNA polymerase, and purified by gel extraction (see section 2.9.1). A specific concentrations of the amplified DNA fragments with diluted pK18*mobsacB* that cut with EcoRI, HindII were mixed with Gibson assembly matrix mix and incubated for 1 hr at 50 °C. Gibson assembly reaction mixture carried out in three enzymatic steps in same buffer, these include an exonuclease that facilitates the annealing of fragments that have compatible overlapping region by creating single stranded 3' overhangs. After that, a DNA polymerase is responsible for filling gaps between the annealed fragments. Finally, a DNA ligase ligates the DNA fragments in the assembled DNA

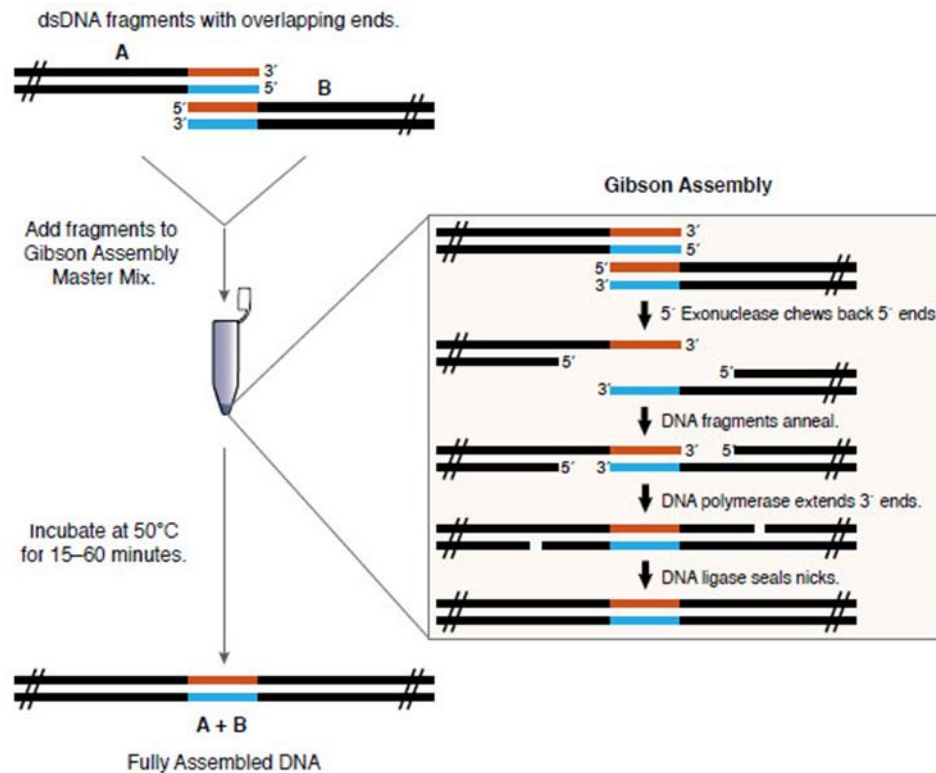


Figure (2. 1): The principle of the Gibson assembly method reaction that occur in single buffer. Image was taken from (<https://www.neb.com/products/e5510-gibson-assembly-cloning-kit>).

2.11 Microscopy

2.11.1 Fluorescence/brightfield microscopy

The bacterial cell images (Brightfield and fluorescent) were taken using a Nikon Eclipse E600 microscope equipped with a 100 x objective lens (Nikon). All images were taken with this magnification. A Prior Lumen 200PRO metal halide lamp was used for illumination of epifluorescence, in combination with the appropriate filter block for each fluorophore. Images were captured using a Hamamatsu ORCA-ER camera and SimplePCI software (version 5.3.1 Hamamatsu). All fluorescent images were taken through the green channel at maximum (255) gain in Simple PCI. The same protocol was applied for Brightfield microscopy but without using of epifluorescence. Each image that captured clarified by double sharpen passes and two smooth image enhancement passes. The bacterial cells were immobilised using 1% agarose/CaHEPES pads that were pipetted onto microscope slides and

allowed to set for 5 mins. The agarose/CaHEPES pads used for timelapse videos and other experiments were prepared with reservoirs at the ends of the slides and filled with sterile distilled water to avoid dehydration of pad. In all experiments, 10 μ l of sample was pipetted onto the centre of the pad and covered with a 22 x 22mm (0.13-0.17 mm thick) coverslip.

2.11.2 Timelapse microscopy for *Bdellovibrio* entry into prey cells

In order to measure the time that *Bdellovibrio* (wild type and mutant) strains take to complete the invasion of prey cells, time lapse microscopy was used with special parameters. Small scale infection of predatory cultures were prepared by using *E. coli* S17-1 or *E. coli* S17-1 (pZMR100) as a prey for the *Bdellovibrio* strains. 10 μ l of this infection was taken and immobilized on a 0.3% agarose/CaHEPES pad containing reservoirs filled with sterile distilled water to avoid dehydration of the pad. SimplePCI software was used for revisiting the same six fields of view (FOV) every 60 seconds and captured in a brightfield image. The videos lasted for 1-2 hours, and for each strain at least two independent repeats were performed.

2.11.3 Localisation of mCherry-tagged proteins during the predatory life cycle

The cellular location of mCherry-tagged proteins during the predatory life cycle is necessary to help to identify the possible function of tagged protein and the time at which the fluorescent tag appears can be examined by using fluorescent microscopy at specific time point. A 50 ml of *Bdellovibrio* mCherry-tagged strain culture was setup 16 hrs prior to the fluorescent time course (50 ml CaHEPES, 3 ml of previously grown *E. coli* S17-1 (pZMR 100), 1 ml *Bdellovibrio* mCherry-tag strain, 50 μ g ml⁻¹ Kanamycin) together with 50 ml *E. coli* S17-1 (pZMR100) in YT broth supplemented with 50 μ g ml⁻¹ of kanamycin. Both cultures were incubated at 29°C and 37°C with 200 rpm shaking for 16 hr respectively. The *Bdellovibrio* culture was checked for full lysis then centrifuged for 20 mins at 5525 x *g* at 29°C.

The supernatant was removed and the pellet re-suspended with 5ml of CaHEPES and incubated for 30 mins at 29 °C with 200rpm shaking. After that, supernatant was poured off and the pellet re-suspended in 5 ml of CaHEPES. During that time, 10 ml of *E. coli* culture was centrifuged for 10 mins at 5525 x g, the supernatant removed and the pellet re-suspended and back diluted to O.D 1 with CaHEPES and incubated for 30 mins at 29 °C with 200 rpm shaking. After the 30 mins incubations was finished, the infection was started by adding 3 ml of CaHEPES containing 50 µg ml⁻¹ Kanamycin, 4 ml of *E. coli* and 5 ml *Bdellovibrio* to a Falcon tube. Immediately, 10 µl of infection culture was taken and immobilized on a 1% agarose/CaHEPES pad and returned the culture to the incubator for the next time points. Images were taken using a fluorescent microscope Nikon Eclipse E600 microscope at 0min, 15 mins, 30 mins, 45 mins, 1 hr, 2hrs, 3 hrs and 4 hrs of starting of the infection.

2.11.4 Staining bacteria with Hoechst (DAPI) stain

In order to visualise the DNA of *B. bacteriovorus* cells (HD and HI) and *E. coli*, samples of these strains were grown under standard conditions and stained with Hoechst 33372 stain (DAPI) at a concentration of 1 µg ml⁻¹. The DAPI stain was mixed with the sample and incubated for 15 mins at room temperature and then examined using the Nikon Eclipse E600 microscope with typical fluorescence exposure time of 0.05 second.

2.12 Protein measurements for bacterial cells

2.12.1 Lowry Assay to match the numbers of *Bdellovibrio* by total protein contents

As predatory attack phase *Bdellovibrio* cells are very small and the yield of cells depends on prey cell density, and because HI cell cultures of *Bdellovibrio* show varies of shapes, it is impossible to match cultures by optical density of an OD₆₀₀ measurement. One of the more accurate method is a Lowry assay that measures

the total protein content of a sample for by matching the cell number (Lowry et al. 1951). *Bdellovibrio* protein content was measured and calculated by the Lowry assay with Markwell's modification to include solubilising membrane proteins (Markwell et al. 1978).

This assay measured the change in colour of Folin reagent in the presence of copper bound proteins at OD₇₅₀ by using the spectrophotometer. The Lowry assay was used as all the *B. bacteriovorus* $\Delta bd0742$ and $\Delta bd0742$ suppressor strains used in this study were unable to form plaques on prey-overlay plates. The total protein content of *Bdellovibrio* was calibrated with a standard curve of known quantities of bovine serum albumin (BSA).

The Lowry assays were carried out by preparing of stock solution of 1 mg ml⁻¹ of BSA in AnalaR water then added the following volumes (0 μ l, 20 μ l, 40 μ l, 60 μ l, 80 μ l and 100 μ l of BSA) to a cuvette to a total volume of 500 μ l with sterile AnalaR water. During this time either the HD or HI of *Bdellovibrio* culture was prepared. In the case of *B. bacteriovorus* HI cultures, 500 μ l of HI strain culture grown under standard conditions was centrifuged at 17000 $\times g$ at 29°C for 2 mins then the supernatant removed and re-suspended in 500 μ l sterile AnalaR water. While, in case of the predatory cultures of *Bdellovibrio*, a 50 ml culture of predatory strain grown on *E. coli* S17-1 for 24 hrs prior the experiment was filtered twice through and centrifuged for 20 mins at 5525 $\times g$. After centrifugation, the supernatant was poured off and the pellets re-suspended in 500 μ l CaHEPES. 100 μ l of this was centrifuged again at 17000 $\times g$ at 29°C for 3 mins then the supernatant removed and the pellet re-suspended in 100 μ l sterile AnalaR water. After that 20 μ l and 50 μ l of samples either predatory strain or HI strain were pipetted in cuvettes and filled up to 500 μ l with sterile AnalaR water. At this stage, Lowry solution C was prepared by mixing 100 parts Lowry A solution to 1 part Lowry B solution (see Appendices for recipes). 1.5 ml of solution C was added to each cuvette with mixed by pipette very well and all cuvette incubated

in dark for 20 mins. Following the first incubation period, 150 µl of Folin reagent solution (1 part of Folin: 1 part of AnalaR water) was added to each cuvette and mixed very well by pipetting and all cuvettes incubated in the dark for 45 mins. After 45 mins, the OD₇₅₀ values for BSA and samples were measured and the total protein content in *Bdellovibrio* culture determined using the standard curve.

2.13 *B. bacteriovorus* assays:

2.13.1 Host-independent attachment assay

The attachment assay was used to assess the ability of HI mutant strains of *Bdellovibrio* to attach to *E. coli* S17-1 prey cells. A 2.5 ml HI strain culture was grown under standard conditions in PY to OD₆₀₀ 1.5-2.0 was centrifuged at 17000 x *g* at 29°C for 3 mins then the supernatant was removed and re-suspended into 1 ml CaHEPES. A 10 ml *E. coli* S17-1 grown under standard conditions was centrifuged for 5 mins at 5525 x *g* and back-diluted to OD₆₀₀ 1.0 in CaHEPES. For recovering cells, both *Bdellovibrio* and *E. coli* cultures were incubated for 30 mins at 29 °C then infection was started by mixing 1 ml HI *B. bacteriovorus* culture, 1.5 ml *E. coli* S17-1 and 1 ml CaHEPES in tube at 29°C with 200 rpm shaking for 1 hour. During the 1 hr of incubation, samples were taken at specific time points 10 mins, 15 mins, 30 mins, 45 mins and 1 hr and immobilized on 1% agarose/CaHEPES. The attachment percentages were then analysed by taken microscopy pictures.

2.13.2 Host-independent predation assay

To examine the ability of *B. bacteriovorus* $\Delta bd0742$ and $\Delta bd0742$ suppressor strains to prey on *E. coli* S17-1, *A. baumannii*, *P. putida*, *S. marcescens* and *S. typhimurum* predation assays were conducted for these strains in comparison to a wild type HI *Bdellovibrio* control strain, HID26. Strains were matched by total protein content using Lowry protein assay (see Section 2.12.1). A HI cell suspension equivalent to 500 µg of total protein was taken of *B. bacteriovorus*

Δbd0742, *Δbd0742* suppressor alongside wild-type *HID26* strains added to 10 ml of different types of prey (*E. coli* S17-1, *A. baumannii*, *P. putida*, *S. marcescens* and *S. typhimurum* grown under standard conditions and back diluted to OD₆₀₀ 1.0 in CaHEPES. *E. coli* S17-1, *A. baumannii*, *P. putida*, *S. marcescens* and *S. typhimurum* were enumerated on YT agar at 0 hr, 24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs using Miles and Misra method (see Section 2.7.2).

2.14 Bioinformatic analysis

For bioinformatics analyses, the following programmes were used, unless stated otherwise: for identifying regions of sequence similarity NCBI BLAST (<http://blast.ncbi.nlm.nih.gov>) was used.

For global alignment of protein sequences, EMBOSS Needle alignment (http://www.ebi.ac.uk/Tools/psa/emboss_needle) was used. To identify the domains with motifs for any protein with cartoon structure (http://smart.embl.de/smart/show_motifs.pl) was used. For looking at the genomic context for any gene in *B. bacteriovorus* HD100 with its surrounding neighbourhood genes (<http://hactar.shef.ac.uk/xbase/>) was used.

2.15 Media recipes used in this study

All media and buffers were made using deionised water.

2.15.1 YT broth (per litre) for prey cells/conjugation strains growth

5 g NaCl
5 g Difco Bacto Yeast Extract
8 g Difco Bacto Tryptone
Adjusted to pH 7.5 with 2 M NaOH and solidified with Sigma-Aldrich Select agar A5054 at 10 g L⁻¹).

2.15.2 SOC broth (per litre) for transformation *E. coli* strains

20 g Difco Bacto Tryptone
5 g Difco Bacto Yeast Extract
0.58 g NaCl
0.186 g KCl
2.03 g MgCl₂
2.46 g MgSO₄
3.6 g Glucose

2.15.3 SPY agar (per litre) for generating HI *Bdellovibrio* isolates, streaking HI *Bdellovibrio* strains and HI way conjugation

10 g Difco Bacto Peptone
3 g Difco Bacto Yeast Extract
10 g Sigma-Aldrich Select agar A5054
Adjusted to pH 6.8 with 2 M NaOH

2.15.4 PY broth (per litre) for HI *B. bacteriovorus* strains growth

10 g Sigma-Aldrich Fluka Broadbean Peptone
3 g Difco Yeast Extract
Adjusted to pH 6.8 with 2 M NaOH

2.15.5 YPSC- bottom and top agar (per litre) for growth of HD *B. bacteriovorus* strains growth

1 g Difco Bacto Yeast Extract
1 g Difco Bacto Peptone
0.5 g Anhydrous Sodium Acetate
0.25 g MgSO₄·7H₂O
Adjusted to pH 7.6 with 2 M NaOH
(YPSC-top agar solidified with Sigma-Aldrich Select agar A5054 at 6 g L⁻¹; YPSC-bottom agar solidified with Sigma-Aldrich Select agar A5054 at 10 g L⁻¹).
Added sterile CaCl₂ from a 25 g L⁻¹ stock to give 0.25 g L⁻¹ after autoclaving.

2.15.6 CaHEPES buffer (per litre) for growth of HD *B. bacteriovorus* strains

5.94g HEPES
0.294 g CaCl₂·2H₂O
Adjusted to pH 7.6 with 2 M NaOH

2.16 Buffers used in this study

2.16.1 Buffers for production of chemically competent *E. coli* cells

2.16.1.1 TFB1

30 mM Potassium acetate
10 mM CaCl₂·2H₂O
50 mM MnCl₂·4H₂O
100 mM RbCl
Adjusted to pH 5.8 with 1 M acetic acid, 15% (v/v) glycerol added and made up to required volume with deionised water. Then filter-sterilised through a 0.2 µm filter.

2.16.2 TFB2

10 mM MOPS

75 mM CaCl₂·2H₂O

10 mM RbCl

Adjusted to pH 6.5 with KOH, 15% (v/v) glycerol added and made up to required volume with deionised water. Then filter-sterilised through a 0.2 µm filter.

2.16.3 Buffers for Lowry assay:

2.16.3.1 Solution A

2% (w/v) Na₂CO₃

0.4% (w/v) NaOH

0.16% (w/v) C₄H₄O₆Na₂·2H₂O (sodium tartrate dihydrate)

1% (w/v) SDS

Made up to required volume with deionised water and pH not adjusted.

2.16.3.2 Solution B

4% (w/v) CuSO₄·5H₂O

Made up to required volume with deionised water and pH not adjusted.

2.16.4 Buffers for agarose gel electrophoresis

2.16.4.1 10x TBE (per litre): 108 g Tris-HCl, 55 g Boric Acid, 40 ml 0.5 M EDTA pH 8.0

2.16.4.2 10x loading buffer: 15% (w/v) Ficoll 400, 0.4% (w/v) Orange G dye.

2.16.4.3 3x loading buffer: 900 µl 10x loading buffer, 2.1 ml 20% (w/v) glycerol.

2.16.4.4 DNA markers: 20 µl NEB DNA ladder stock (500 µg ml⁻¹), 120 µl 3x loading buffer, 160 µl TE (10 mM Tris-HCl 2 mM EDTA, pH 8.0), 100 µl 20% (w/v) glycerol.

3 Chapter three: Testing predation ability of suppression of diguanylate cyclase Δ *dgcB* (*bd0742*) deletion mutant and how Δ *bd0742* suppressor strains emerged

3.1 Introduction to GGDEF proteins in *Bdellovibrio*

Cyclic di-guanosine monophosphate (c-di-GMP) is a bacterial second messenger that regulates complex processes in bacteria (such as virulence factor, biofilm formation, and motility) (Chen et al. 2016). The synthesis and degradation of c-di-GMP are controlled by di-guanylate cyclase activity (DGC) and phosphodiesterase activity (PDE) respectively (Tal et al. 1998). Di-guanylate cyclase activity (DGC) is responsible for combining of two GTP molecules that occur in dimerized GGDEF domains to synthesise c-di-GMP, while degradation of c-di-GMP happens in dimeric HD-GYP or EAL domains with phosphodiesterase activity (PDEs).

Bdellovibrio has five GGDEF domain proteins that are predicted to participate in c-di-GMP signalling. These are Bd0367 (DgcA), Bd0742 (DgcB), Bd1434 (DgcC), Bd3125 (CdgA) and Bd3766 (DgcD). Three of these proteins: Bd0367, Bd0742 and Bd3766 have a GGDEF domain plus one or more accessory domains at the N-terminus that might act as either an activator or an inhibitor of c-di-GMP synthesis by interaction with a second protein. Bd1434 and Bd3125 proteins seemed initially to have a GGDEF domain alone with no recognisable extra domain (from SMART database, <http://smart.embl-heidelberg.de/>), (Hobley et al. 2012). The GGDEF domain of Bd3125 is a degenerate GVNF and Hobley and co-workers found it acts as a receptor rather than a producer for the c-di-GMP.

Hobley and co-workers (2012) deleted/ interrupted genes encoding all c-di-GMP synthetic proteins in *Bdellovibrio* and worked to find what phenotype can result from their deletion in both HD and HI lifestyles. They found that impaired predatory growth, especially in prey entry stage, occurs only for Δ *bd0742* (*DgcB*) and Δ *bd3125* (*CdgA*) mutants and this suggested that both of them belong to the same regulatory cascade which might be that *bd0742* signals to *bd3125* through

c-di-GMP synthesis. Also, when Hobley and co-worker (2012) deleted the *bd3766* gene, they found that $\Delta bd3766$ mutant had no obvious phenotype.

bd0742 (dgcB) is one of the four genes in *B. bacteriovorus* that encodes for active GGDEF proteins that function as diguanylyl cyclases that can synthesise the c- di GMP molecules. The two adjacent genes to *bd0742* are in the same orientation on the *B. bacteriovorus* chromosome; they are *bd0741 (pgi)* and *bd743 (rpoE)*. *bd0741* encodes Glucose-6-phosphate isomerase, an enzyme that plays a role in the glycolysis pathway whereas *bd0743*, encodes the RNA polymerase sigma-E factor an enzyme that plays a role in DNA-templated transcription, initiation (Figure 3-1) (<http://hactar.shef.ac.uk/xbase/>) (Lambert et al. 2012).

The deletion of *bd0743* gene resulted in a mutant strain that is smaller and slower in swimming than wild type but still able grow in host- dependent and host-independent cultures (Lambert et al. 2012). Gene *bd0742* is predicted in silico to be transcribed from within an operon with *bd743 (rpoE)* downstream (<http://operons.ibt.unam.mx/OperonPredictor/>).

Bd0742 is essential for the predation ability of *Bdellovibrio*, and its deletion resulted in *B. bacteriovorus* being unable to grown Host- dependently on *E. coli* prey after 48 hrs of testing but growing obligatorily axenically (Hobley et al. 2012)

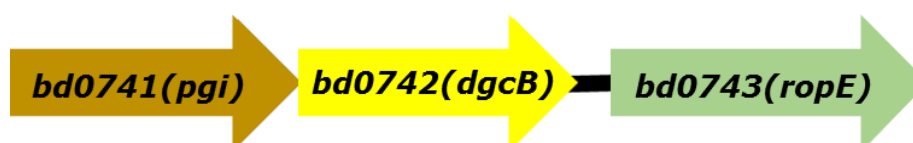


Figure 3. 1: The genomic context of *B. bacteriovorus* *bd0742* gene (<http://hactar.shef.ac.uk/xbase/>).

Bd0742 expression was examined by RT-PCR, and it expressed in a continuous manner throughout *Bdellovibrio* predatory life cycle (unpublished data with permission from Dr. Carey Lambert) shown with permission in Figure (3-2).



Figure 3. 2: Reverse-transcriptase PCR showing transcriptional expression of *bd0742* throughout the predatory cycle. RNA was isolated at the indicated time points during one round of synchronous *Bdellovibrio* HD100 infection of *E. coli* host prey cells, and primers specific to the gene were used to amplify (RT-PCR, 27 cycles) 100 bp internal fragment from each transcript if present. AP = Attack phase (free-swimming *Bdellovibrio*); 15, 30, 45 = minutes since start of infection (attachment to and rounding of *E. coli* host cell into a bdelloplast structure); 1h, 2h, 3h, 4h = hours since start of infection (host cell resources being degraded and used for *Bdellovibrio* growth into a filament, followed by septation into multiple progeny and eventually lysis from host cell); Ec = *E. coli* S17-1 RNA (no *Bdellovibrio* control); gen = *B. bacteriovorus* HD100 genomic DNA (positive control); NT = No template control; L = NEB 100 bp DNA ladder. (Unpublished data with permission from Dr. Carey Lambert)

RT-PCR was performed on total RNA (sample matched 10 ng/ μ L) isolated from the attack phase and different HI strains (HID2, HID13, HID22 and HID26). Figure (3-3) showed that *bd0742* expression is higher in all HI strains than the attack phase and this agreed with Microarray data.

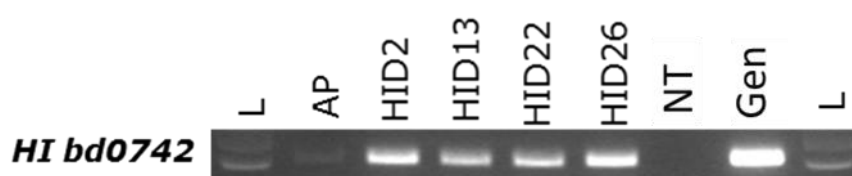


Figure 3. 3: Reverse-transcriptase PCR showing relative transcription expression of the *B. bacteriovorus* HD100 *bd0742* during attack phase and host-independent growth. RNA was isolated from *B. bacteriovorus* HD100 attack phase (AP) cells and four host-independent strains (HID2, HID13, HID22, HID26) at OD600 0.6 and the RNA concentration was matched to 10 ng μ L⁻¹. Primers specific to gene were used to amplify (RT-PCR, 30 cycles) an approximately 110 bp internal fragment from each transcript if present. Gen = *B. bacteriovorus* HD100 genomic DNA (positive control); NT = no template control; L = NEB 100 bp DNA ladder. (Unpublished data with permission from Dr. Carey Lambert)

The transcription of *bd0743* was examined by RT-PCR, and it was expressed mostly continuously during the *Bdellovibrio* life cycle with a drop at 4 hrs shown in Figure (3-4) (Lambert et al. 2012).



Figure 3. 4: Reverse-transcriptase PCR showing transcriptional expression of *bd0743* throughout the predatory cycle. RNA was isolated at the indicated time points during one round of synchronous *Bdellovibrio* HD100 infection of *E. coli* host prey cells, and primers specific to the gene were used to amplify (RT-PCR, 25 cycles) 100 bp internal fragment from each transcript if present. AP = Attack phase (free-swimming *Bdellovibrio*); 15, 30, 45 = minutes since start of infection (attachment to and rounding of *E. coli* host cell into a bdelloplast structure); 1h, 2h, 3h, 4h = hours since start of infection (host cell resources being degraded and used for *Bdellovibrio* growth into a filament, followed by septation into multiple progeny and eventually lysis from host cell); L = NEB 100 bp DNA ladder (Lambert et al. 2012).

The RT-PCR analysis to detect if there is co-transcription between *bd0741* and *bd0742* was run by the summer student Sarah Basford under Simon King Master student supervision, and the results showed that there is no shared operon found between these two genes.

Bd0742 (DgcB) is a 320 amino acid protein that is annotated as a GGDEF domain protein. Bd0742 also contains a Forkhead-associated (FHA) domain (which runs from amino acids 66-116) that are found in the N-terminus of the protein followed by the GGDEF domain (which runs from amino acids 146-319). The FHA domain is known from other bacteria to be involved in interactions with other proteins through binding threonine (pThr) residues of other proteins (Figure 3-5) (Pennell et al. 2010, Hopley et al. 2012).



Figure 3. 5: The domains of Bd0742 protein. The protein consists of a forkhead-associated domain (FHA) at the N-terminus followed by the GGDEF domain (Image taken from SMART database, <http://smart.embl-heidelberg.de/>).

3.2 Deletion of bd0742 (dgcB)

Before I started my PhD study in the Sockett laboratory, Dr Hobley a postdoctoral researcher and Master student Rowena Fung made the deletion of *bd0742* (*dgcB*) to investigate the role of Bd0742 alongside the other GGDEF genes. They asked if Bd0742 is required for invasion of *E. coli* S17-1 by *B. bacteriovorus* HD100 in the predatory lifecycle. A kanamycin insertion deletion construct Δ *bd0742:Kan* was conjugated into *B. bacteriovorus*, recombining initially by single crossover on a suicide plasmid and maintained as a double crossover by kanamycin selection. Later a silent deletion strain was also made Δ *bd0742* and the resultant single crossover *Bdellovibrio* plaques from conjugation were screened for potential *Bdellovibrio* Δ *bd0472* mutants in both host-dependent (HD) and host-independent (HI) with the sucrose-suicide selection. All host-dependent attempts to obtain predatory *B. bacteriovorus* Δ *bd0472* on *E. coli* S17-1 prey were unsuccessful (56 populations were screened, and merodiploid populations only were obtained from all the different conjugations), while four host-independent of Δ *bd0742* isolates were obtained from 43 isolates tested. This means that *bd0742* is essential for the predatory life cycle but not for the host-independent cycle (Hobley et al. 2012). This revealed that the deletion of *bd0742* forced the *Bdellovibrio* to grow as a Host-independent (HI) strain and Hobley and co-workers (2012) retested the ability of the HI *B. bacteriovorus* Δ *bd0742* mutant strain to invade and prey on *E. coli* S17-1 for 48hrs and they found that HI *B. bacteriovorus* Δ *bd0742*

mutant strain was unable to lyse *E. coli* S17-1 whereas wild type HI *B. bacteriovorus* was able lyse *E. coli* S17-1.

3.3 How deletion of *bd0742* produced the Δ *bd0742* suppressor strains

Dr. Hobley and Masters Student Simon King worked further on the HI *B. bacteriovorus* Δ *bd0742* mutant strain again to test the ability of HI *B. bacteriovorus* Δ *bd0742* mutant strain to prey on *E. coli* S17-1 by leave the HI mutant cells for a longer period than 48 hrs with prey. King set up 21 cultures on *E. coli* S17-1 prey in Ca/HEPES buffer from three separate HI *B. bacteriovorus* Δ *bd0742* mutant strain HI isolates versus three cultures of WT HI as controls. They found that after 9 hrs that WT HI control cultures started to clear by killing the prey while HI *B. bacteriovorus* Δ *bd0742* mutant strain cultures had no signs of bdelloplast formation in their cultures. After 24 hrs, WT HI control cultures were fully cleared from *E. coli* S17-1 prey but HI *B. bacteriovorus* Δ *bd0742* mutant strain cultures were filled with prey.

After 120 hr incubation however, 4 of the 21 cultures of HI *B. bacteriovorus* Δ *bd0742* mutant strains started to kill prey, and bdelloplast formation could be seen and these strains were named *Δbd0742 suppressor* strains whereas the rest of the 17 of HI *B. bacteriovorus* Δ *bd0742* mutant strain cultures showed no bdelloplasts and no prey killing after the same incubation time.

The presence of the original deletion of *bd0742* in the suppressor strain was retested by PCR, and confirmed. The same experiment was repeated on two separate occasions using luminescent *E. coli* S17-1 prey in 96 well plate using 18 cultures from two separate HI *B. bacteriovorus* Δ *bd0742* mutant isolates, and this time after 72 hr no significant decrease in luminescence could be observed which indicated that the HI *B. Bacteriovorus* Δ *bd0742* mutant strain is non-predatory and that rare suppressor mutants may be isolated.

When I started my work on HI *B. bacteriovorus* $\Delta bd0742$ mutant strain, I derived some new $\Delta bd0742$ suppressor strains when I let HI *B. bacteriovorus* $\Delta bd0742$ mutant strain incubated for extended period with *E. coli* S17-1 and *A. baumannii*. From all the three sources of $\Delta bd0742$ suppressor strains that were produced from HI *B. bacteriovorus* $\Delta bd0742$ mutant strain extended growth on *E. coli* S17-1 or latterly in my hands on *E. coli* S17-1 and *A. baumannii*, I prepared genomic DNA and sent for the whole genome sequences. The results of this more extensive genome sequencing will discuss later.

In this chapter, I reported the ability of suppression arose in the genome of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain from *E. coli* S17-1 after extending predation assay and also asking about the probability of suppression arose in the genome HI *B. bacteriovorus* $\Delta bd0742$ mutant strain upon using different types of prey other than *E. coli* S17-1 including *A. baumannii*, *P. putida*, *S. marcescens* and *S. typhimurum*. As will become obvious later- differences were found at the more extended time points of predation assays.

Also, I tested if prolonged incubation of overlay plates of this strain with different prey lawns can produce differences like the liquid cultures and start to produce plaques or not. In addition, I will report if HI *B. bacteriovorus* $\Delta bd0742$ mutant Strain has the ability to attach properly to prey cell or not comparing to wild type *B. bacteriovorus* HID26. Also, I will report when and how the suppression emerge from HI *B. bacteriovorus* $\Delta bd0742$ mutant strain during the predation assay. Moreover, I will discuss the results of the genome sequencing and what is the strikingly common factor in the genome of $\Delta bd0742$ suppressor strains that plays vital role leading these strains to arise.

3.4 Specific Research Aims

1. To investigate if there is any co-transcription between *bd0742* and *bd0743*.
2. To test predation and subsequently ability of suppression to occur in the genome of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain when incubated in liquid cultures with *E. coli* S17-1 for extended time periods with compared to HID26 the wild-type predatory control.
3. To test the ability of suppression to occur in the genome of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain when incubated in liquid cultures with some human pathogen for extended time periods with compared to HID26 the wild-type predatory control.
4. To test the ability of suppression to occur in the genome of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain when incubated for extended time periods on different prey lawns through formation plaques.
5. To test the attachment ability of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain to prey on *E. coli* S17-1 compared to the wild type control HID26 strain at specific time points during 1 hr of infection.
6. To explore how and when the suppression starts to become obvious in HI *B. bacteriovorus* $\Delta bd0742$ mutant strain populations and results in production of purifiable suppressor strains during the predation assay that runs for 120 hrs.

3.5 Expected outcome

1. Suppression occurs in the genome of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain when incubated with *E. coli* but, requires extended time.
2. Suppression occurs in the genome of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain when incubated with some human pathogens but, requires extended time.
3. Suppression occurs in the genome of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain when incubated for extended time period (21 days) with *E. coli* and some human pathogens on lawns plates through formation plaques.

4. The ability of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain attachment not affected by deletion of *bd0742* and strain able to attach properly to prey cells.
5. The suppression in HI *B. bacteriovorus* $\Delta bd0742$ mutant strain genome start to become obvious in 96 hrs of the predation assay.

3.6 Results

3.6.1 The *bd0742 (dgcB)* gene is not cotranscribed from an operon with *bd0743 (rpoE)*

RT-PCR was performed in order to identify whether *bd0742* and *bd0743* are co-transcribed in an operon using matched amount of RNA (10 ng/μL) isolated from *B. bacteriovorus* HD100 strain during 1 hr of the predatory lifecycle, where transcription levels were previously known to be high for both genes (Hobley and Lambert 2012 unpublished data). There was no continuous transcript as indicated by a 1110 bp product band which mean that *bd0742* and *bd0743* are not collocated in an operon (Figure 3-6).

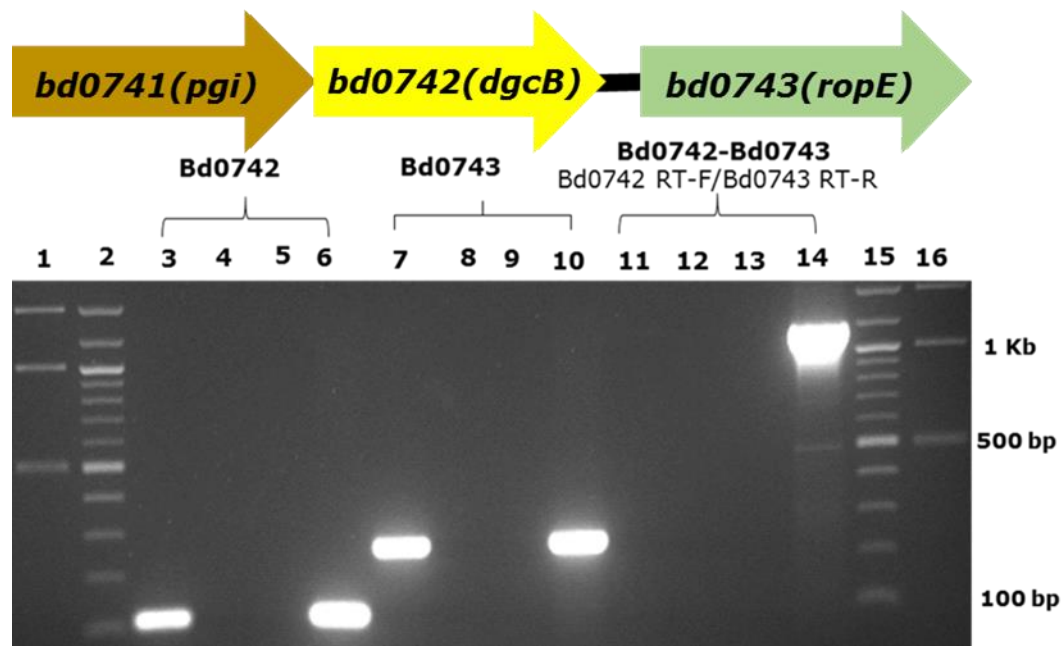


Figure 3. 6: RT-PCR shows that the *B. bacteriovorus* genes *bd0742 (dgcB)* and *Bd0743 (rpoE)* are not co-transcribed. Lanes 1 and 16: NEB 1000 bp ladder; Lanes 2 and 15: NEB 100 bp ladder; Lanes 3, 7 and 11: predator and prey total RNA at 1 hr; Lanes 4, 8 and 12: *E. coli* S17-1 RNA; Lanes 5, 9 and 13: No template; Lanes 6, 10 and 14: *B. bacteriovorus* HD100 genomic DNA. Lane 14 giving a 1110 bp band, not found in lane 11 is indicative that there is no co-transcription between *bd0742* and *bd0743* genes.

3.6.2 Suppression of *Δbd0742* deletion in HI *B. bacteriovorus Δbd0742* mutant strain genome occurred when incubated with *E. coli* S17-1 or *A. baumannii* prey for an extended time periods, in liquid culture.

Previously, the predation ability of HI *B. bacteriovorus Δbd0742* mutant strain was tested by Hobley and co-worker and they found that this strain is non-predatory when left with prey for 48 hrs (Hobley et al. 2012). Therefore, in this section and next section I test the possibility of suppression arose in the HI *B. bacteriovorus Δbd0742* mutant strain when incubated with different types of prey cells including human pathogens.

To examine the ability of suppression to occur in HI *B. bacteriovorus Δbd0742* mutant strain genome when incubated with *E. coli* and on *A. baumannii* for extended time period, predation assays were conducted using a starting *E. coli* S17-1 or *A. baumannii* stationary phase inoculum of OD₆₀₀ 1.0 (10 ml containing 4.22x10⁸ and 7.17x10⁸ per ml for *E. coli* and *A. baumannii* respectively) with HI *B. bacteriovorus Δbd0742* mutant strain and HID26 strains matched by Lowry protein assay (500 mg protein per strain). Lowry method was used to match cultures as no plaque counts are possible for HI *B. bacteriovorus Δbd0742* mutant strain (see Section 2.12.1 and CFU are not reliable). The assays were performed for 120 hrs for both strains in triplicate.

As shown in Figure (3-7-B and C), HI *B. bacteriovorus Δbd0742* mutant strain was unable to lower the number of *E. coli* S17-1 until suppression arose and become obvious and then the *Bdellovibrio* population started to kill *E. coli* (~ two log drop in CFUs of *E. coli* S17-1) at 96 hrs or more compared with the HID26 wild type strain (which is a positive predatory control) which rapidly decreased the number of *E. coli* S17-1 after only 24 hrs (~ three log drop in CFUs of *E. coli* S17-1) (Figure 7-4 A and C).

With regards to *A. baumannii* prey, HI *B. bacteriovorus* $\Delta bd0742$ mutant strain was unable reduced the number of *A. baumannii* until suppression arose and become obvious and start to kill *A. baumannii* ($\sim$ three log drop in CFUs of *A. baumannii*) at 72 hrs for two biological repeats but suppression did not occur in the third repeat (Figure 3-8 B and C) compared to WT HID26 strain (a positive predatory control) which rapidly decreased the number of *A. baumannii* after only 48 hrs ($\sim$ three log drop in CFUs of *A. baumannii*) for all three biological repeats (Figure 3-8 A and C).

These results matched with those previously found by Hobley and her co-workers about deletion of *bd0742* abolish the predation ability of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain on prey cells. Knowing that possible suppression in the genome of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain may be occurring from Simon King and David Milner work. The prey lysates resulting from possible suppression in genome of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain, that led to emerge new Suppressor strains that able to lyse *E. coli* S17-1 and *A. baumannii* after 72 hrs and 96 hrs, were tested directly if they were still alive by filtering out residual prey and debris and adding a new *E. coli* prey for them and they lysed this prey. The genomic DNAs were prepared from the lysates of possible Suppressor strains cells and sent for genome sequencing and their results will explained at the end of this chapter

3.6.3 Suppression of *Δbd0742* deletion in HI *B. bacteriovorus Δbd0742* mutant strain did not arise when incubated with *P. putida*, *S. marcescens* or *S. typhimurum* prey even after extended time periods, in liquid culture.

To investigate the ability of suppression to occur in HI *B. bacteriovorus Δbd0742* mutant strain genome when incubated with other prey types: *P. putida*, *S. marcescens* and *S. typhimurum*, predation assays were carried out using a starting of *P. putida*, *S. marcescens* and *S. typhimurum* inoculum of OD⁶⁰⁰ 1.0 (10 ml containing 1.53X10⁹, 1.53X10⁹, 1.53X10⁹ *P. putida*, *S. marcescens* and *S. typhimurum*) with HI *B. bacteriovorus Δbd0742* mutant strain and HID26 strains matched by Lowry protein assay (500 mg protein per strain). Lowry method was used to match cultures as no plaques counts are possible for *B. bacteriovorus Δbd0742* (see Section 2.12.1 and CFU are not reliable). The predation assay was performed for 120 hr for all strains in triplicate.

As it is apparent from Figure (3-9 B and C) that no suppression took place or was selected for in HI *B. bacteriovorus Δbd0742* mutant strain so there was no reduction in the number of *P. putida* after 120 hrs compared with HID26 (Figure 3-9 A and C) which was able to lower the number of *P. putida* after 72 hrs (~ 2.5 log drop in CFUs of *P. putida*).

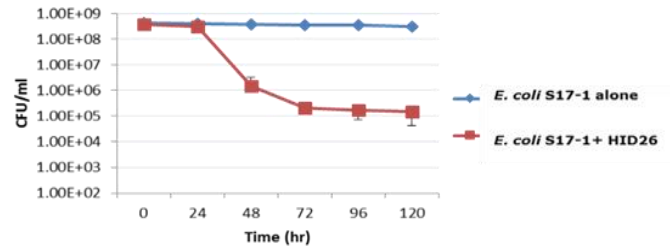
With respect to *S. marcescens* as prey, no suppression took place or was selected for in HI *B. bacteriovorus Δbd0742* mutant strain genome so there was no drop in *S. marcescens* cells number after 120 hr Figure (3-10 B and C) compared with HID26 which is (a positive predatory control) and was able to decrease the number of *S. marcescens* after 72 hrs (~ two log drop in CFUs of *S. marcescens*) (Figure 3-10 A and C).

Concerning *S. typhimurum* as prey, no suppression also took place or was selected for in HI *B. bacteriovorus Δbd0742* mutant strain genome therefore no reduction in number of *S. typhimurum* after 120 hrs (Figure 3-11 B and C) as compared to positive predatory control HID26 which was decreased the number of *S.*

typhimurum after 72hrs (~ two log drop in CFUs of *S. typhimurum*) (Figure 3-11 B and C).

These results fitted with results of Hobley and co-workers who pointed out that deletion of *bd0742* enforced HI *B. bacteriovorus* $\Delta bd0742$ mutant strain to be non- predatory. Additional to that, these results also means that no suppression can occurred when HI *B. bacteriovorus* $\Delta bd0742$ mutant strain is incubated with *P. putida*, *S. marcescens* or *S. typhimurum*.

A: Control



B: B1,2,3 Test (all three repeats)

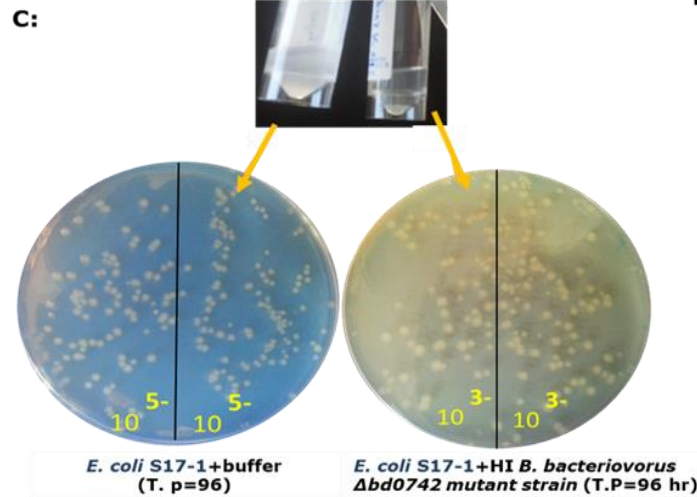
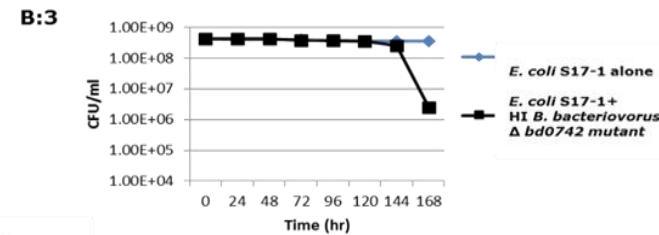
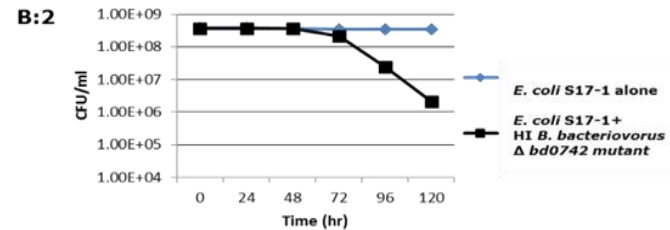
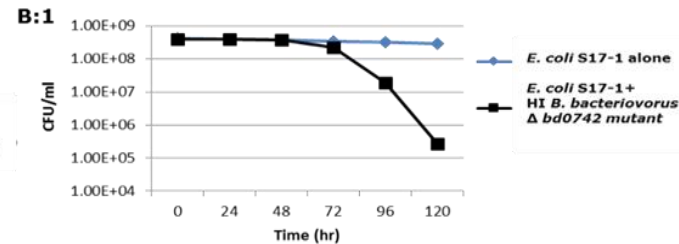


Figure 3. 7: The results of testing possibility of suppression occurrence in genome of HI *B. bacteriovorus* Δbd0742 mutant strain during predation assays tested against wild-type (WT) HI *B. bacteriovorus* (HID26) strain as the predatory control on *E. coli* S17-1 prey (from 3 biological repeats in separate weeks). The error bars represent 1 SD from the mean.

- (A) Control predation of *B. bacteriovorus* WT HI *B. bacteriovorus* (HID26) strain on *E. coli* S17-1 prey was examined by drop in number of *E. coli* over 120 hrs. In liquid cultures, HID26 started to reduce the number of *E. coli* after 24hrs (from three biological repeats).
- (B) 1,2,3: test predation ability of HI *B. bacteriovorus* Δbd0742 mutant strain on *E. coli* S17-1 prey was examined by drop in number of *E. coli* over 120 hrs. In liquid cultures, HI *B. bacteriovorus* Δbd0742 mutant strain unable reduce the number of *E. coli* until the suppression took place at 96 hrs or more.
- (C) Tubes of *E. coli* S17-1+ buffer control and *E. coli*+ HI *B. bacteriovorus* Δbd0742 mutant strain infection and their corresponding serial dilution plates at 96 hrs.

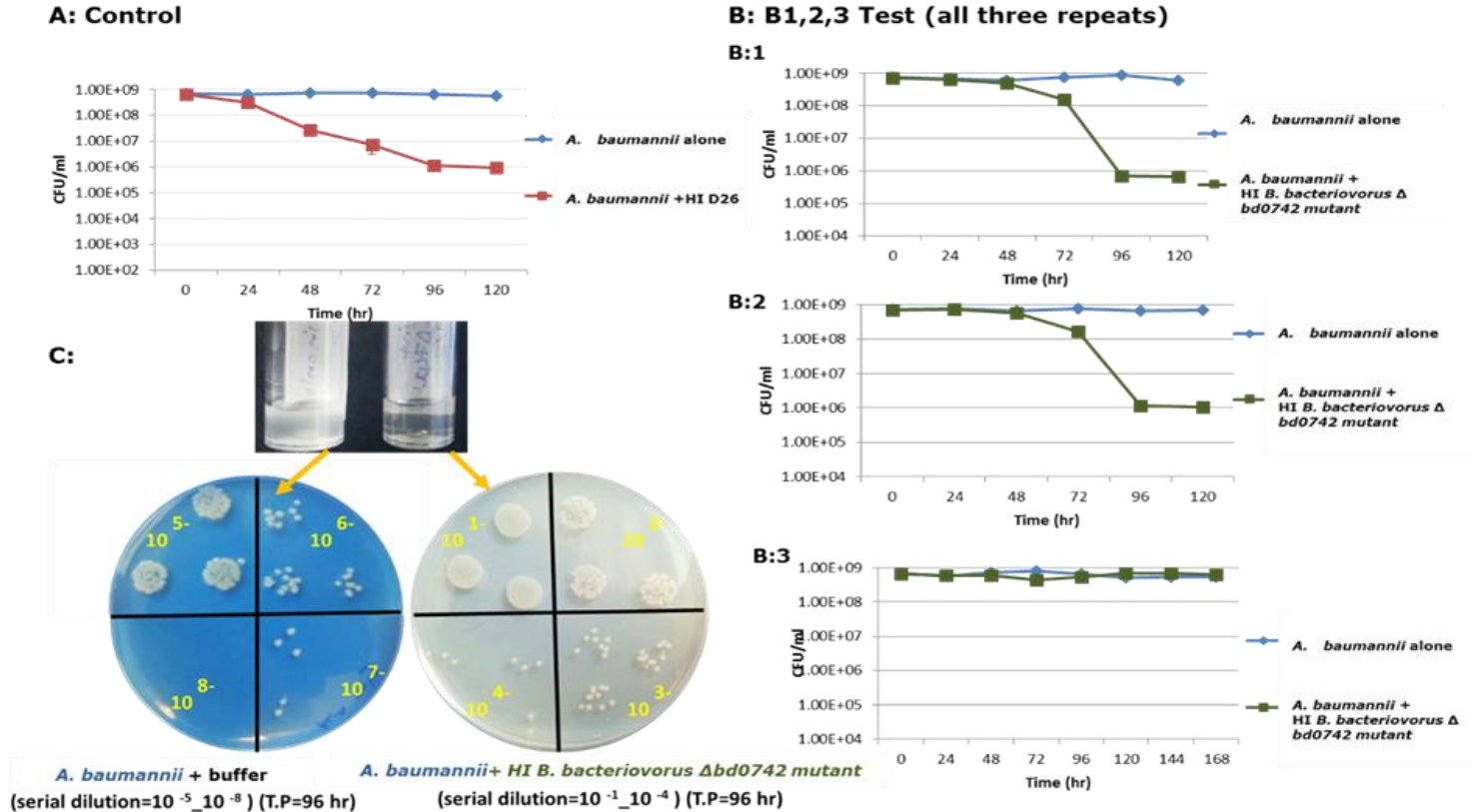


Figure 3. 8: The results of testing possibility of suppression occurrence in genome of HI *B. bacteriovorus* Δbd0742 mutant strain during predation assays tested against wild-type (WT) HI *B. bacteriovorus* (HID26) strain as the predatory control on *A. baumannii* prey (from 3 biological repeats in separate weeks). The error bars represent 1 SD from the mean.

- (A) Control predation of *B. bacteriovorus* WT HI *B. bacteriovorus* (HID26) strain on *A. baumannii* prey was examined by drop in number of *A. baumannii* over 120 hrs. In liquid cultures, HID26 started to reduce the number of *A. baumannii* after 24hrs (from three biological repeats).
- (B) 1,2,3: test predation ability of HI *B. bacteriovorus* Δbd0742 mutant strain on *A. baumannii* prey was examined by drop in number of *A. baumannii* over 120 hrs. In liquid cultures, HI *B. bacteriovorus* Δbd0742 mutant strain unable reduce the number of *A. baumannii* until the suppression took place at 72 hrs.
- (C) Tubes of *A. baumannii* + buffer control and *A. baumannii* + HI *B. bacteriovorus* Δbd0742 mutant strain infection and their corresponding serial dilution plates at 96 hrs.

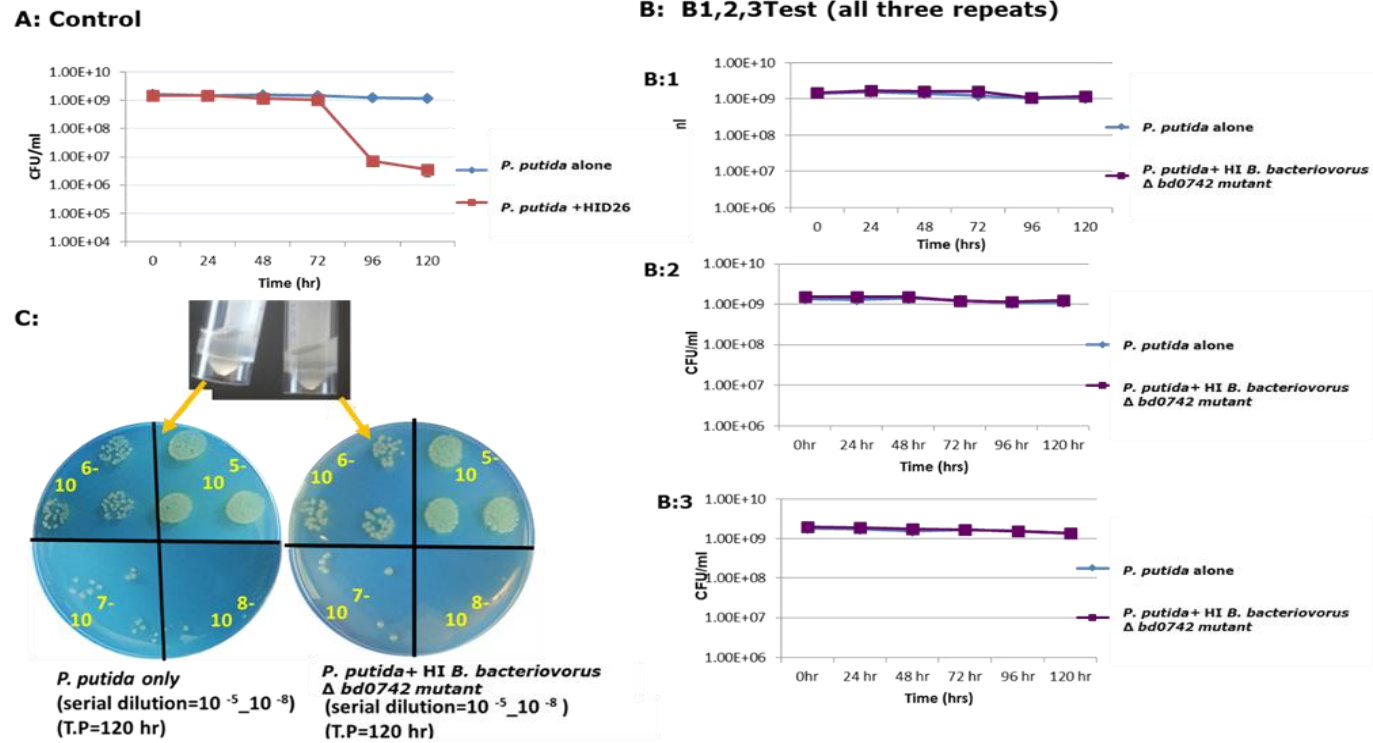


Figure 3. 9: The results of testing possibility of suppression occurrence in genome of HI *B. bacteriovorus* Δbd0742 mutant strain during predation assays tested against wild-type (WT) HI *B. bacteriovorus* (HID26) strain as the predatory control on *P. putida* prey (from 3 biological repeats in separate weeks). The error bars represent 1 SD from the mean.

- (A) Control predation of *B. bacteriovorus* WT HI *B. bacteriovorus* (HID26) strain on *P. putida* prey was examined by drop in number of *P. putida* over 120 hrs. In liquid cultures, HID26 started to reduce the number of *P. putida* after 72 hrs (from three biological repeats).
- (B) 1,2,3: test predation ability of HI *B. bacteriovorus* Δbd0742 mutant strain on *P. putida* prey was examined by drop in number of *P. putida* over 120 hrs. In liquid cultures, HI *B. bacteriovorus* Δbd0742 mutant strain unable reduce the number of *P. putida*. The suppression did not happen so there were no decrease in *P. putida* cells number.
- (C) Tubes of *P. putida* + buffer control and *P. putida* + HI *B. bacteriovorus* Δbd0742 mutant strain infection and their corresponding serial dilution plates at 120 hrs.

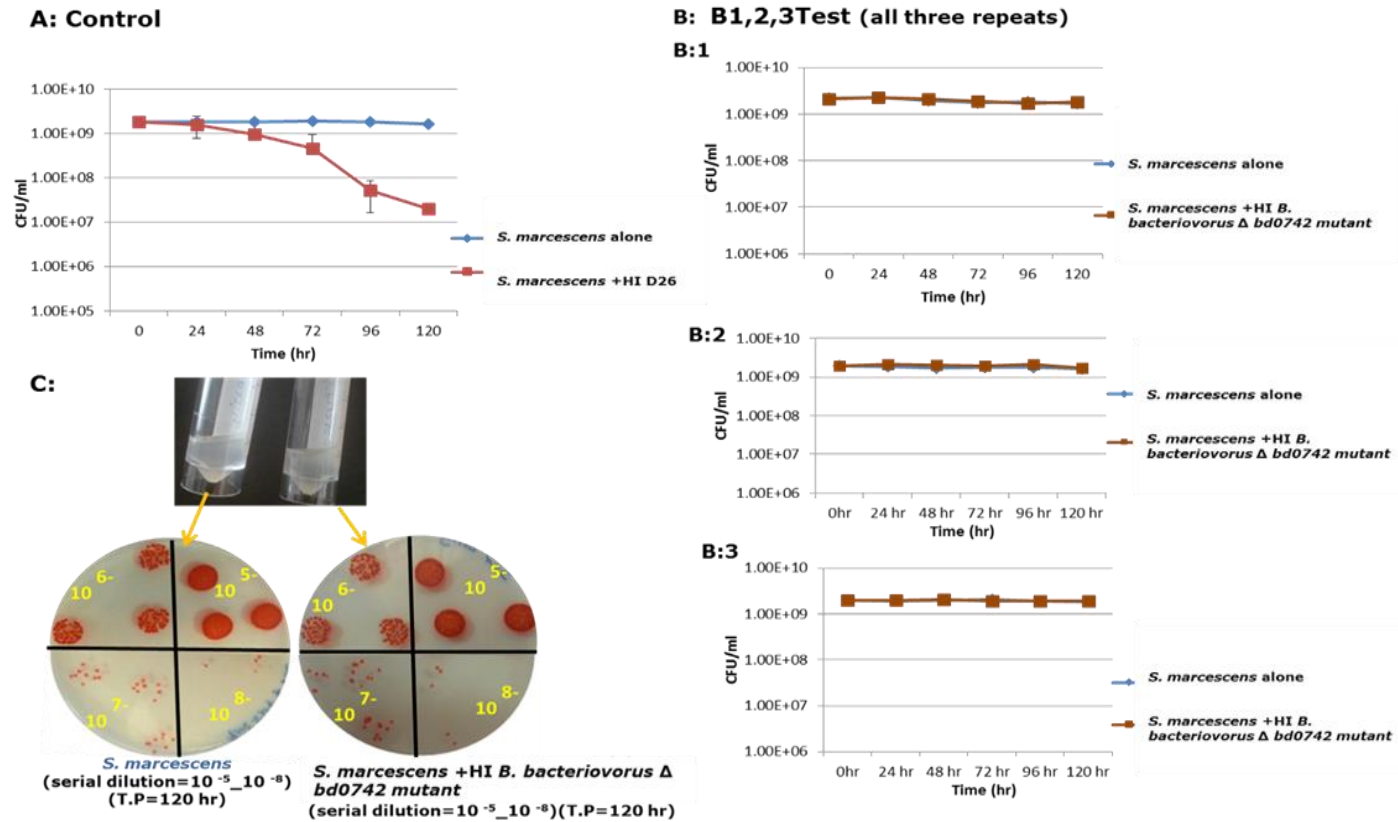


Figure 3. 10: The results of testing the possibility of suppression occurrence in genome of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain during predation assays tested against wild-type (WT) HI *B. bacteriovorus* (HID26) strain as the predatory control on *S. marcescens* prey (from 3 biological repeats in separate weeks). The error bars represent 1 SD from the mean.

- (A) Control predation of *B. bacteriovorus* WT HI *B. bacteriovorus* (HID26) strain on *S. marcescens* prey was examined by drop in number of *S. marcescens* over 120 hrs. In liquid cultures, HID26 started to reduce the number of *S. marcescens* after 72 hrs (From three biological repeats).
- (B) 1,2,3: test predation ability of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain on *S. marcescens* prey was examined by drop in number of *S. marcescens* over 120 hrs. In liquid cultures, HI *B. bacteriovorus* $\Delta bd0742$ mutant strain unable reduce the number of *S. marcescens*. The suppression did not happen so there were no decrease in *S. marcescens* cells number.
- (C) Tubes of *S. marcescens* + buffer control and *S. marcescens*+ HI *B. bacteriovorus* $\Delta bd0742$ mutant strain infection and their corresponding serial dilution plates at 120 hrs.

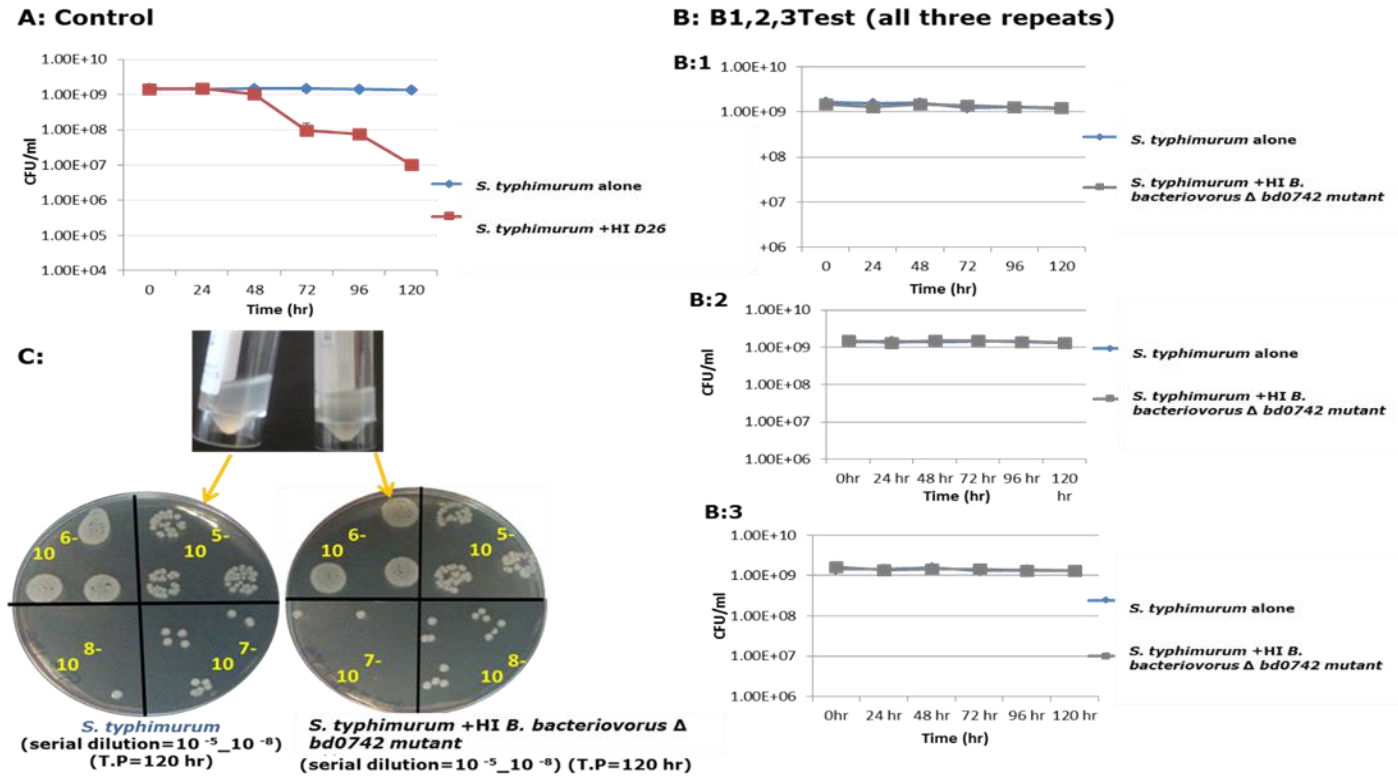


Figure 3. 11: The results of testing the possibility of suppression occurrence in genome of HI *B. bacteriovorus* Δbd0742 mutant strain during predation assays tested against wild-type (WT) HI *B. bacteriovorus* (HID26) strain as the predatory control on *S. typhimurium* prey (from 3 biological repeats in separate weeks). The error bars represent 1 SD from the mean.

- (A) Control predation of *B. bacteriovorus* WT HI *B. bacteriovorus* (HID26) strain on *S. typhimurium* prey was examined by drop in number of *S. typhimurium* over 120 hrs. In liquid cultures, HID26 started to reduce the number of *S. typhimurium* after 72 hrs (from three biological repeats).
- (B) 1,2,3: test predation ability of HI *B. bacteriovorus* Δbd0742 mutant strain on *S. typhimurium* prey was examined by drop in number of *S. typhimurium* over 120 hrs. In liquid cultures, HI *B. bacteriovorus* Δbd0742 mutant strain unable reduce the number of *S. typhimurium*. The suppression did not happen so there were no decrease in *S. typhimurium* cells number.
- (C) Tubes of *S. typhimurium* + buffer control and *S. typhimurium* + HI *B. bacteriovorus* Δbd0742 mutant strain infection and their corresponding serial dilution plates at 120 hrs.

3.6.4 Testing if suppression in genome of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain can occur on plates of different types of prey lawn.

The ability of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain to produce plaques were previously tested by Dr Hobley and co-workers, and they were found that the deletion of the *bd0742* led *B. bacteriovorus* to lose its ability to produce plaques following incubation the plates for 7 days and the ability to produce plaques returned back when complemented with wild type *bd0742* in *B. bacteriovorus* $\Delta bd0742$. As I have shown in (Figure 3-7, 3-8, 3-9, 3-10 and 3-11), suppression happened in liquid cultures for some types of prey after extended incubation periods. For that reason, I turned to the usual plaque assay to try to study if suppression can occur on solid surface (plates of prey lawns).

Therefore, here I tested the ability of suppression to arise in genome of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain to occur and kill the prey in lawns plates and produce plaques, when plates of this strain were incubated at 29 °C for 21 days using different types of prey in top agar lawns (as seen in liquid cultures of *B. HI B. bacteriovorus* $\Delta bd0742$ mutant strain with some prey when the liquid cultures were left for more than 48 hrs). Even after this extended incubation (21 days) no suppression occurred in genome of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain on plates as they were unable to produce the plaques on different prey lawns (Figure 3-12). This indicates that the suppression only take place in liquid cultures on some types of prey but not on the prey lawn plates.

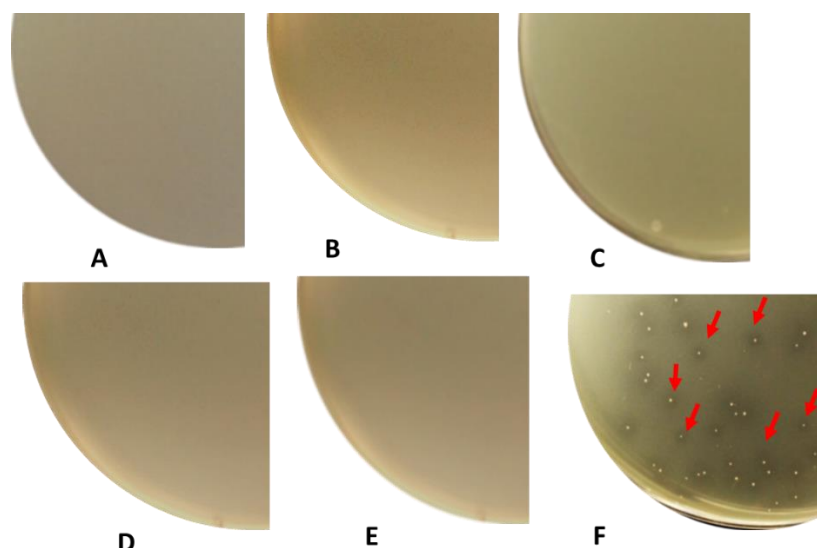


Figure 3. 12: The plaque plates assay to test the occurrence of suppression in HI *B. bacteriovorus* $\Delta bd0742$ mutant strain on double layer YPSC overlay plates containing different prey (A: *E. coli* S17-1 , B: *A. baumannii*, C: *P. putida*, D: *S. marcescens* and E: *S. typhimurum* F: WT HI D26 the control strain). No suppression occurred in HI *B. bacteriovorus* $\Delta bd0742$ mutant strain genome so no prey killed and no plaques formed on the prey lawns after incubation for 3 weeks comparing with control strain the WI HI D26.

3.6.5 Testing the attachment ability of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain compared to the wild type control *HID26*

B. bacteriovorus $\Delta bd0742$ mutant strain was unable to lyse *E. coli* in a lawn on YPSC overlay plates and took a long time (due to suppressor accumulation/ arising) to lyse some types of prey in liquid culture. Introduction of wild type *bd0742* by *in cis* complementation described by Hobley et al (2012) found that this restored the plaque forming ability on the prey lawn plates. Attaching to the prey in liquid culture versus on the prey lawn is physically different so I tested where the defect in attachment ability of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain. Therefore, the nature of the predatory defect of the HI *B. bacteriovorus* $\Delta bd0742$ mutant strain was analysed further by microscopy, using an attachment assay for estimating the percentage of HI cells attaching to *E. coli* S17-1 by

addition of the HI *B. bacteriovorus* $\Delta bd0742$ mutant strain and wild-type HID26 strain to *E. coli* S17-1 and incubated for 1 hr.

This assay demonstrated that HI *B. bacteriovorus* $\Delta bd0742$ mutant cells had some ability to attach to the *E. coli* S17-1 cells but at a lower percentage compared to HID26 which had high percentage as shown in Table (3-1) and this was observed for all time points.

Table 3-1: Shows the Host-independent attachment assays for HI *B. bacteriovorus* $\Delta bd0742$ mutant and the wild type control (HID26) strains. The percentage of attached and unattached of HI *B. bacteriovorus* $\Delta bd0742$ mutant and WT HID26 strains to *E. coli* S17-1 at specific time points (10mins, 15 mins, 30 mins and 45 mins) after adding the prey cells were counted from three biological repeats (the cells no.=282 for HI *B. bacteriovorus* $\Delta bd0742$ mutant strain and the cells no.=305 for HID26 strain).

Time (mins) after mixing	<i>$\Delta bd0742$</i>		Wild type HID26	
	<i>E. coli</i> without attached <i>Bdellovibrio</i>	<i>E. coli</i> with attached <i>Bdellovibrio</i>	<i>E. coli</i> without attached <i>Bdellovibrio</i>	<i>E. coli</i> with attached <i>Bdellovibrio</i>
10	94.22%	5.78%	79.57%	20.43%
15	90.76%	9.24%	67.80%	32.20%
30	88.79%	11.21%	72.41%	27.59%
45	86.88%	13.12%	64.26%	35.74%

Although HI *B. bacteriovorus* $\Delta bd0742$ mutant cells could attach to the outside of a prey cell but they could not invade the prey cells to form bdelloplasts after 1hr (Figure 3-13-A and table 3-2) while the wild-type HID26 strain successfully invaded 38.71% *E. coli* cells to form bdelloplasts after 1hr (Figure 3-13-B and table 3-2). Furthermore 18.39% of *E. coli* cells were attached to by HI *B. bacteriovorus* $\Delta bd0742$ mutant strain and 22.22% of *E. coli* cells were attached to, but not invaded by HID26 cells (table 3-2).

Table 3-2: Shows the Host-independent attachment assays for HI *B. bacteriovorus* $\Delta bd0742$ mutant and the wild type control (HID26) strains. The percentage of attached, unattached *B. HI B. bacteriovorus* $\Delta bd0742$ mutant and WT HID26 strains to *E. coli* S17-1, and bdelloplasts formed after 1 hr from adding the prey cells, were counted from three biological repeats (the cells no.=310 for HI *B. bacteriovorus* $\Delta bd0742$ mutant strain and the cells no.=279 for HID26 strain).

Strain	<i>E. coli</i> without attached <i>Bdellovibrio</i> (after 1 hr)	<i>E. coli</i> with attached <i>Bdellovibrio</i> (after 1 hr)	Invaded <i>E. coli</i> bdelloplast (after 1 hr)
Wild-type HID26	39.07%	22.22%	38.71% (108/279)
$\Delta bd0742$	81.61%	18.39%	0.00% (0/310)

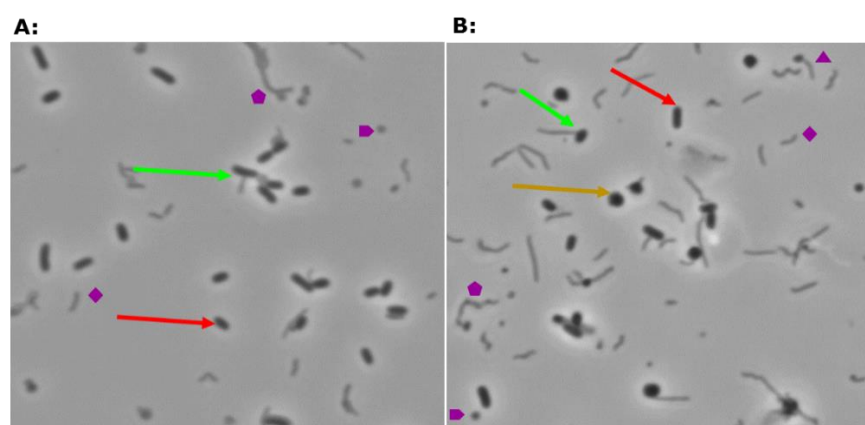


Figure 3. 13: The Field of view for the Host-independent attachment assays. A: HI *B. bacteriovorus* $\Delta bd0742$ mutant strain **B:** The wild type control HID26 after 1 hr. After 1 hr, HI *B. bacteriovorus* $\Delta bd0742$ mutant strain has low percentage of the attached cell to the prey and no round bdelloplasts formed while, HID26 has high percentage of attached cells and formed bdelloplast. Red arrow (*E. coli* unattached with *Bdellovibrio*), bright green arrow (*E. coli* attached with *Bdellovibrio*) and yellow arrow (Bdelloplast). Also, visible is the diversity of HI *B. bacteriovorus* cells shapes for both strains

(♦ vibroid, ⬠ serpentine and ■ small round *Bdellovibrio*) and occasionally autobdelloplast ▲ HI-only structures (discussed in later chapter)

3.6.6 How and when the suppression in HI *B. bacteriovorus* $\Delta bd0742$ mutant strain starts in the liquid predation culture to produce the $\Delta bd0742$ suppressor strains

In order to get visual qualitative evidence of how and when the suppression started to happen during incubation of liquid predation cultures, the predation assays were done for HI *B. bacteriovorus* $\Delta bd0742$ mutant strain and the microscopic images were taken starting from 48 hrs, every day at the same time, till the end of the predation (120 hrs). The images taken at 48 hrs revealed that many of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain (red arrows) were attached to the prey cells and few bdelloplasts (bright green arrows) were formed (Figure 3-14-A). The images that taken at 72 hrs, from beginning of experiment, were showed many bdelloplasts (bright green arrows) which mean that suppressor taking over although some prey were still not invaded (Figure 3-14-B) and at 96hr, the images showed that the prey is cleared and a lot of new progeny of *Bdellovibrio* (yellow arrow) were released with few bdellopast (bright green arrows) remaining in the cultures meaning that the suppressor strain is now dominant in the population (Figure 3-14-C).

Clearly at the beginning of co-incubation of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain (Figure 3-14) there was no predation but later predation occurred. The images of *Bdellovibrio* qualitatively support what is seen for prey death. In later cultures active predation and replication of *B. bacteriovorus* predominates. This tells me that suppressor strains have arisen.

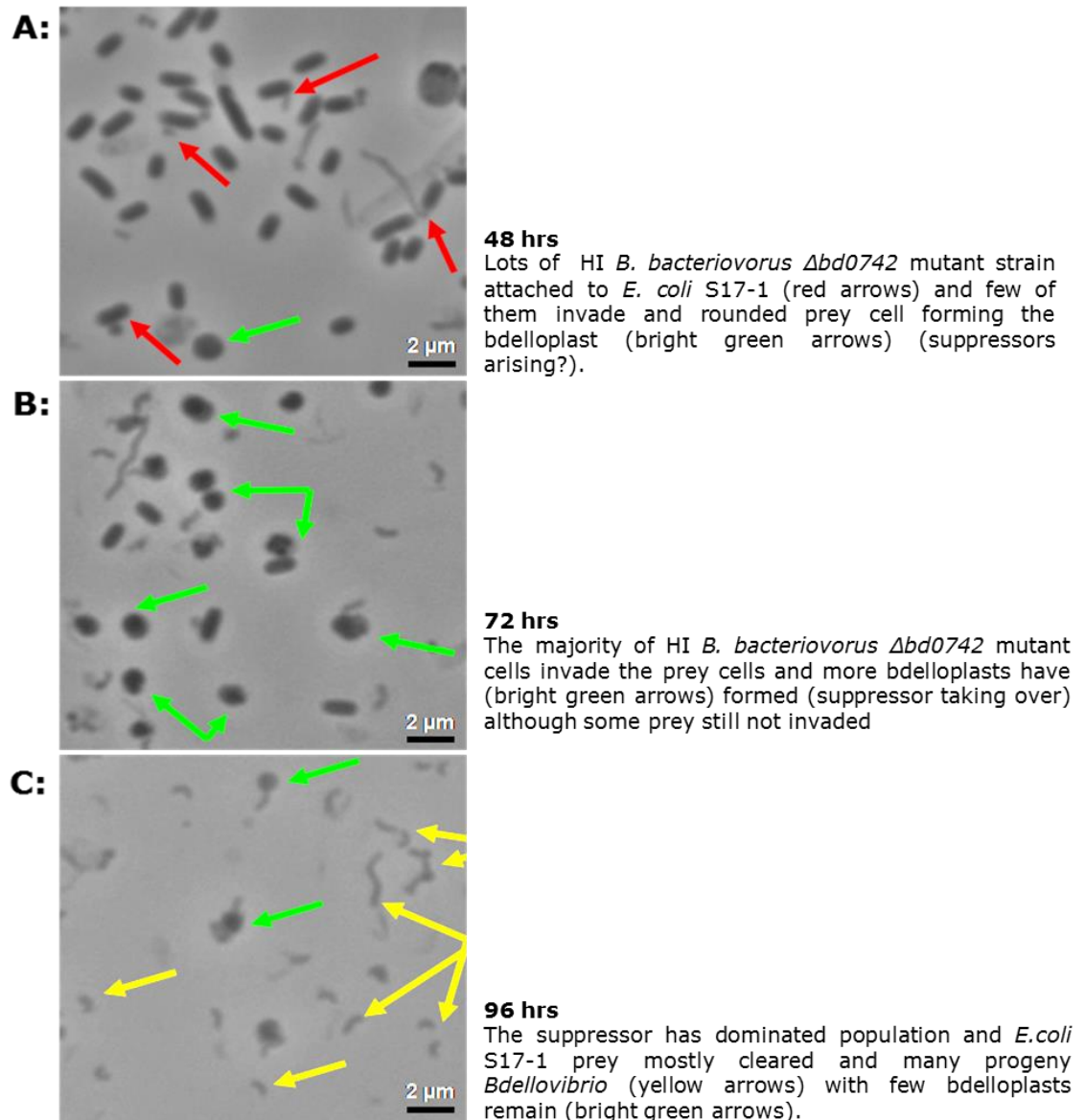


Figure 3. 14: Qualitative examination of the Fields of view for the predation assays of HI *B. Bacteriovorus* $\Delta bd0742$ mutant strain taken over the 120 hrs on *E. coli* S17-1 prey. After 48 hrs, HI *B. Bacteriovorus* $\Delta bd0742$ mutant strain has high number of *Bdellovibrio* cells (red arrows) attached to prey and low percentage of bdelloplasts formed (bright green arrows). After 72 hrs, more bdelloplasts are formed (bright green arrows). At 96 hrs of predation, few bdelloplast are left (bright green arrows) and a lots of new free progeny of *Bdellovibrio* (yellow arrows) are visible.

3.6.7 Genome sequencing showed surprise point mutations in the *Bdellovibrio* genomes of “ Δ bd0742 suppressor strains”

After the ability of HI *B. Bacteriovorus* Δ bd0742 mutant strain cultures to prey on different prey were tested and several Δ bd0742 suppressor strains from different prey were obtained, it was necessary to find out what changes may have happened in the genome making them predatory and able to prey on some prey but not on others. As mentioned previously, Simon King and David Milner had obtained some suppressor strains and the genomic DNA was prepared from these suppressor strains. When suppressor strains were obtained in this study, the cultures were grown in a large volumes, filtered twice following predation and genomic DNA was prepared from them for genome sequencing. The results of the genome sequencing showed it was possible that different changes in separate *B. bacteriovorus* Δ bd0742 suppressor strains might have been contaminating these cultures.

The genomic DNA from predatory cultures of all Δ bd0742 suppressor strains were prepared, and sent for genome sequencing to discover the possible cause(s) of predation by Δ bd0742 suppressors strains (Table 3-3). The original isolate of the HI *B. bacteriovorus* Δ bd0742 mutant strain was also sent for genome sequencing to reconfirm that this strain does not have the same point mutation(s) that may be present in the Δ bd0742 suppressor strains.

The overall results showed that the original HI *B. bacteriovorus* Δ bd0742 mutant and all Δ bd0742 suppressor strains had the same point mutation in bd0467. In contrast, all Δ bd0742 suppressors, but not the original *B. bacteriovorus* Δ bd0742 had a point mutation in the same gene *bd3766* DNA encoding different amino acid changes from similar 100 bp region in the *bd3766*. In addition, Δ bd0742 suppressor strains HD1 and HD2 had an extra point mutation in the bd0134 gene.

3.6.8 Genomic changes seen initially for original HI *B. bacteriovorus* $\Delta bd0742$ mutant and $\Delta bd0742$ suppressor strains

All (original HI *B. bacteriovorus* $\Delta bd0742$ mutant and $\Delta bd0742$ suppressors) strains genome had the same point mutation in the *bd0467* gene in the same amino acid (R135G). $\Delta bd0742$ suppressor strains HD1, HD2 and HD3, HD4 and HD5 had the same point mutation affecting the same amino acid in *bd3766* causing LL439L while strains HD3, HD4 and HD5 had an apparently second point mutation in *bd3766* changing amino acid (F394C) addition to (LL439L).

Table 3-3: shows the summary key which illustrates the names of *bd0742* suppressor strains, their dates of isolation, name of students who isolated these strains and how these suppressors were produced.

Name of suppressor strain	Date of isolation	Name of student isolated strain	How the strain produced
<i>Δ742 suppressor HD1</i>	2012	Simon King/Master student	King prepared numbers of HI <i>B. bacteriovorus</i> $\Delta bd0742$ mutant strain liquid cultures using <i>E. coli</i> S17-1 in Ca/HEPES buffer. Few cultures able to lyse the prey while most are not.
<i>Δ742 suppressor HD2</i>			
<i>Δ742 suppressor HD3</i>	2013	David Milner/PhD student	Milner prepared number of liquid cultures of HI <i>B. bacteriovorus</i> $\Delta bd0742$ mutant strain using <i>E. coli</i> S17-1 in Ca/HEPES buffer that incubated for extended periods. Few of cultures able to lyse the prey.
<i>Δ742 suppressor HD4</i>			
<i>Δ742 suppressor HD5</i>			
<i>Δ742 suppressor HD6</i>	2016	Asmaa Al-Bayati/ PhD student	In this study, Al-Bayati tested the ability of HI <i>B. bacteriovorus</i> $\Delta bd0742$ mutant strain using different prey. Al-Bayati prepared numbers of <i>B. bacteriovorus</i> $\Delta bd0742$ liquid cultures using different of prey in Ca/HEPES buffer, and found that <i>B. bacteriovorus</i> $\Delta bd0742$ able to lyse <i>E. coli</i> S17-1 and <i>A. baumannii</i> only.
<i>Δ742 suppressor HD7</i>			

Although, V437 was listed in annotation as a V437 the overall change is only LL439L, whereas both of my more recent Δ *bd0742 suppressors* strains had a single point mutation in *bd3766* which encodes a different amino acid change than in the other Δ *bd0742 suppressor* strains (Q429L). Δ *bd0742 suppressor* strains HD1 and HD2 strain had an extra point mutation in *bd0134* in amino acid (T355I), and all details of the results of genome sequences showed in the Table (3-4). This last observation about *bd0134* confirmed a preliminary observation by Simon King in 2012.

Table 3-4: initial results of genome sequencing of HI *B. Bacteriovorus* Δ bd0742 mutant strain and Δ 742 suppressor mixed strain cultures illustrating the gene that has the point mutation, what amino acid and nucleotide change in their genome, prey used to isolated these strains and whether they are able to produce plaques on lawns.

Name of suppressor strain	Gene with point mutation	Amino acid changed	Nucleotide changed	Prey used to isolated strain	Suppressor strain producing plaque
<i>B. bacteriovorus</i> Δ bd0742	<i>bd0467</i>	R135G	Cgg/Ggg	None	No
Δ 742 suppressor HD1 (Simon)	<i>bd3766</i>	LL439L	ctgctc/ctc	<i>E. coli</i> S17-1	No
	<i>bd0134</i>	T355I	aCc/aTc		
	<i>bd0467</i>	R135G	Cgg/Ggg		
Δ 742 suppressor HD2 (Simon)	<i>bd3766</i>	LL439L	ctgctc/ctc	<i>E. coli</i> S17-1	No
	<i>bd0134</i>	T355I	aCc/aTc		
	<i>bd0467</i>	R135G	Cgg/Ggg		
Δ 742 suppressor HD3* (David)	<i>bd3766</i>	F394C	tTc/tGc	<i>E. coli</i> S17-1	No
	<i>bd3766</i>	LL439L	ctgctc/ctc		
	<i>bd0467</i>	R135G	Cgg/Ggg		
Δ 742 suppressor HD4* (David)	<i>bd3766</i>	F394C	tTc/tGc	<i>E. coli</i> S17-1	No
	<i>bd3766</i>	LL439L	ctgctc/ctc		
	<i>bd0467</i>	R135G	Cgg/Ggg		
Δ 742 suppressor HD5* (David)	<i>bd3766</i>	F394C	tTc/tGc	<i>E. coli</i> S17-1	No
	<i>bd3766</i>	LL439L	ctgctc/ctc	<i>E. coli</i> S17-1	
	<i>bd0467</i>	R135G	Cgg/Ggg	<i>E. coli</i> S17-1	
Δ 742 suppressor HD6 (Asmaa)	<i>bd3766</i>	Q429L	cAa/cTa	<i>E. coli</i> S17-1	No
	<i>bd0467</i>	R135G	Cgg/Ggg		
Δ 742 suppressor HD7 (Asmaa)	<i>bd3766</i>	Q429L	cAa/cTa	<i>A. baumannii</i>	No
	<i>bd0467</i>	R135G	Cgg/Ggg		

*The strains that are mixed populations.

After the genome sequencing were came back, I amplified each of the *bd3766* genes that containing single or apparently double point mutations that were found in *bd3766* from *Δbd0742* suppressor strains to test if they are able to produce more or less c-di-GMP using swimming test in *E. coli* MG1655. The results of my *bd3766* gene sequencing for these clones discovered that the all suppressor strains that apparently had both F394C and LL439L point mutations in *bd3766* were a mixed population and they could be purified and each strain had either F394C or LL439L point mutations but not both of them in the same strain (figure 3-15 and 3-16).

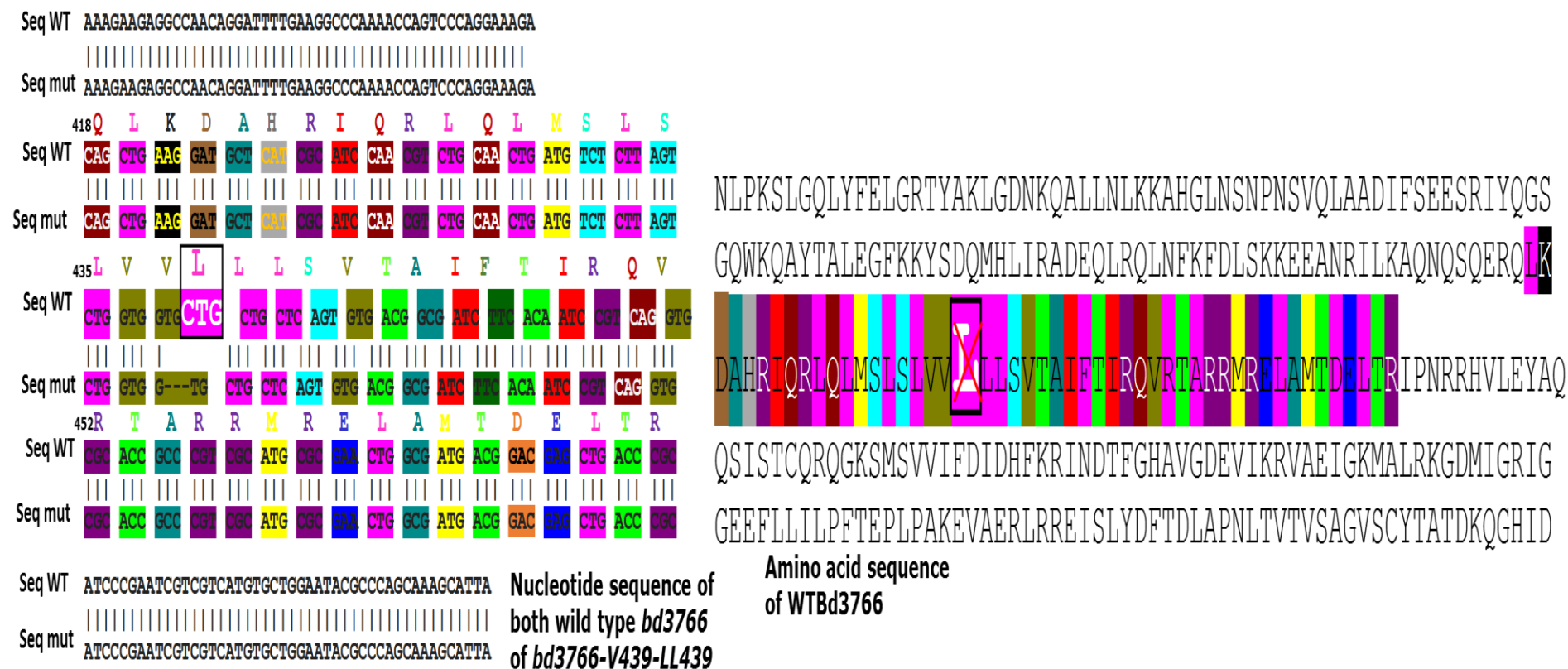


Figure 3. 15: The alignment sequences of the wild type *bd03766* with *bd03766* of *Δbd0742* suppressor strains that have point mutation LL439L. The sequences of them revealed that there is miss annotated V437 deletion in the raw results of the genome sequencing and the real point mutation that these strains have is only LL439L only.

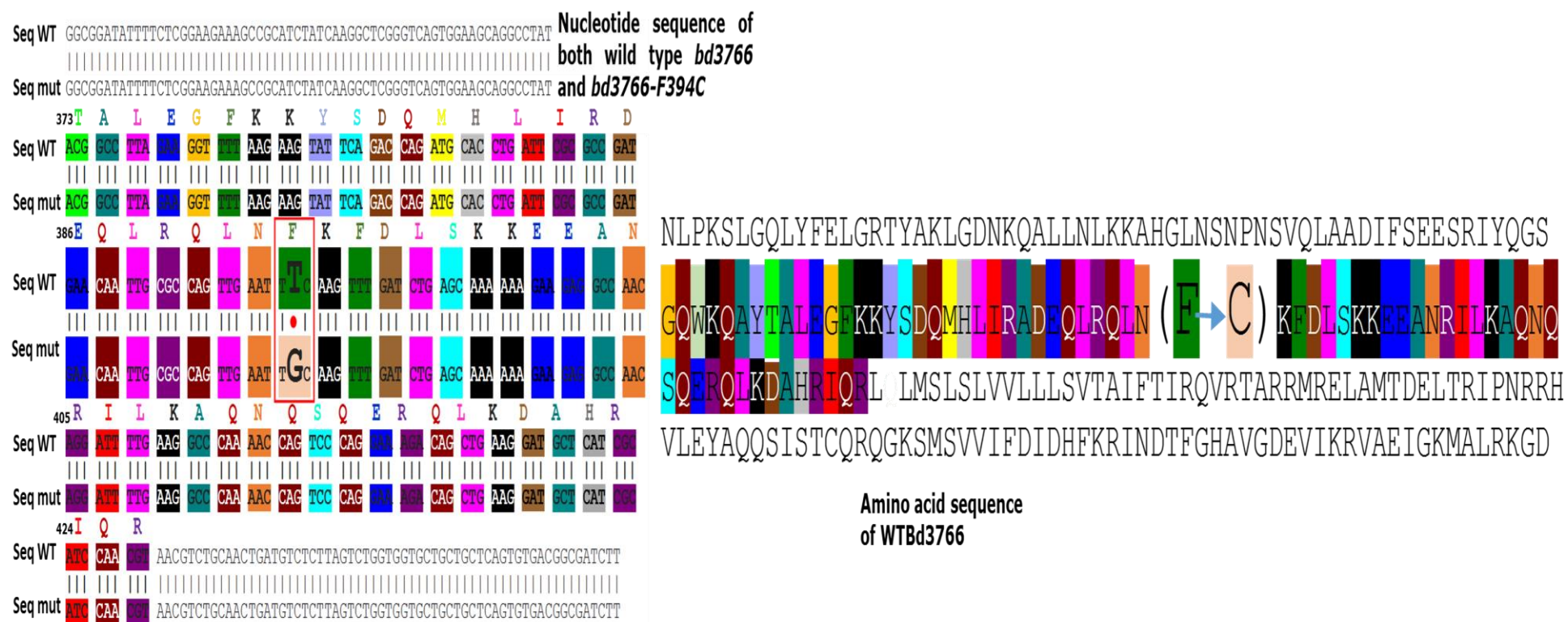


Figure 3. 16: The alignment sequences of the wild type *bd03766* with *bd03766* of *Δbd0742* suppressor strains that have point mutation F394C. The sequencing revealed that there is either LL439L point mutation as in (Figure 3-13) point mutation or there is only F394C but not both of them together in the same strain.

3.7 Discussion

3.7.1 Deletion of *bd0742* abolished predation ability of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain but did not eliminate the attachment ability.

The deletion of *bd0742* abolished early (<24 hrs) predation of *E. coli* by *Bdellovibrio* because as expected Bd0742 is one of the GGDEF domain protein that participates in c-di-GMP molecules synthesis that signals to Bd3125 for predation to start. Hobley et al. (2012) reported that *bd0742* is essential for the predatory life cycle. Bd0742 was suggested to regulate penetration of prey through controlling the activity/expression of host hydrolytic and prey modifying/degrading proteins (Hobley et al. 2012). Also, they stated that Bd0742 may function as a receiver for sensing by the *Bdellovibrio* in their vicinity or attachment to prey. The findings of predation assays of HI *B. Bacteriovorus* $\Delta bd0742$ mutant strain on different types of prey agreed with Hobley and co-worker previous results.

My attachment data showed that HI *B. Bacteriovorus* $\Delta bd0742$ mutant strain have the ability to attach to the prey cells less than the WT HID26 (control strain) and they are unable to enter to form the bdelloplast even after one hour comparing with WT HID26 strain. This means that HI *B. bacteriovorus* $\Delta bd0742$ mutant strain has the ability to attach to prey cells but can not enter and population requires long time until the point mutation arises to enter and form bdelloplast and lysed the prey cells.

3.7.2 Why suppression in genome of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain can occur in some types of prey

The predation ability of HI *B. Bacteriovorus* $\Delta bd0742$ mutant strain was tested using various Gram- negative human pathogens (*E. coli* S17-1, *A. baumannii*, *P. putida*, *S. marcescens* and *S. typhimurum*) in this chapter (Figure 3-7, 3-8,3-9, 3-10 and 3-11). The results of the predation showed that HI *B. bacteriovorus* $\Delta bd0742$ mutant strain was unable to prey on any type of prey cells, but when cultures of these prey were incubated for extended periods with HI *B. bacteriovorus* $\Delta bd0742$ mutant strain, suppression in genome arose resulted in drop in number of *E. coli* S17-1 and *A. baumannii* but the suppression did not occur with other types of prey.

This result indicated that the suppression makes *Bdellovibrio* act as a selective predator because they were able to kill *E. coli* and *A. baumannii* but not *P. putida*, *S. marcescens* and *S. typhimurum*. It could be possible that a secondary mutation occurred in one of the other 4 GGDEF genes found in *Bdellovibrio* that might be compensate for the absent *bd0742* by overexpressing one GGDEF gene or altered the activity of the encoded protein which means that deletion of one of GGDEF genes may be rescued by changes other GGDEF genes (Solano et al. 2009).

Therefore, to answer this question the genomic DNAs from the prey lysate cultures of *Bdellovibrio* that lysed *E. coli* and *A. baumannii* were named as suppressor strains purified stocked and DNA sent for the genome sequencing (Table 3-2 and 3-3). The result of genome sequence revealed that in all strains there were a secondary mutation in other GGDEF gene which is Bd3766 and it happened when left in liquid culture with *E. coli* and *A. baumannii* but not happened with other type of prey. So far the results of suppression led to another question why the suppression only happened in *E. coli* and *A. baumannii* not with other prey. This may be due to the differences in content of cell envelope of these prey than the other or might be something secreted like enzymes from other prey which prevent the suppression happening.

3.7.3 Why suppression did not occurred in the prey lawn plates but did when HI *B. bacteriovorus* $\Delta bd0742$ mutant strain was incubated for extend periods as liquid culture

Hobley et al. (2012) showed that the deletion of *bd0742* gave a strain that unable produce plaque and plaque formation ability was restored upon and complementation of this deletion. Hobley and co-workers also found that if conjugated plasmid of mutagenized motif GGAFF plasmid instead of GGDEF was put into this mutant plaque formation was not restored which means that the GGDEF domain of Bd0742 is important for predation and invasion of the prey in liquid and on solid surfaces. Furthermore, Bd0742 protein has at N-terminus a forkhead domain that might be sensing the prey which will activated Bd0742 via this domain (Hobley et al. 2012). Suppression did not arise on prey lawns plates even after long incubation period on different prey this might be due to high osmolality on solid surface comparing to liquid culture or there was no physiological signal to activate the suppression to occur in genome and this mean that HI *B. bacteriovorus* $\Delta bd0742$ mutant strain still without suppression and unable to sense prey when attached or attaching to it in lawn grown preys on solid media.

3.8 Conclusions

1. I have found that the suppression HI *B. bacteriovorus* $\Delta bd0742$ mutant strain take place in some types of prey *E. coli* and *A. baumannii* but not with other prey.
2. I found that suppression had not happened in HI *B. bacteriovorus* $\Delta bd0742$ mutant strain genome on prey overly lawns and was not able to produce plaques.
3. Also, I found that HI *B. bacteriovorus* $\Delta bd0742$ mutant strain able to attach to prey cells but slightly less than the control HI D26.
4. I found that the suppression in the genome of *B. bacteriovorus* $\Delta bd0742$ started to be dominant at 72 hrs with *A. baumannii* and at 96 hrs with *E. coli* after start of the culture mixing- predator and prey.
5. Genome sequencing discovered that there were point mutations in *bd3766* in the genome of lysate that able to kill *E. coli* and *A. baumannii*.

3.9 Future work

1. investigating the gliding ability of *B. bacteriovorus* $\Delta bd0742$ strain to give a clue why this strain is unable to produce plaques on a different type of prey lawn.
2. Also, doing a long entry video to see if *B. bacteriovorus* $\Delta bd0742$ strain able to attach properly to prey or their attachment is irreversible.
3. Measure c-di-GMP levels inside *Bdellovibrio* produced by complemented $\Delta bd0742$ with wild-type *bd0742* versus suppressor strains.
4. Try and measure the level of c-di-GMP when wild-type *Bdellovibrio* binds prey cells compared to level in suppressors strains.

3.10 Road map of my project

Deletion of the *bd0742* resulted in strains that grew only as a HI having lost the ability to do prey-invasion/predation.

When these strains were left in cultures with *E. coli* S17-1 for longer than 48 hrs suppression occurred in their genome and new strains emerged that were able to kill the *E. coli* S17-1.

The genome sequencing data showed that there were three genes where point mutations occurred in the genome of the $\Delta bd0742$ suppressor strains.

These mutations are in the following genes:

1. *bd0467* (encoding transposase like enzyme):

The point mutation (Cgg/Ggg) encoded R135G

2. *bd0134* (encoding AsKα a key enzyme at the top of the DAP/Lysine synthesis pathway (responsible for cell wall cross-link materials or other amino-acids): The point mutation (aCc/aTc) encoded T355I

3. *bd3766* (*dcgD*) one of the GGDEF domain proteins in the *Bdellovibrio*: the point mutations were (tTc/tGc) encoded F394C, (cAa/cTa) encoded Q429L and (ctgctc/ctc) encoded LL439L.

I report my testing of each point mutation in Chapter 4 for *Bd467*, Chapter 5 for *Bd0134* and Chapter 7 for *bd3766*. Chronologically, I began with *Bd0134* to learn molecular genetics (in doing this I also tested other DAP synthesis genes and cell wall roles), I present them in this order.

At the end of my PhD I did little more work on wall and invasion with gene *bd0635*, reported in Chapter 8.

Figure 3. 17: Road map of my project

4 Chapter four: Testing the effect of point mutation in the transposase-like gene (*bd0467*) on the predation and plaque formation ability of *Bdellovibrio*

4.1 Introduction

As I mentioned in the Chapter three, the HI *B. bacteriovorus* $\Delta bd0742$ mutant strain was sent for genome sequencing and the results of the sequencing for HI *B. bacteriovorus* $\Delta bd0742$ mutant strain showed that one of the point mutations that this strain contains encoded R135G (Cgg/Ggg) from the *bd0467* gene. As I explain below, the predicted function of Bd0467 may be unlikely to be associated with suppressing a GGDEF mutant, but I tested it to rule it in or out. To assess if the point mutation in *bd0467* gene could have any effect on the enhancing predation ability of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain to prey on the other bacteria, this gene was tagged and deleted to see what is the function of this gene and what is the effect of deletion of this gene on *B. bacteriovorus* in both HD and HI lifestyle.

Bd0467 is a 208 amino acid protein that was annotated initially as a conserved hypothetical protein that it is predicted to have a role transposition and DNA-binding (<http://www.uniprot.org/uniprot>). The genomic context showed that the two adjacent genes to *bd0467* are in opposite orientation. The upstream gene is *bd0466* that encodes alanine racemase that plays a role in conversion of L-alanine to D-alanine which used for cross-linking of peptidoglycan by binding D-Alanine from one peptide stem with meso-diaminopimelic acid (DAP) of a neighbouring peptide stem (Also dependent on pyridoxal 5'-phosphate as a coenzyme) (Figure 4-1). The GC content of *bd0467* is lower when compared to the GC content of neighbouring genes. Possibly, this gene which may be recently incomer in the genome (Figure 4-1).

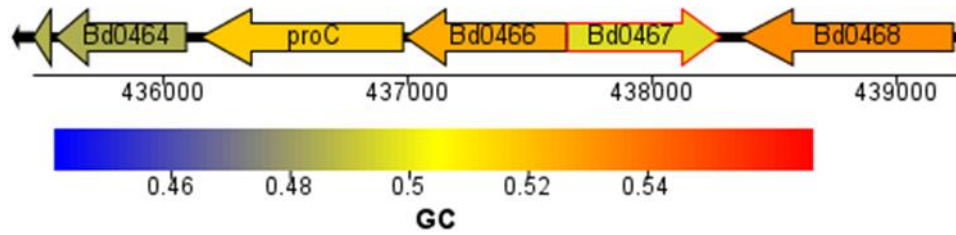


Figure 4. 1: The genomic context of *B. bacteriovorus* *bd0467* gene with usage codon. The GC content of *bd0467* is lower when compared to the GC content of neighbouring genes. Possibly, this gene which may be recently incomer in the genome (<http://hactar.shef.ac.uk/xbase/>).

The other gene, *bd0468*, a N-acetylglucosamine (GlcNAc) deacetylase that is responsible for the modification of peptidoglycan by N-deacetylation (Lambert et al. 2016). *Bd0467* is conserved in prey- invasive *B. bacteriovorus* Tiberius but not in epibiotic *B. exovorus*. There are three nucleotides between the end of *bd0466* and the start of *bd0467* is likely to be three base pairs GTA as shown in Figure (4-2).



Figure 4. 2: The base pairs found between *bd0466* and *bd0467*. There are three nucleotides between the end of *bd0466* and the start of *bd0467*.

BLAST analysis initially showed that that *Bd0467* has no known conserved domains. (Figure 4-3); however Pfam sequence search predicted that *Bd0467* belongs to an Y1_TnpTransposase IS200 like family with $E=0.00032$ and has Y1_Tnp domain that started from 92–170 bp (<http://pfam.xfam.org/search/sequence>). Phyr2 server showed also that *Bd0467* has homologous to IS200-like Transposase (www.sbg.bio.ic.ac.uk/phyre2/).

Transposases are often parts of mobile DNA and so might vary more often than housekeeping genes.

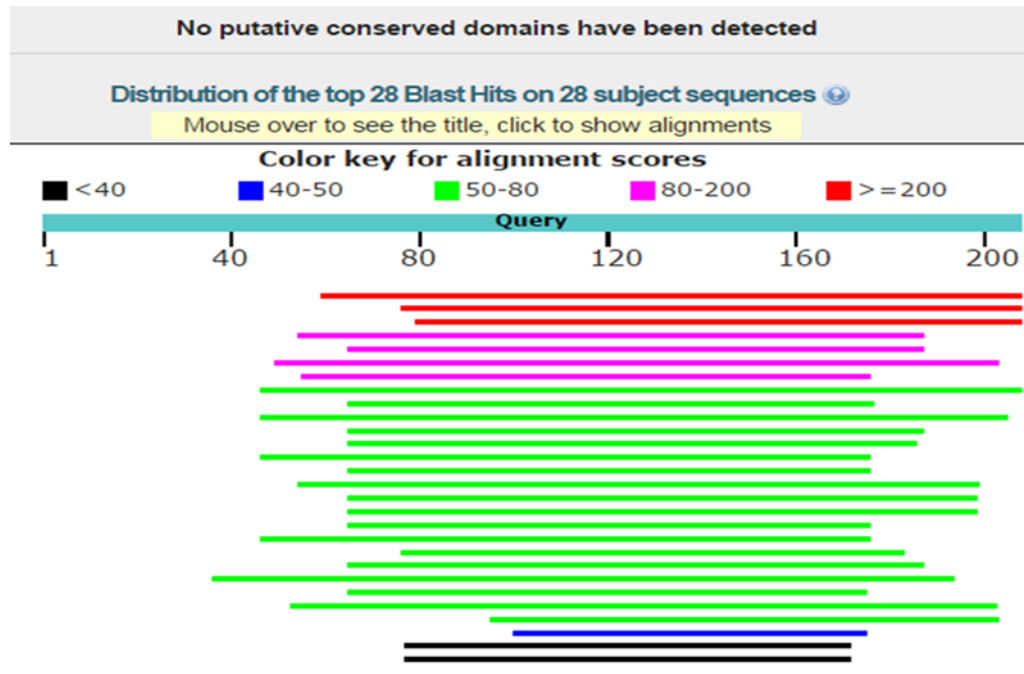


Figure 4. 3: The conserved domains of Bd0467 (<https://blast.ncbi.nlm.nih.gov/>).

The CELLO Prediction revealed that this protein location is cytoplasmic with 2.060 score (<http://cello.life.nctu.edu.tw/>). The occurrence of *bd0467* in deltaproteobacteria was tested using STRING neighbourhood studies (Figure 4-3). The presence of *bd0467* was not identified in the *Proteobacteria* and only found in *Bdellovibrio* as would be expected for a transposases, possibly associated with mobile transposon DNA. The Pfam sequences and the Blast analysis showed that R135 residue is not conserved in other bacteria which mean that this residue is not important for transposition of this protein.

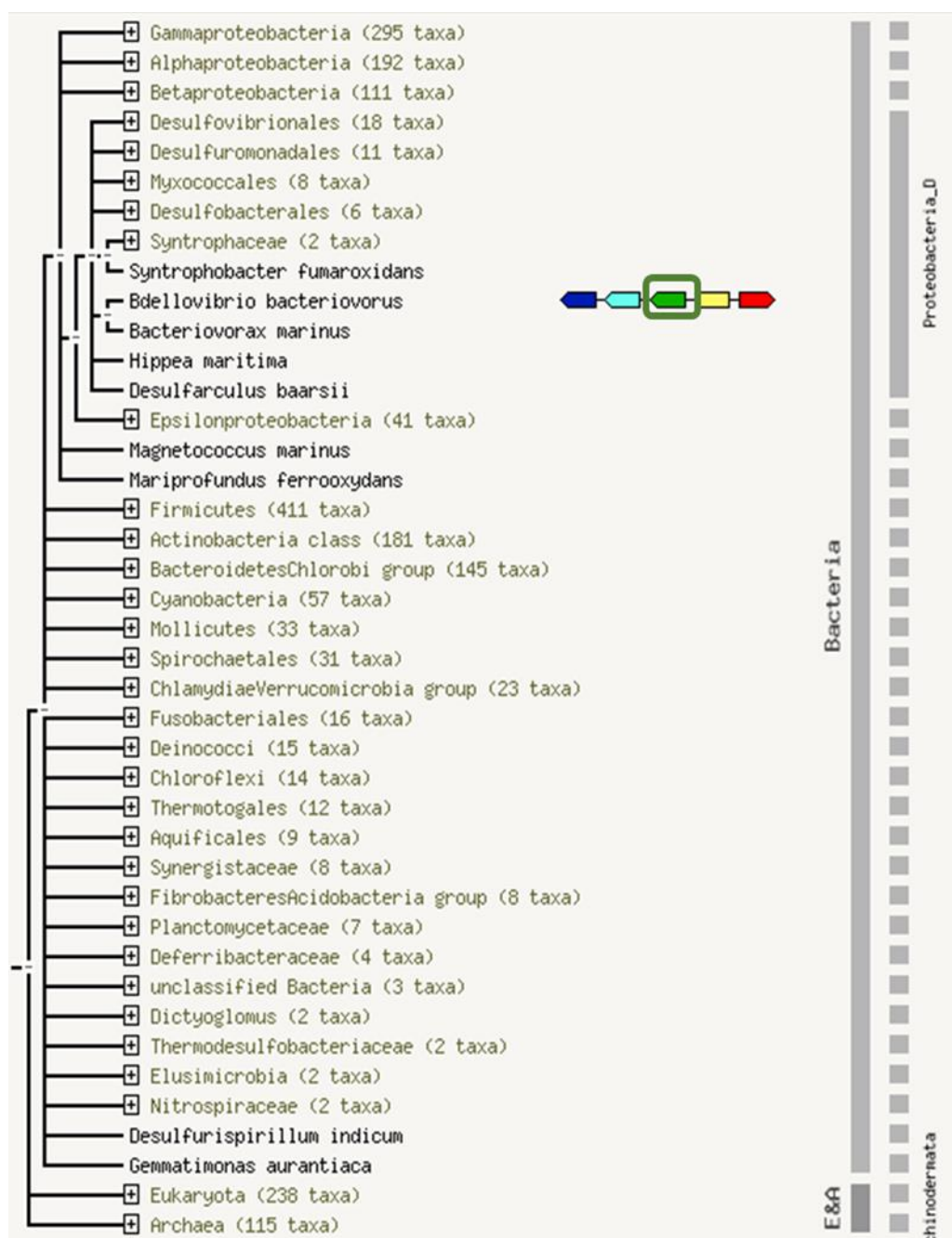


Figure 4. 4: The occurrence of STRING *bd0467* in all bacteria phylal. *bd0467* is unusual in deltaproteobacteria group versus typical housekeeping arrangement of other bacteria (<http://string-db.org/cgi/neighborhood>) as expected for a transposase.

4.2 Specific Research Aims

1. To examine the transcription of *bd0467* of *B. bacteriovorus* throughout the predatory lifecycle and Host-independent lifestyle seeing if it is similar to *bd0742* expression.
2. To assess the cellular localisation of Bd0467 during the predatory lifecycle seeing if it is expressed at same time point of Bd0742.
3. To assess the cellular localisation of Bd0467 during host- independent growth at same time as Bd0742.
4. To isolate an in frame deletion strain of Bd0467 and assay the phenotypes of the mutant strain, seeing if it related to predation ability of *Bdellovibrio*.

4.3 Expected outcome

1. *bd0467* will be transcribed continuously during the predatory lifecycle of *B. bacteriovorus* like the *bd0742*.
2. Fluorescent tag of Bd0467 will be visible in attack phase and all stages of the predatory cycle at same time of the Bd0742 expression and in host-Independent cells.
3. *bd0467* suppress the absence of *bd0742* will restore of the predation.
4. The deletion of *bd0467* gene possible and if a deletion strain is obtained, is the deletion strain still able to perform the predatory lifecycle.

4.4 Results:

4.4.1 Transcription of *bd0467* in host- dependent (HD) and host-independent (HI) lifestyle

The expression of *bd0467* during the predatory life cycle of *Bdellovibrio* was examined using RNA samples isolated at indicated time points of the predatory life cycle of *B. bacteriovorus* strain during one round of synchronous *Bdellovibrio* infection of *E. coli* host cells (Figure 4-5). The expression of *bd0467* was weak in phase attack and started to rise at 15 mins till end of the predatory lifecycle of *Bdellovibrio*. This expression pattern suggests that this gene product may have an important role in predation and intracellular growth of *Bdellovibrio* (Figure 4-5). The transcription of *bd0467* is opposite to the *bd0742* transcription which is on in all time point of the predatory lifecycle (see Chapter 3, Section 3.1, Figure 3-3).



Figure 4. 5: Reverse-transcriptase PCR showing transcriptional expression of *bd0467* throughout the predatory cycle. RNA was isolated at the indicated time points during one round of synchronous *Bdellovibrio* HD100 infection of *E. coli* host cells, and primers specific to the gene were used to amplify (RT-PCR, 27 cycles) an approximately 112 bp internal fragment from each transcript if present. AP = Attack phase (free-swimming *Bdellovibrio*); 15, 30, 45 = minutes since start of infection (attachment to and rounding of *E. coli* host cell into a bdelloplast structure); 1h, 2h, 3h, 4h = hours since start of infection (host cell resources being degraded and used for *Bdellovibrio* growth into a filament, followed by septation into multiple progeny and eventually lysis from host cell); Ec = *E. coli* S17-1 RNA (no *Bdellovibrio* control); gen = *B. bacteriovorus* HD100 genomic DNA (positive control); NT = No template control; L = NEB 100 bp DNA ladder.

Microarray data found that *bd0467* expression is 4.67 fold higher upon entry to bdelloplast than growing HI. Therefore, RT-PCR was performed on total RNA (sample matched 10 ng/ μ L) isolated from the attack phase and different HI strains

(HID2, HID13, HID22 and HID26). Figure (4-6) showed that *bd0467* expression is higher in all HI strains than the attack phase and this agreed with Microarray data.

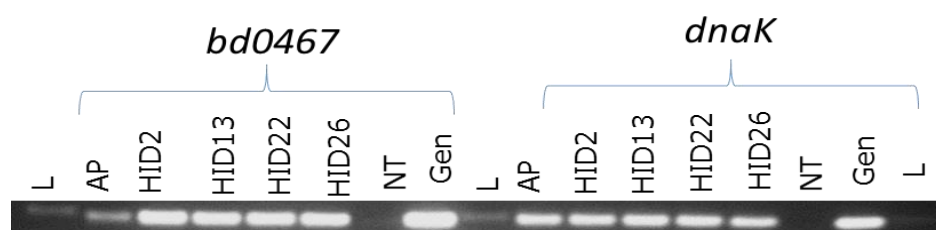


Figure 4. 6: Reverse-transcriptase PCR showing relative transcription expression of the *B. bacteriovorus* HD100 *bd0467* during attack phase and host-independent growth. RNA was isolated from *B. bacteriovorus* HD100 attack phase (AP) cells and four host-independent strains (HID2, HID13, HID22, HID26) at OD600 0.6 and the RNA concentration was matched to 10 ng μl^{-1} . Primers specific to gene were used to amplify (RT-PCR, 30 cycles) an approximately 112 bp internal fragment from each transcript if present. Gen = *B. bacteriovorus* HD100 genomic DNA (positive control); NT = no template control; L = NEB 100 bp DNA ladder.

4.4.2 Localisation of Bd0467-mCherry assessed by fluorescently tagged mcherry

To assess the cellular location of Bd0467- mCherry during the predatory lifecycle, generating a fluorescently tagged *bd0467* strain. Bd0467-mCherry was constructed by overlapping 25 bp of the 624-bp ORF fragment of *bd0467* lacking the stop codon and 25 bp from mCherry gene in pK18mobsacB plasmid using *E. coli* DH5 α as a host using Gibson assembly cloning method (see Section 2.10.3). pK18::Bd0467- mCherry plasmid tagged protein was fluorescent (Figure 4-7) and cytoplasmically located.

Bd0467- mCherry construct was transformed into *E. coli* S17-1 and conjugated from donor *E. coli* strain S17-1 into *B. bacteriovorus* by single crossover with maintenance on the plasmid pK18 mobsacB by kanamycin selection. The observed

fluorescence in *E. coli* prior to conjugation helped to verify the functional fusion of *bd0467* to mCherry gene on the plasmid.

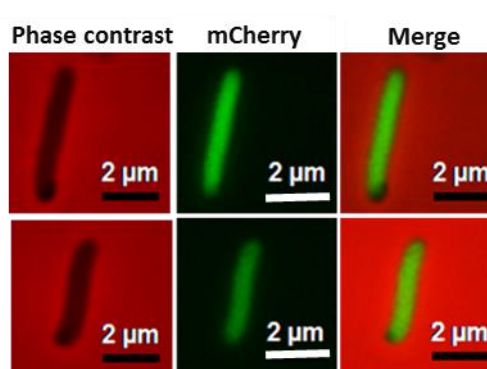


Figure 4. 7: Heterologous expression of pK18 Bd0467 -mCherry in *E. coli* DH5α in stationary phase that were grown in YT broth for 16 hours with appropriate antibiotic.

The resultant strain of *B. bacteriovorus* Bd0467-mCherry showed no fluorescent expression at attack phase and not at any time point of the predatory lifecycle and location of Bd0467-mCherry could not be assessed (Figure 4-8). The genomic DNA was prepared from this strain and sent for sequencing to confirm the presence of mCherry. The result of the sequencing proved the presence of the gene with tagged mCherry and the reason for lack of visible fluorescence of this strain suggest that production of the protein is due to very low so it is not measurable and this is expected from HD lifecycle RT-PCR results shown in Figure (4-5). This result contrast to Bd0742-mCherry that expressed during all stages of the predatory lifecycle (Hobley et al. 2012).

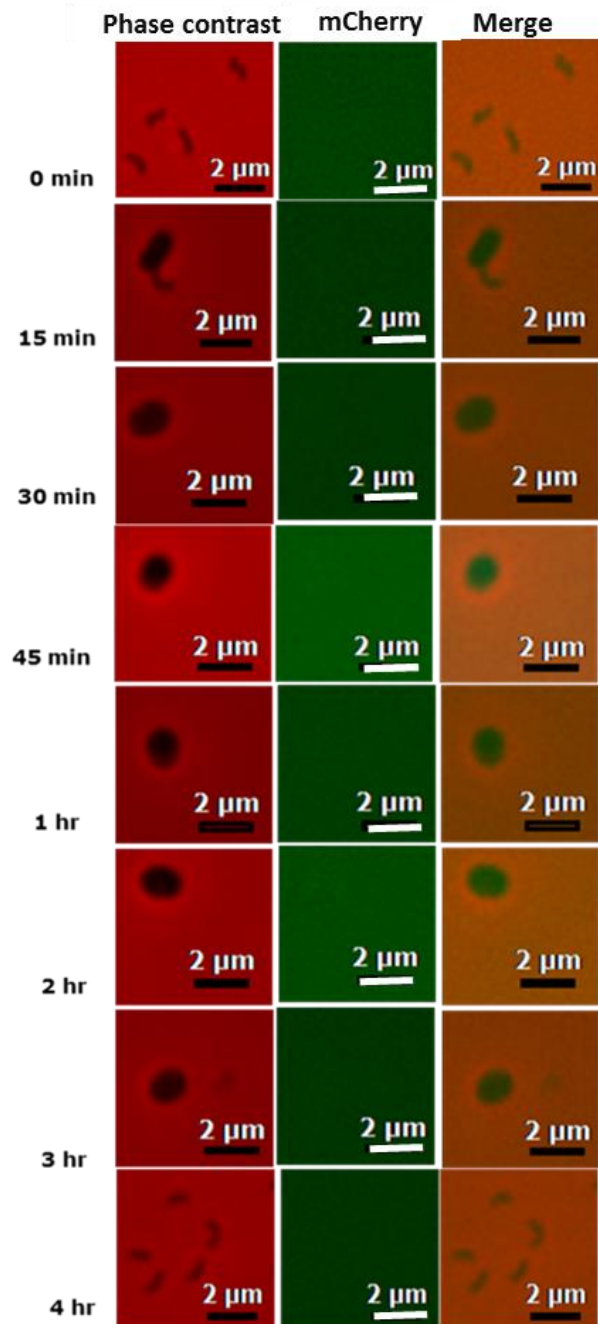


Figure 4. 8: The absence of fluorescence pattern of *B. bacteriovorus* HD100 *bd0467-mCherry* during the whole predatory lifecycle on *E. coli* pZMR100. 1.9
Second exposure with maximum gain.

4.4.3 Bd0467-mCherry was visualized fluorescence in rounded and growing HI *Bdellovibrio* cells

To examine if Bd0467-mCherry displayed any fluorescent signal in host-independent growing cells and if so their location in HI cells, isolates of the *B. bacteriovorus* HD100 *bd0467-mcherry* strain were generated and grown as HI. The HI *B. bacteriovorus bd0467-mCherry* was visualized showing faint fluorescence mostly in round cells (self-auto-bdelloplast possibly rearranging DNA in them during self-replication cannibalism) than other types of cell shapes (Figure 4-9). This may indicate that *bd0467* may plays a role during the host independent growth of *Bdellovibrio* possibly rearranging the genome by transposition. This can be further investigated in when obtain the deletion strain of this gene.

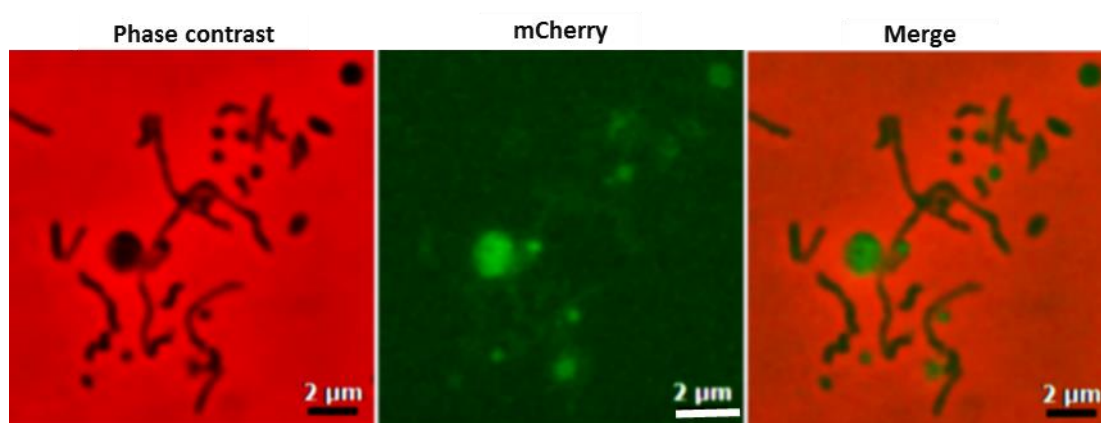


Figure 4. 9: HI *B. bacteriovorus* Bd0467-mCherry strain. Bd0467-mCherry expressed in Host- Independent growth phase mostly in rounded cells. The images were taken with 0.8 second fluorescent exposure and maximum gain.

4.4.4 Deletion of *bd0467* successfully obtained

Even though Bd0467 is a predicted transposase and the fluorescence weakly expressed only in HI cells and not in HD lifestyle, further evidence was sought about whether *bd0467* had a role in the predation ability of the original HI *B. bacteriovorus* $\Delta bd0742$ mutant strain, so to understand the function of *bd0467* gene and if it has any hidden role in the predation of *B. bacteriovorus* especially

after the genome sequences for HI *B. bacteriovorus* $\Delta bd0742$ mutant strain returned back with point mutation in this gene, a silent knockout deletion strain of *bd0467* was required as a quick answer for these questions. A deletion plasmid for *bd0467* construct was made using Gibson assembly cloning method (see Section 2.10.3) to combine the 1 kb region upstream of the gene of interest and the plasmid pK18*mobsacB*. After that $\Delta bd0467$ construct was confirmed by sequences and transformed to *E. coli* S17-1 then conjugated in *B. bacteriovorus*, recombining by single crossover and maintained by kanamycin selection.

The resultant *Bdellovibrio* plaques from conjugation were grown as a host-dependent cultures, and then screened for gene replacement and plasmid loss using 5% sucrose. All the exconjugants were sensitive to kanamycin and 60 pre-lysate culture were screened using Dream Taq PCR for initial conformation of obtaining deletion strain of this gene. Some of the exconjugants gave band of knockout 791 bp which mean that they were potential deletion strains. PCR fragments of 2050 bp amplified from the genomic DNA at the deletion site of the candidate $\Delta bd0467$ strain was sent for sequencing across the gene deletion site to confirm that the remaining DNA sequence was identical to wild type DNA of *B. bacteriovorus* HD100 with only the *bd0467* gene missing. The deletion strain could be isolated in the host dependent lifecycle and this strain was still predatory (Figure 4-10) and as expected from Figure (4-5 and 4-8) so these data did not give any significant effects on predation of *Bdellovibrio*.

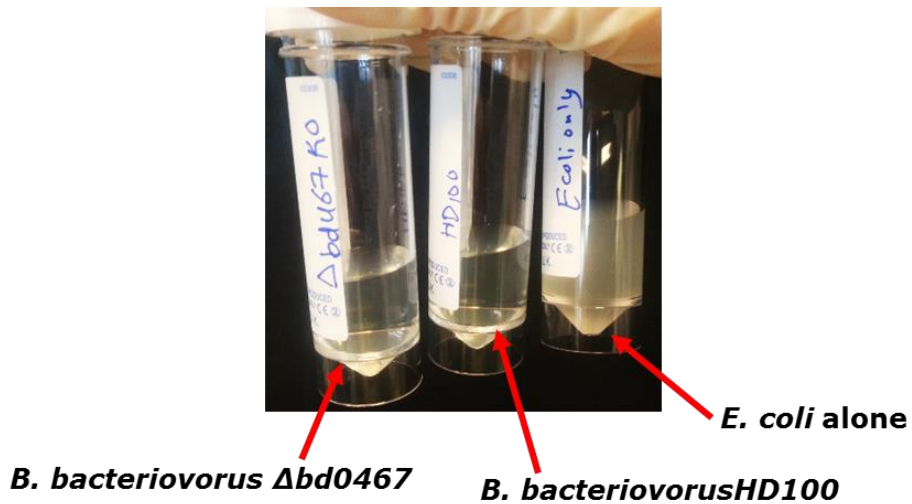
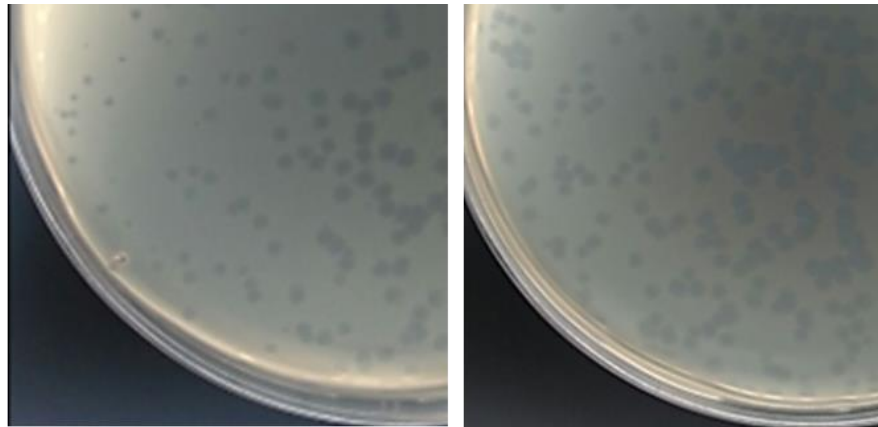


Figure 4. 10: Predatory lysates of *B. bacteriovorus* Δ *bd0467* and *B. bacteriovorus* HD100 strains with *E. coli* S17-1 control after 24 hrs. Deletion of Δ *bd0467* from the genome did not affect the predation ability of *B. bacteriovorus* Δ *bd0467* and it is still predatory and able to kill the prey *E. coli* S17-1 after 24 hrs compared to the control strain *bacteriovorus* HD100 strains that also able to lyse the prey *E. coli* S17-1 within the 24 hrs.

B. bacteriovorus Δ *bd0467* strain is still able to produce plaques like *B. bacteriovorus* HD100 (Figure 4-11) within incubation 6 days and *B. bacteriovorus* Δ *bd0467* produce plaques near same size of *B. bacteriovorus* HD100 not bigger plaques to said that that the point mutation in *bd0467* might be suppressed the genome in absence of Bd0742. This gives a strong evidence that the HI *B. bacteriovorus* Δ *bd0742* mutant strain has lost their ability to produce plaques from deletion of *bd0742* gene and not from the point mutation in *bd0467* gene that was identified in original strain of HI *B. bacteriovorus* Δ *bd0742* mutant.



***B. bacteriovorus* Δ bd0467 strain *B. bacteriovorus* HD100 strain**

Figure 4. 11: The plaque plates of predatory *B. bacteriovorus* HD100 and *B. bacteriovorus* Δ bd0467 strains on double layer YPSC overlay plates containing prey *E. coli* S17-1 in the top layer. Deletion of *bd0467* does not affect the ability of *B. bacteriovorus* Δ *bd0467* strain to produce plaques.

4.4.5 Deletion of *bd0467* is not essential for host -independent growth

Due to no obvious defect in morphology or predation of *B. bacteriovorus* Δ bd0467 strain that was obtained in the host dependent lifecycle, HI *B. bacteriovorus* Δ bd0467 was generated in order to test if deletion of *bd0467* has any effect on HI growth of this strain remembering that Bd0467 is expressed in HI growth (Figure 4-9).

A growth curve (monitoring OD₆₀₀ nm) from an axenically growing culture for HI Δ bd0467 strain in PY media was performed by selecting three isolates from HI Δ bd0467 and three from the wild-type *B. bacteriovorus* HD100 derivative HI strains (*HI D13*, *HI D22* and *HI D26*) used as controls (Figure 4-12). Each culture of HI was back- diluted in PY broth to OD₆₀₀ 0.1 and transferred to a 96-well microtitre plate. After that the plate was incubated at 29°C with 200 rpm shaking and the growth of HI strains were measured for 72 hrs every 30 mins using BMG Fluostar plate- reader.

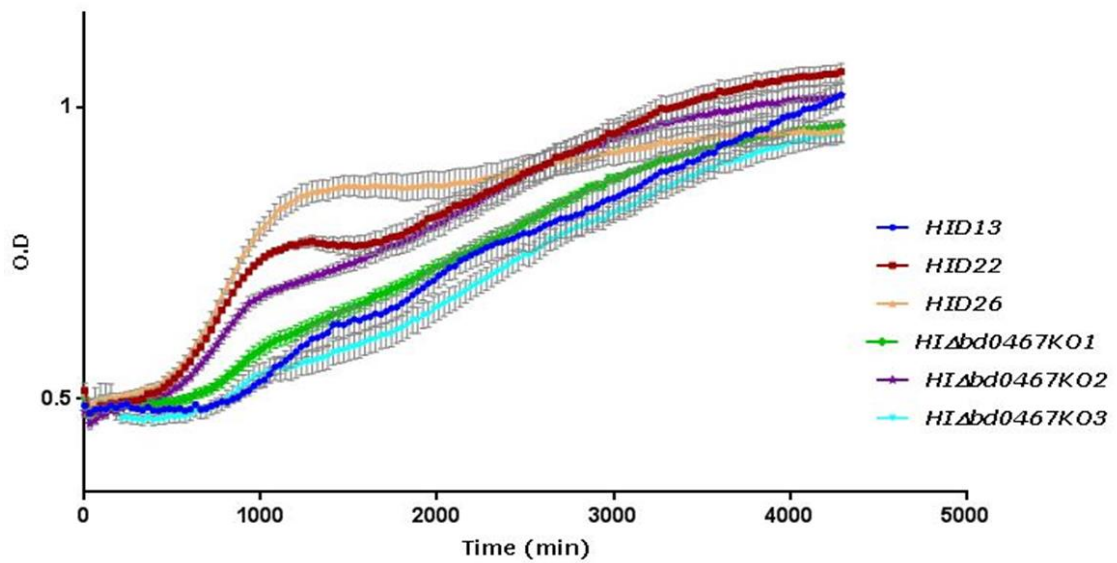


Figure 4. 12: Host -independent growth rates meaned for three *HI B. bacteriovorus* $\Delta bd0467$ strains against three wild type strains (D13, D22 and D26) controls. HI cultures diluted to O.D₆₀₀ of 0.1. The measurement of each HI strains occur every 30 mins for 72 hrs (Three biological repeats).

The results showed that all HI $\Delta bd0467$ isolates did not show any noteworthy differences in growth rate compared to the wild- type HI isolates (Figure 4-12). It is known that WT HI strains show a range of growth rates (Chang et al. 2011) but the mutant rate was within this range and mostly HI $\Delta bd0467$ cells had the same shape as WT HI *B. bacteriovorus* (Figure 4-12). Both WT HI *B. bacteriovorus* and HI $\Delta bd0467$ strains still form the round autobdelloplast which mean that the *bd0467* not essential for round autobdelloplast formation, but might be culturing and varying chromosomes in WT HI *B. bacteriovorus* (Figure 4-13).

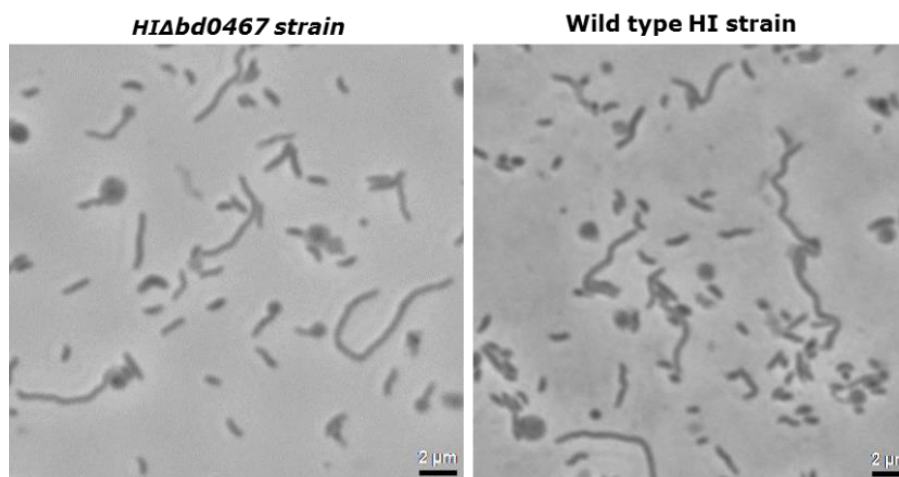


Figure 4. 13: HI *B. bacteriovorus* $\Delta bd0467$ and wild-type HI strains isolates under phase contrast microscopy. HI $\Delta bd0467$ isolates show similar diverse cell shapes of the wild-type Host-independent.

4.5 Discussion

Because *bd0467* has transposase homology, this gene could have recently been acquired by genome and the GC content showed that *bd0467* has lower GC content than neighbour genes (Figure 4-1). The Blast search showed that Bd0467 found in *B. bacteriovorus* HD100 and also in *B. bacteriovorus* tiberious; but Pfam sequence search and Phyr2 server predicted that Bd0467 belongs to Y1_TnpTransposase family.

The transcriptional analysis of the *bd0467* gene across the predatory lifecycle (Figure 4-5) showed very low levels of expression. In the HI growth mode it was shown that the transcription of *bd0467* gene is upregulated in comparison with *B. bacteriovorus* HD100 attack phase cells (Figure 4-6). The low level of expression demonstrated by RT-PCR of *bd0467* was confirmed by Fluorescence microscopy which showed that there was no visible Bd0467-mCherry fluorescence seen in any time point of the predatory lifecycle at all (Figure 4-8). This might be due to the very low level of production of this protein in the predatory growth mode. Genomic DNA from this strain was prepared and sequenced and demonstrated that there was no mutation detected that might have affected function of the Bd0467-

mCherry fluorescent tag). However, the fluorescence could be seen in HI cells where the protein seems to be produced in higher concentration (Figure 4-9) and this matched the HI RT-PCR result for this gene.

The Knockout deletions of the *bd0467* gene produced a strain that was still able to grow as a predator (Figure 4-10). Furthermore, deletion of this gene also had no effect on the ability of *Bdellovibrio* $\Delta bd0467$ strain to produced plaques on double layer YPSC overlay plates containing prey *E. coli* S17-1 in the top layer (Figure 4-11). The normal plaque formation ability of *Bdellovibrio* $\Delta bd0467$ indicated that this gene has no effect on the predation of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain and does not play a role in plaque formation that was also lost in HI *B. bacteriovorus* $\Delta bd0742$ mutant strain as this strain is still predatory and able to form plaques (Figure 4-11). In addition, deletion *bd0467* did not affect the HI growth of *Bdellovibrio* (Figure 4-12 and 4-13).

4.6 Conclusions

This chapter has investigated *Bdellovibrio* Bd0467 protein which is Transposase that might be acquired by the *Bdellovibrio* genome. From all the results of this chapter we can conclude the following:

1. The localisation of fluorescent Bd0467 during the predatory cycle was not detectable and this might be explained and matched with the low expression of *bd0467* in HD lifecycle. This contrast to Bd0742 that was expressed in all time points of the predatory lifecycle.
2. Deletion of *bd0467* has no significant effect on the predatory lifecycle of *Bdellovibrio* and also, this deletion had no obvious effect can see in HI lifestyle of the *Bdellovibrio*. This gives strong evidence that the point mutation found in *bd0467* (that was discovered by the genome sequencing of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain strain) has no effect on predation ability of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain that were abolished by deletion of *bd0742*.
3. Deletion of *bd0467* has no effect on ability of *Bdellovibrio* to produce plaques on double layer YPSC overlay plates containing prey *E. coli* S17-1 in the top layer, this give clear indication that the point mutation that found in *bd0467* has no effect on ability of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain to produce plaques on prey lawn.
4. *bd0467* is not ameliorated in the genome as the point mutation in R135G of this gene is Cgg become Ggg.

4.7 Future work

There is no future work needed relating Bd0467 to the HI *B. bacteriovorus* *Δbd0742* mutant strain but it was interesting that Bd0467 was showed the highest expressed (almost exclusively only) in the round cells of the HI grown Bd0467mcherry strain. As you will see in later Chapters 6 for Bd0047 and Bd0129 (DapB and DapE) and Chapter 8 for CpoB (Bd0635). Sockett laboratory now seeing these as possible "self/auto-bdelloplast" structures where interior predation of self-material produces new attach phase *Bdellovibrio*. The activity of the transposase like gene-product in those structures could allow new genome rearrangements to occur which could be selected to give new fitness to emerging attack phase from these structures. Studying that would be interesting in combination with other genes that might be play role in self/auto-bdelloplast" structures.

5 Chapter Five: Testing the roles of *Bdellovibrio* Aspartokinases (ASK α and ASK β) and exploring the effect of point mutation in *aska* gene on the predation and plaque formation ability of *Bdellovibrio*

5.1 Introduction

As discussed in chapter three, some of *Δ bd0742* suppressor strains genome contains a point mutation in *bd0134* gene that encodes an aspartokinase; a key enzyme in the DAP/Lysine pathway that plays a role in cell wall synthesis (see Chapter 1, section 1.13). Therefore, to assess the role of Bd0134 in *Bdellovibrio*, this protein was fluorescently tagged and the gene deleted in the *B. bacteriovorus* genome. Additionally, to evaluate if the point mutation in *bd0134* gene affects the predation ability of the *Δ bd0742* suppressor strains to prey on other bacteria or form plaques, a *B. bacteriovorus* *bd0134-T355I* strain was generated where *bd0134* was fully replaced in the genome with the site-directed mutant version (T355I).

Furthermore, the work in this chapter expanded to include another aspartokinase in *Bdellovibrio*. *Bdellovibrio* has two aspartokinase enzymes, ASK α and ASK β encoded by *bd0134* and *bd0528* respectively (Lo et al. 2009).

Bd0134 is a 460 amino acid protein that was annotated initially as aspartokinase III; a lysine sensitive protein. The genomic context showed that *bd0134* lies between two genes, one in the same orientation and the other in the opposite orientation. The upstream gene is *bd0133*, annotated as a hypothetical protein. The downstream gene is *bd0135* which also annotated as a hypothetical protein. (Figure 5-1) (<http://hactar.shef.ac.uk/xbase/>).

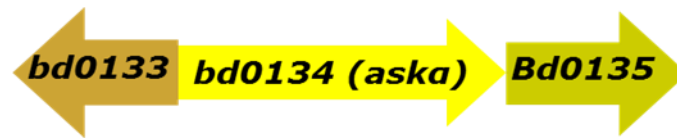


Figure 5. 1: The genomic context of *B. bacteriovorus* *bd0134* gene (<http://hactar.shef.ac.uk/xbase/>).

Bd0528 is a 395 amino acid protein that was annotated initially as an aspartate kinase protein. The genomic context showed that *bd0528* located between two genes, one in the same orientation as *bd0134* and the other in the opposite orientation. The upstream gene is *bd0527*, annotated as a hypothetical protein. The downstream gene is *bd0529*, annotated as a Murein transglycosylase (MltD) protein responsible for degradation of cell wall murein by cleaving beta-1,4-glycosidic bond between N-acetylmuramic acid and N- acetylglucosamine (van Asselt et al. 1999) (Figure 5-2) (<http://hactar.shef.ac.uk/xbase/>).

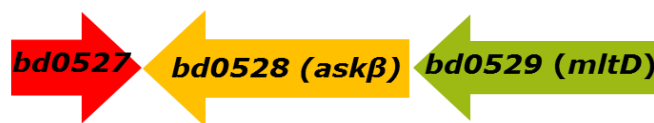


Figure 5. 2: The genomic context of *B. bacteriovorus* *bd0528* gene (<http://hactar.shef.ac.uk/xbase/>).

BLAST analysis shows that both Bd0134 and Bd0528 are conserved in *B. exovorus*, *B. bacteriovorus* *Tiberius*, *Bdellovibrio* *sp. ArHS* and *Bdellovibrio bacteriovorus* *W*. Both Bd0134 and Bd0528 have a conserved AA-Kinase domain and belong to lysC superfamily (Figure 5-3 and 5-4) (<https://blast.ncbi.nlm.nih.gov/>).

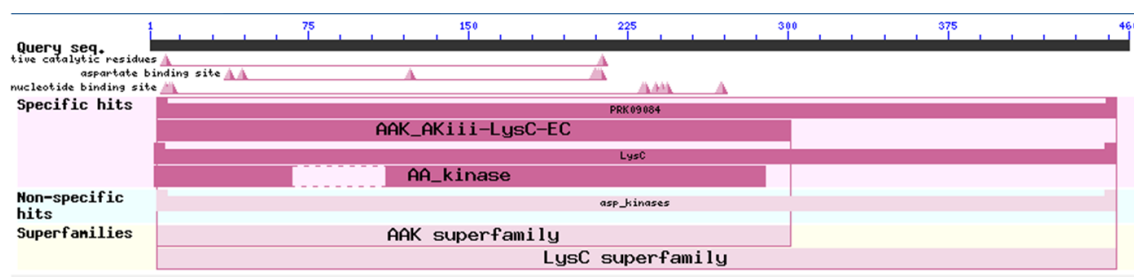


Figure 5. 3: Blast analysis showing the conserved domains of Bd0134 (<https://blast.ncbi.nlm.nih.gov/>).

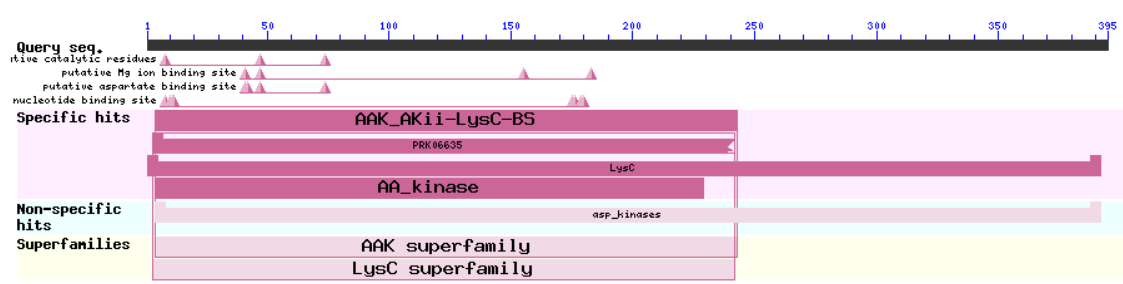


Figure 5. 4: Blast analysis showed the conserved domains of Bd0528 (<https://blast.ncbi.nlm.nih.gov/>).

By using the CELLO website (<http://cello.life.nctu.edu.tw/>), the subcellular localisation of Bd0134 and Bd0528 was predicted and the results (Figure 5-5) show that Bd0134 and Bd0528 have a cytoplasmic location with predicted scores of 4.533 and 2.493 respectively.

LOCALIZATION	RELIABILITY	RELIABILITY
Cytoplasmic	0.952	0.388
Cytoplasmic	0.828	0.459
Cytoplasmic	0.958	0.393
Cytoplasmic	0.807	0.482
Cytoplasmic	0.987	0.771
Cytoplasmic	4.533 *	2.493 *
Periplasmic	0.172	1.031
OuterMembrane	0.155	0.894
InnerMembrane	0.083	0.393
Extracellular	0.057	0.189

Figure 5. 5: The prediction of Bd0134 and Bd528 locations by CELLO. The location of Bd0134 (red box) and Bd0528 (green box) are in the cytoplasm (<http://cello.life.nctu.edu.tw/>).

The shared operons for upstream or downstream genes of *bd0134* and *bd0528* were examined using (<http://operons.ibt.unam.mx/OperonPredictor/>), and it was predicted that *bd0134* is encoded in an operon with *bd0135* downstream and *bd0528* is encoded in an operon with *bd0529* (*mltD*) upstream. This is investigated further in this chapter.

5.2 Specific Research Aims

1. To examine the transcription of *B. bacteriovorus* *bd0134* and *bd0528* throughout the predatory life cycle and HI lifestyle.
2. To investigate if there are shared operons between *bd0134* and *bd0135* and if there are shared operons between *bd0528* and *bd0529* of *B. bacteriovorus*.
3. To examine the transcription of *bd0528* throughout the predatory life cycle using RNA prepared from the *B. bacteriovorus* $\Delta bd0134$ strain.
4. To assess the cellular localisation of *Bd0134* and *Bd0528* during the predatory life cycle.
5. To assess the cellular localisation of *Bd0134* and *Bd0528* during the host-independent lifestyle.
6. To isolate an in-frame deletion strain of *bd0134* and *bd0528* and investigate the phenotypes of the mutant strains in both HD and HI lifestyle.
7. To analyse complementation of the *B. bacteriovorus* *HD100* $\Delta bd0134$ mutant with WT *bd0134* using the pK18*mobsacB* vector.
8. To test the ability of *B. bacteriovorus* *HD100* *bd0134-T355I* to prey on *E. coli* and some human pathogens compared to the wild type control *B. bacteriovorus* *HD100* at extended time periods.
9. To test the ability of *B. bacteriovorus* *HD100* *bd0134-T355I* to produce plaques on prey lawns compared to *B. bacteriovorus* *HD100*.

10. To test the effect of adding the extra copy of wild type *bd0134* on the plaque formation of HI *B. bacteriovorus* $\Delta bd0742$ mutant and $\Delta bd0742$ suppressor strains.

5.3 Expected outcome

1. Transcription of *bd0134* and *bd0528* will be expressed in continuous manner during the predatory life cycle of *B. bacteriovorus*.
2. Deletion of *bd0134* is possible and if a deletion strain can be obtained, the deletion strain should still be able to perform the full predatory process.
3. Deletion of *bd0528* is possible and if a deletion strain can be obtained, the deletion strain should still be able to perform the full predatory process.
4. Deletion of *bd0134* will be viable but may affect on host- independent cell wall synthesis and growth of the deletion strain will be defective compared to wild-type host- independent *Bdellovibrio*.
5. Point mutation *Bd0134-T355I* will not affect the predation ability of *B. bacteriovorus* HD100 to prey on diverse Gram-negative prey, including pathogens.
6. Point mutation *Bd0134-T355I* will not influence the ability of *Bdellovibrio* to form plaques on *E. coli* prey lawn plate compared to the wild type *B. bacteriovorus* HD100.
7. An extra copy of wild-type *bd0134* in HI *B. bacteriovorus* $\Delta bd0742$ mutant and $\Delta bd0742$ suppressor strains will restore plaque formation in these strains.

5.4 Results:

5.4.1 Bioinformatic analysis of *B. bacteriovorus* Bd0134 (ASK α) and (ASK β)

Little is known about *B. bacteriovorus* DAP synthesis genes *bd0134* and *bd0582* that are predicted to control cell wall synthesis or modification, thus bioinformatic analysis was used to identify these genes. The protein sequence of Bd0134 and Bd0528 from the Gram-negative *B. bacteriovorus* HD100 genome was aligned with ASK α sequences from the Gram-negative *E. coli* (B4024) to assess conservation of important residues. Figure (5-6) has been annotated with protein structural information obtained from the *E. coli* ASK α crystal structure (Marco-Marín et al. 2003, Kotaka et al. 2006).

One of the interesting residues in Figure (5-6) alignment was K8, a crucial residue for catalysing the phosphoryl transfer through interaction with ADP directly and the aspartate side chain indirectly, and this residue is conserved in both Bd0134 and Bd0528. D202 residue is responsible for organising and orientating the catalytic group and is also conserved in both Bd0134 and Bd0528. In *E. coli*, when the amino acids are mutated to K8R or D202E, this led to a reduction of the catalytic activity of this enzyme (Marco-Marín et al. 2003, Kotaka et al. 2006).

Kotaka et al. (2006) found that T45, E119 and R198 residues participated in the binding of aspartate to the N-lobe of the catalytic domain (found in Bd0134 and Bd0528). In contrast, the existence of Y420 in the ACT2 domain at the C- terminus acts as a hindering factor that blocks binding of lysine to this domain. The authors also suggested that L104 and S107 residues in each monomer take part in tetramer formation of ASK α of *E. coli*.

It is apparent from this alignment that both ASK α of *E. coli* and *B. bacteriovorus* share high sequence similarity (46.0% sequence identity and 64.6% similarity, NEEDLE global alignment). However, ASK α of *E. coli* and Ask β of *B. bacteriovorus*

share less sequence similarity (22.8% sequence identity and 37.3% similarity, NEEDLE global alignment).

ASK α of *E. coli* and ASK α of *B. bacteriovorus* have shared the same motif sequences D, P and R (position 230-232) whereas; Ask β of *B. bacteriovorus* has modified motif sequence D, P and N in the same position (Omori and Komatsubara 1993). On the other hand, all of them have conserved the same active site (K8, F9, G10 and G 11 in N-terminal domain) except bd0528 that has Y instead of F in position 9 (Ghislain et al. 1994). Changing amino acids at positions G323D, T344M and S345L resulted in the ASK enzyme becoming insensitive to lysine inhibition (Kikuchi et al. 1999). The phylogenic tree of *Bdellovibrio* aspartokinases showed that *Bdellovibrio* have two different aspartokinases, ASK α (Bd0134) and ASK β (Bd0528) (Figure 5-7).

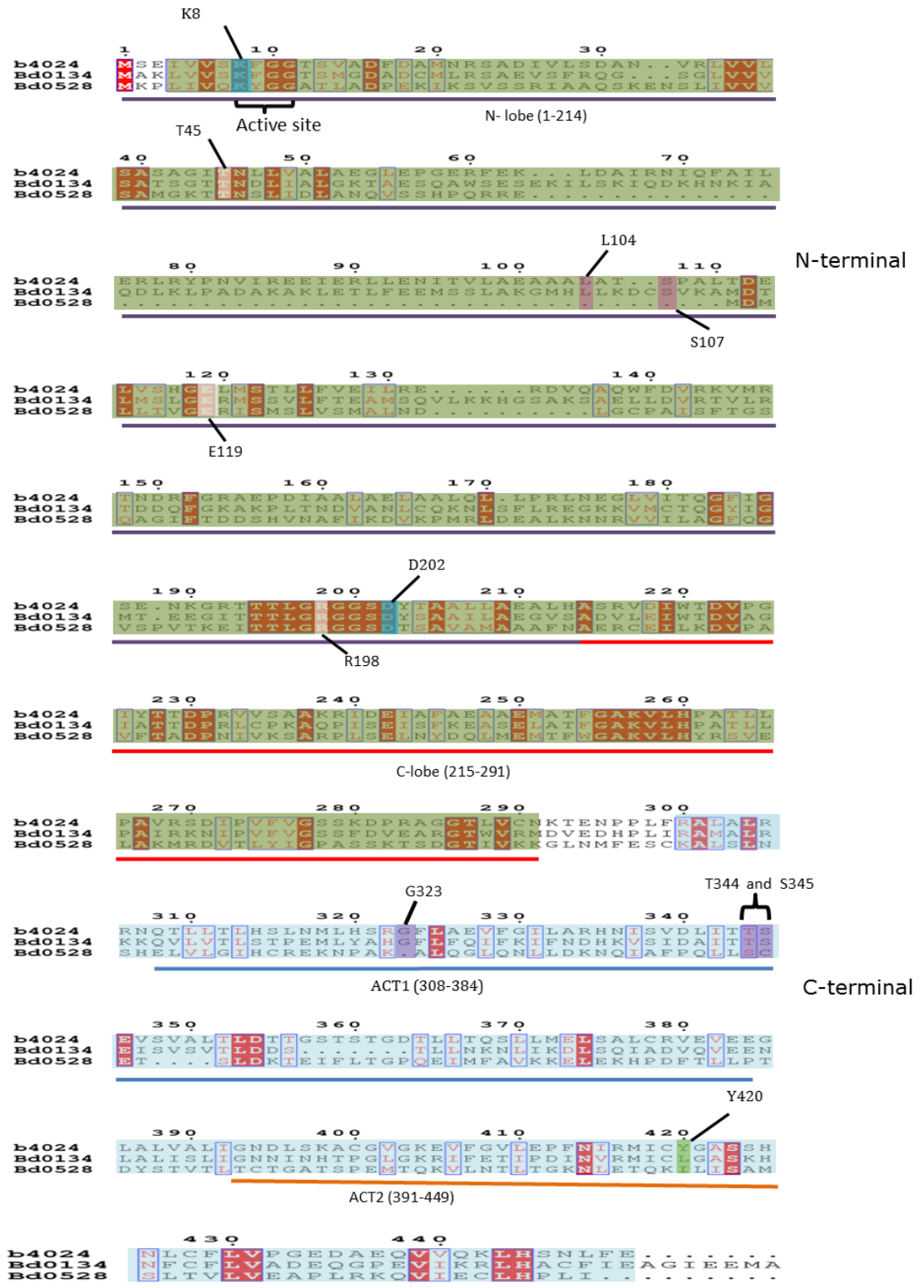


Figure 5. 6: ClustalW alignment of Bd0134 (ASKα) and Bd0528 (ASKβ) of *B. bacteriovorus* and known ASKα (B4024) protein of *E. coli*.

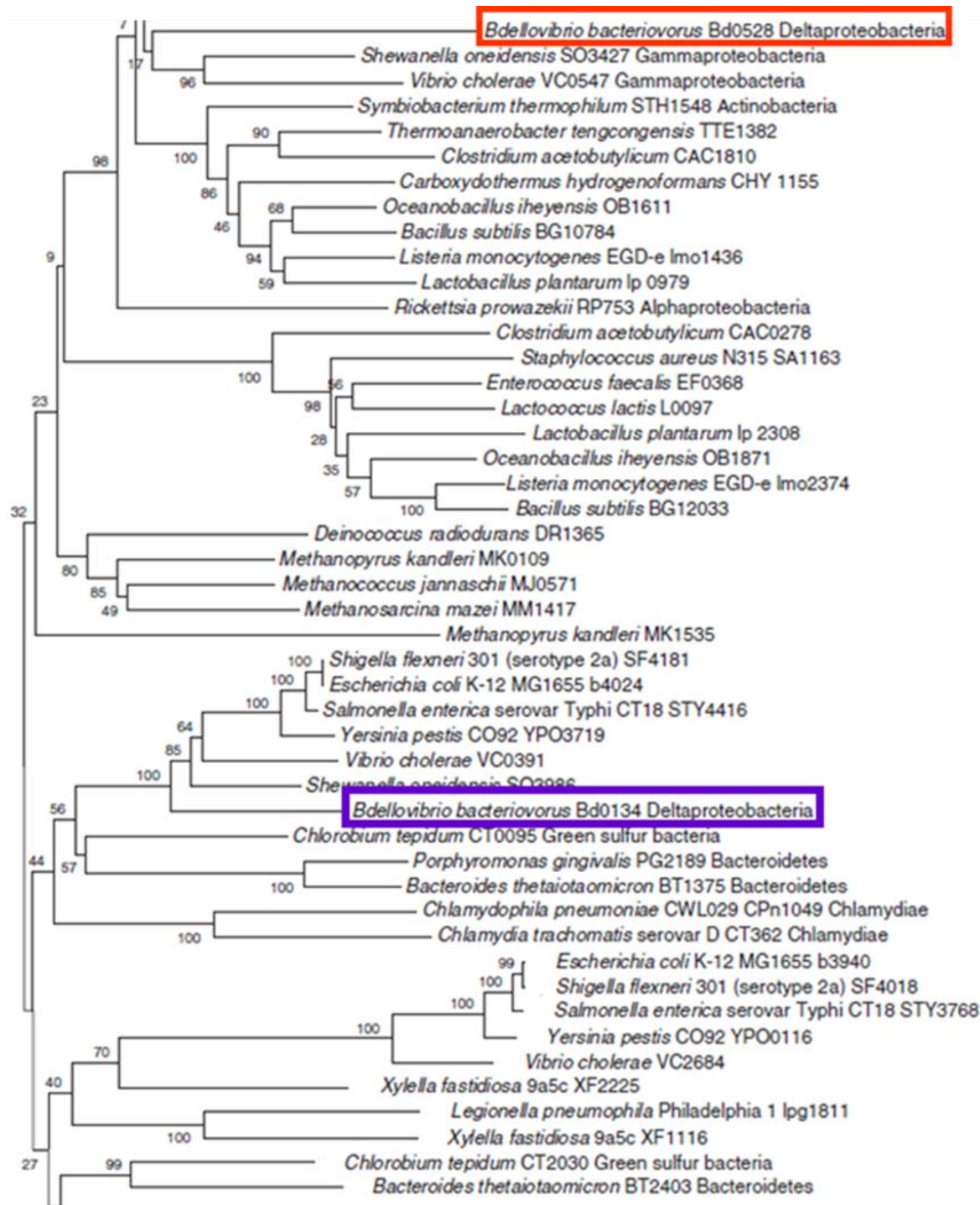


Figure 5. 7: The phylogenetic tree of *Bdellovibrio* aspartokinases showed that *Bdellovibrio* have two different aspartokinases, ASK α (Bd0134) and ASK β (Bd0528). The phylogenetic tree modified from (Nishida and Narumi 2007).

5.4.2 Transcription of *bd0134* (*ask_a*) throughout the predatory life cycle and HI lifestyle.

RT-PCR was performed to examine the expression of *bd0134* gene (*ask_a*) using RNA samples taken from points across a single round of a synchronous *B. bacteriovorus* predatory cycle. Figure (5-8) shows that the highest expression of *bd0134* was seen when *Bdellovibrio* grows within the prey cell as a rise of expression at 15 mins and fall in expression at 4 hrs, at a time when the *Bdellovibrio* is exiting the prey. This *bd0134* expression pattern indicates that this gene might have an important role in the life cycle of *Bdellovibrio*, so it seems possible that *bd0134* plays a role in production and regulation of bdelloplast wall or self-wall during the predatory cycle.



Figure 5. 8: Reverse-transcriptase PCR is showing transcriptional expression of *bd0134* throughout the predatory cycle. RNA was isolated at the indicated time points during one round of synchronous *Bdellovibrio* HD100 infection of *E. coli* host prey cells. Primers specific to the gene were used to amplify (RT-PCR, 30 cycles) a 98 bp internal fragment from each transcript if present. AP = Attack phase (free-swimming *Bdellovibrio*); 15, 30, 45 = minutes since the start of infection (attachment to and rounding of *E. coli* host cell into a bdelloplast structure); 1h, 2h, 3h, 4h = hours since start of infection (host cell resources being degraded and used for *Bdellovibrio* growth into a filament, followed by septation into multiple progeny and eventually lysis from the host cell); Ec = *E. coli* S17-1 RNA (no *Bdellovibrio* control); gen = *B. bacteriovorus* HD100 genomic DNA (positive control); NT = No template negative control; L = NEB 100 bp DNA ladder.

To test if *bd0134* was expressed and if it has any role in the HI growth mode of *Bdellovibrio*, RT-PCR was carried out using total RNA matched at 10 ng/μl isolated from the attack phase and different wild-type HI strains (HID2, HID13, HID22 and HID26). It is obvious from Figure (5-9) that the *bd0134* expression level is higher in all HI strains in comparison to attack phase (non- growing cells), and this

matched the results of microarray data of Lambert et al. (2010) that found that the expression of this gene during HI growth is 1.94 fold higher than in attack phase.

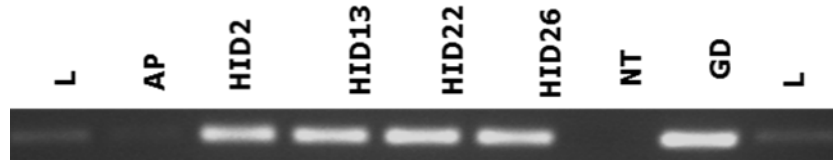


Figure 5. 9: Reverse-transcriptase PCR showing relative transcription expression of the *B. bacteriovorus* HD100 *bd0134* gene during attack phase and host-independent growth. RNA was isolated from *B. bacteriovorus* HD100 attack phase (AP) cells, and four host-independent strains (HID2, HID13, HID22, HID26) at OD₆₀₀ 0.6 and the RNA concentration was matched to 10 ng μl^{-1} . Primers specific to the gene were used to amplify (RT-PCR, 30 cycles) a 98 bp internal fragment from each transcript if present. Gen = *B. bacteriovorus* HD100 genomic DNA (positive control); NT = no template negative control; L = NEB 100 bp DNA ladder.

5.4.3 The *bd0134* gene is in an operon with *bd0135*

To identify if *bd0134* and *bd0135* were co-transcribed in an operon, RT-PCR was performed using matched amounts of RNA (10 ng/ μL) isolated from *B. bacteriovorus* HD100 at one point during the predatory life cycle (3hr). A 1050 bps product indicated that *bd0134* and *bd0135* are in an operon which suggests that they might have associated functions (Figure 5-10).

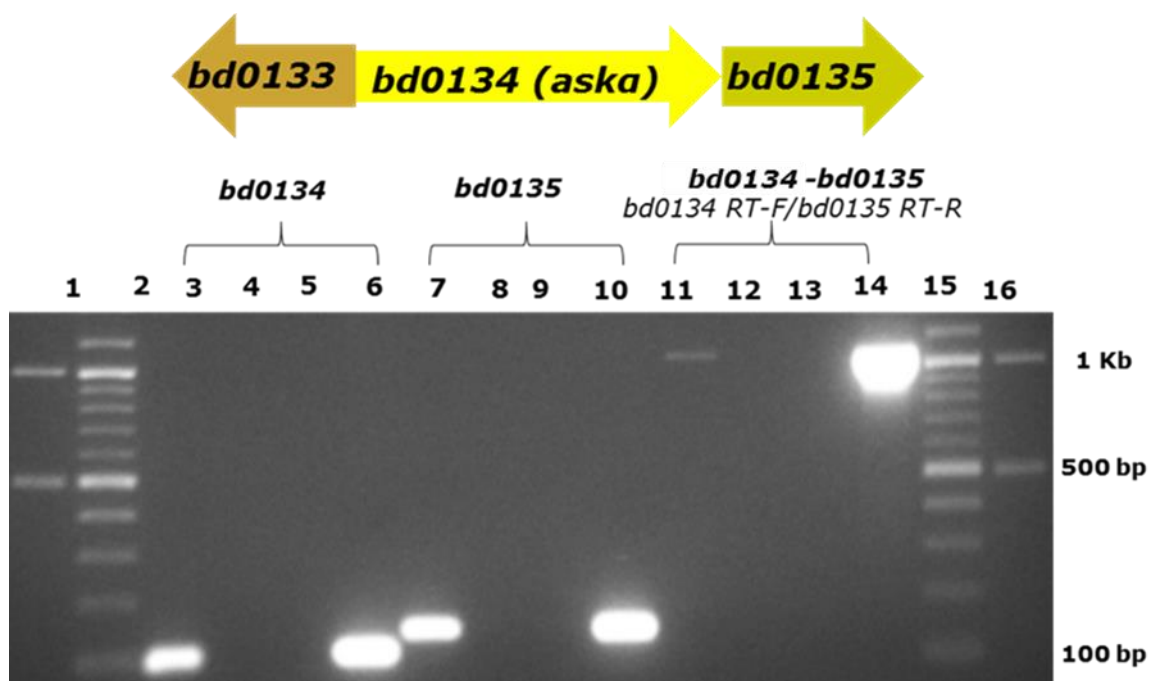


Figure 5. 10: RT-PCR shows that the *B. bacteriovorus* genes *bd0134* and *bd0135* are co-transcribed. Lanes 1 and 16: NEB 1000 bp ladder; Lanes 2 and 15: NEB 100 bp ladder; Lanes 3, 7 and 11: predator and prey total RNA at 3 hr; Lanes 4, 8 and 12: *E. coli* S17-1 RNA; Lanes 5, 9 and 13: No template; Lanes 6, 10 and 14: *B. bacteriovorus* HD100 genomic DNA. A 1050 bp band found in lane 11 indicates that there was co-transcription between *bd0134* and *bd0135* genes.

5.4.4 Location of *E. coli* B4024-mTFP (AsK) in *E. coli*

In order to study *Bdellovibrio* Bd0134-mCherry a control fusion in a different bacterium was useful. Therefore the *E. coli* *b4024* (aspartokinase) gene was tagged with mTFP and expressed in *E. coli* DH5α as a good comparison in a bacterium that elongates its cell wall by binary fission rather than filamentous growth. B4024- mTFP was constructed using the method described in (see Section 2.10.1). The fluorescence was visible in *E. coli* DH5α B4024-mTFP strain and was diffuse throughout the cytoplasm (Figure 5-11).

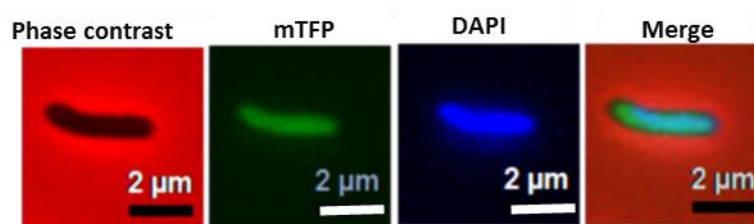


Figure 5. 11: Heterologous expression of pAKF04 B4024 -mTFP in *E. coli* DH5α in stationary phase (grown in YT broth for 16 hours with appropriate antibiotic). The fluorescence is visible spread throughout the cytoplasm within the cell.

5.4.5 Native localisation of Bd0134 (ASKα) assessed by fluorescently tagging with mCherry in *B. bacteriovorus*

To assess the cellular location of Bd0134-mCherry (ASKα) during the predatory lifecycle, we generated a C-terminal fluorescently tagged *B. bacteriovorus* *bd0134* (*aska*) strain. Bd0134-mCherry was constructed by fusion of a 1881-bp ORF fragment of *bd0134* lacking the stop codon, cloned in frame with mCherry in the plasmid pAKF56 using *E. coli* DH5α as a host. pAKF56::Bd0134-mCherry plasmid tagged protein was fluorescent (Figure 5-12) and localized in pattern different from the location of pAKF56::Bd4024- mCherry of *E. coli* near the poles (avoiding the middle of the cell and located near the poles).

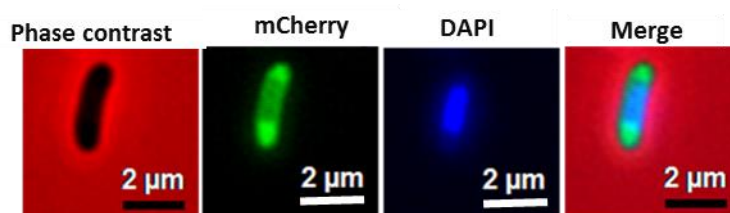


Figure 5. 12: Heterologous expression of pAKF56::Bd0134-mCherry in *E. coli* DH5α in stationary phase (grown in YT broth for 16 hours with appropriate antibiotic). The fluorescence is visible and located near poles.

The Bd0134-mCherry construct was then cloned into the pK18*mob*sacB plasmid and conjugated from donor *E. coli* strain S17-1 into *B. bacteriovorus* resulting in a single crossover into the genome which is maintained by kanamycin selection.

It was noteworthy to mention that the fluorescence of Bd0134-mCherry in *Bdellovibrio* was expressed in all stages of the predatory lifecycle (Figure 5-13). The majority of the fluorescence in attack phase cells localised to both poles, but in some cells, it localised to one pole. The majority of *Bdellovibrio* cells attached to *E. coli* (at 15 min) had bipolar fluorescent patterns. At all other stages (30 min, 45 min, 1 hr, 2 hrs and 3 hrs) of the predatory lifecycle, the pattern of fluorescence was diffuse. At the 4 hrs time point of the life cycle, the pattern of fluorescence was the same as attack phase (mostly bipolar and some monopolar). The fluorescent expression of Bd0134 during the life cycle matched the RT-PCR results which showed a similar pattern to the fluorescent expression. This led to the proposal that Bd0134 has an active role in the regulation of cell wall synthesis or participates in cell wall synthesis during the predatory life cycle.

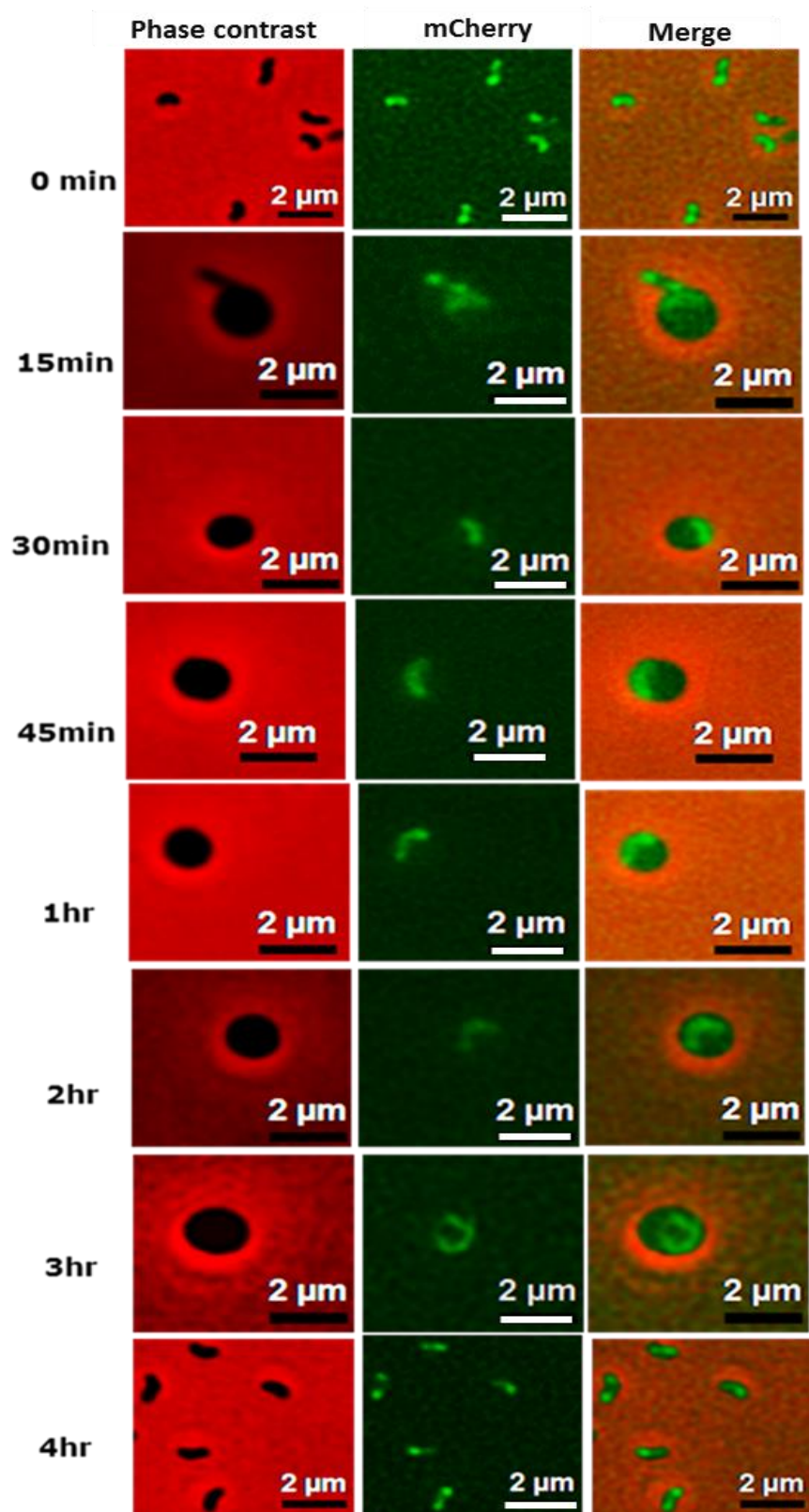


Figure 5. 13: The fluorescence pattern of *B. bacteriovorus* HD100 *bd0134-mCherry* during the whole predatory life cycle on *E. coli*. Fluorescence was acquired with a 1.8 second exposure and maximum gain.

5.4.6 Bd0134-mCherry expressed in the host-independent lifestyle

To investigate if *bd0134-mCherry* was expressed in HI growing cells, *B. bacteriovorus bd0134-mcherry* strain was grown host-independently (HI). The growing HI *B. bacteriovorus bd0134-mCherry* strain were fluorescent within all various cell types and shapes (Figure 5-14), with many of the cells having a round morphology. This indicates that the mCherry tag effects the function of *bd0134*. The expression of the fluorescence agrees with the results of microarray data which showed that Bd0134 expressed during HI growth is 1.98 fold higher than in attack phase (Lambert et al. 2010).

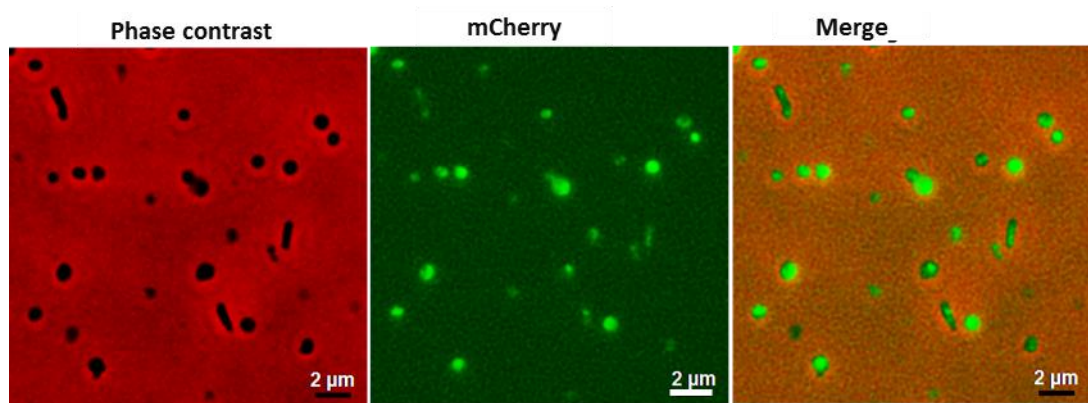


Figure 5. 14: HI *B. bacteriovorus bd0134-mCherry* strain. Bd0134-mCherry expressed in Host- Independent growth phase in all cell types with the presence of many round cells. The images were acquired with 0.5 second fluorescent exposure and maximum gain.

The DAPI stain of the HI Bd0134-mCherry showed that the fluorescent avoids the place of the DNA location mainly in all types of HI cells (Figure 5-15).

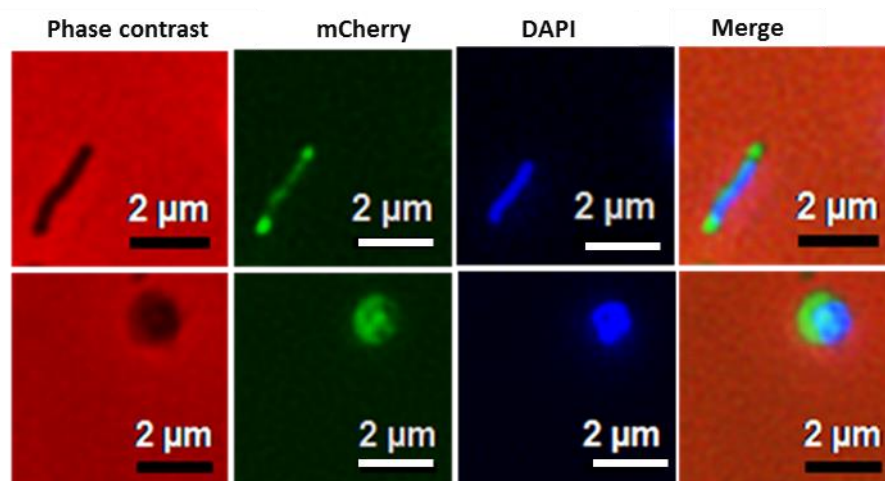


Figure 5.15: HI *B. bacteriovorus* *bd0134-mCherry* cells were stained with DAPI stain. HI cells were stained with DAPI and imaged with 0.5 seconds fluorescent exposure for mCherry imaging and 0.04 seconds for DAPI.

5.4.7 Deletion of *bd0134* was obtained successfully in *B. bacteriovorus* HD100

To investigate the functional role of *bd0134* in *B. bacteriovorus*, a silent deletion strain of *bd0134* was required. The gene encoding the Bd0134 protein was deleted using the method described in (see Section 2.10.2). A PCR fragment of 2574 bp was amplified from the genomic DNA of candidate $\Delta bd0134$ strain and was sequenced to confirm that the remaining DNA sequence was identical to wild type DNA of *B. bacteriovorus* HD100 with only the *bd0134* gene missing.

Additional confirmation to check that the *B. bacteriovorus* $\Delta bd0134$ strain was correct was to check by RT-PCR to see absence of transcription in the deletion strain. RT-PCR was performed on wild type and mutant total RNA from the attack phase cells using primers specific to the deleted internal sequence of the *bd0134* gene and using *dnak* as a positive control (Figure 5-16). The absence of a product for $\Delta bd0134$ indicates that no mRNA transcript for *bd0134* existed and this means that the gene was deleted. The wild type RNA and DNA from *B. bacteriovorus* HD100 were used as positive controls for RT-PCR using the same primers. The

presence of bands using *dnak* primers in all RNAs denotes that amplifiable RNA exists in all samples including the $\Delta bd0134$ strain.

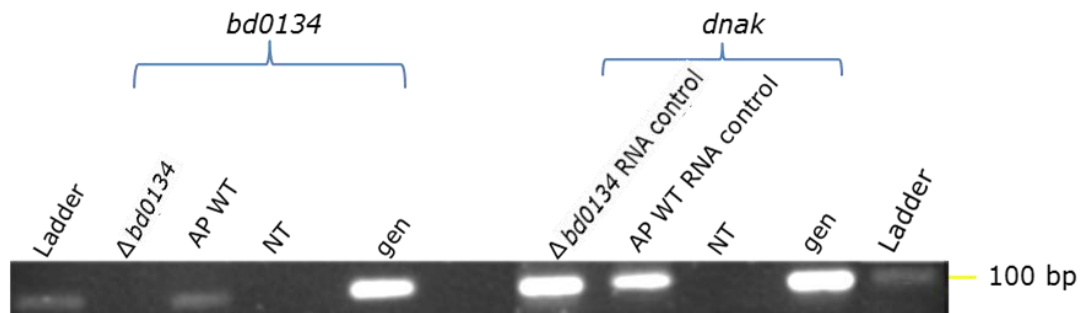


Figure 5. 16: RT-PCR confirming the $\Delta bd0134$ deletion strain. RNA was extracted from the attack phase cells of the potential deletion strain then used as a template for RT-PCR using primers specific for *bd0134* or *dnak* as a control to ensure that RNA was present within the sample. No RT-PCR product for *bd0134* gene was observed with the potential $\Delta bd0134$ strain, while products were observed using primers designed to anneal to *dnak*, confirming that the sample contained amplifiable RNA. Ladder- NEB 100bp ladder; $\Delta bd0134$ - RNA from $\Delta bd0134$ strain; AP WT= RNA from wild-type HD100 strain; NT= no template negative control; gen- HD100 genomic DNA positive control.

5.4.8 The effect of deletion of *bd0134* on the attachment and the invasion time in $\Delta bd0134$ strain

The *B. bacteriovorus* $\Delta bd0134$ strain was isolated as a host-dependent culture (HD), indicating that this strain was still capable of predation. To test if there were any defects in predation, using time lapse phase contrast microscopy, the time that *Bdellovibrio* remained attached to the prey cell outer membrane until entry began (attachment time), and the time that *Bdellovibrio* took to pass through of the prey into the periplasm (invasion time) were measured for both *B. bacteriovorus* HD100 and $\Delta bd0134$ strains (Figure 5-17). Three experiments were performed with different cultures of predator and prey on three different days.

For the attachment time, the result revealed that there was a significant increase in attachment time from 21.05 mins (± 0.70) for the wild type strain to 32.11 mins (± 0.7) for the $\Delta bd0134$ strain ($P < 0.0001$ using Student's T test). This indicates

that the attachment time for $\Delta bd0134$ strain was longer than for *B. bacteriovorus* HD100.

For the invasion time, the time from *Bdellovibrio* beginning to cross the outer membrane until it was no longer visible outside of the prey, was measured. The invasion time increased significantly from 3.24 mins (± 0.13) in the wild type to 3.90 mins (± 0.11) in $\Delta bd0134$ strain compared to the wild-type ($P < 0.0001$ using Student's T test). This means that $\Delta bd0134$ cells invade the prey cells more slowly than *Bdellovibrio* HD100. These data for the attachment time and the invasion time indicate that although $\Delta bd0134$ participates in attachment/invasion processes but it is not essential for the predatory lifecycle of *B. bacteriovorus*.

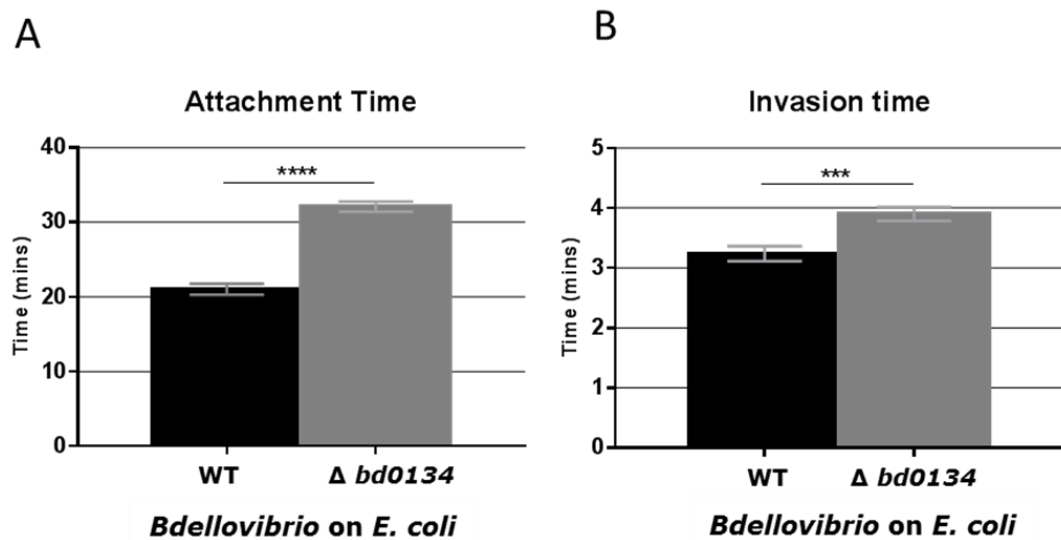


Figure 5.17: Histograms of *B. bacteriovorus* HD100 and $\Delta bd0134$ strains infecting *E. coli* S17-1. Histograms of mean times for attachment (A) and invasion (B) by *B. bacteriovorus* HD100 wild type and $\Delta bd0134$. At least three independent experiments were carried out with error bars showing 95% confidence intervals and statistical analyses using Student's T test shown (****= p , 0.0001, ***= p , 0.001) (no. cells= 100).

5.4.9 Complementation of *B. bacteriovorus* $\Delta bd0134$ strain

The increase of the attachment and the invasion of *B. bacteriovorus* $\Delta bd0134$ strain suggested that *bd0134* plays a role in entry of *Bdellovibrio* into prey cells but is not essential. To fully validate the result from the $\Delta bd0134$ mutation, a complementation construct was generated using pK18mobsacB vector that containing *bd0134* with 975 bp upstream and 100 bp downstream of gene. After that, the complementation vector pK18*bd0134* was conjugated in *B. bacteriovorus* $\Delta bd0134$ strain in the presence of kanamycin. The exconjugants were selected and grown in the predatory cultures and microscopy was performed again to measure the attachment and invasion time. The attachment time of $\Delta bd0134:pK18bd0134$ reduced significantly from 32.11 (± 0.676) mins in $\Delta bd0134$ strain to 28.16 (± 0.248) mins ($P < 0.0001$ using Student's T test) but partially, not fully to the value of 21.05 mins for wild type. While the invasion time did not change significantly from that of the deletion mutant. These results (Figure 5-18) point out that reinsertion of *bd0134* into *B. bacteriovorus* $\Delta bd0134$ mutant caused a slight but significant reduction in attachment time. The results also indicated that invasion time remained nearly the same as that of mutant strain which meant not significantly reduced.

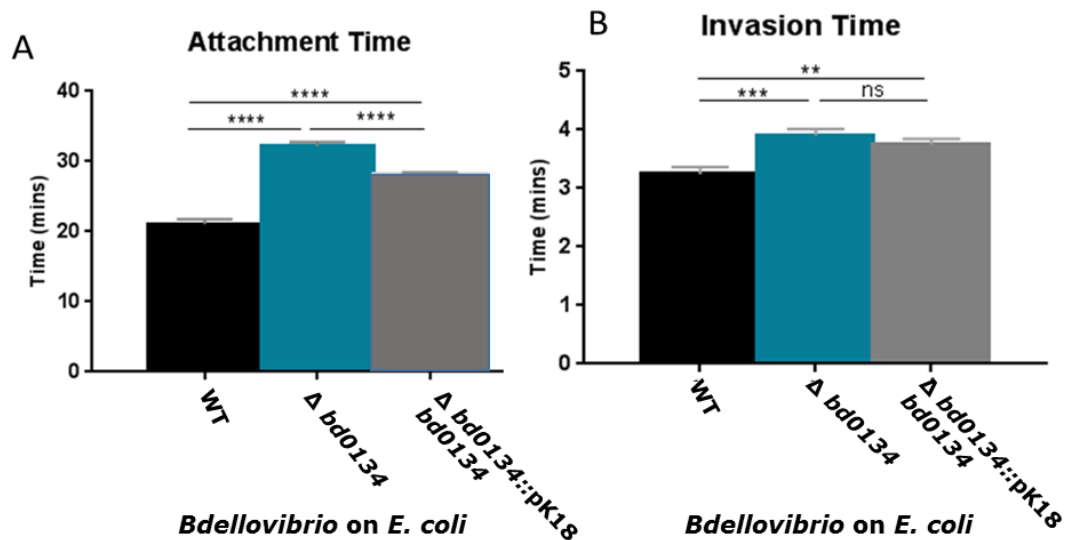


Figure 5. 18: Histograms of *B. bacteriovorus* HD100, $\Delta bd0134$ and $\Delta bd0134::pK18bd0134$ strains infecting *E. coli* S17-1. Histograms of mean times for attachment (A) and invasion (B) by *B. bacteriovorus* HD100 wild type, $\Delta bd0134$ and $\Delta bd0134::pK18bd0134$ strains. At least three independent experiments were carried out with error bars showing 95% confidence intervals and statistical analyses using Student T test shown (****=p, 0.0001, ***=p, 0.001, **=p, 0.01, ns= not significant) (no. cells= 100).

5.4.10 Testing *Bd0134* role in host-independent growth of *B. bacteriovorus*

To assess if deletion of *bd0134* has any effect on the non-predatory lifecycle (HI) of *B. bacteriovorus*, a confirmed host- dependent $\Delta bd0134$ strain was grown on PY medium (no prey cells needed) to isolate HI strains. The HI $\Delta bd0134$ isolates were examined microscopically to find out if there was variation in the cell morphology or length compared with the wild-type host- independent *Bdellovibrio* (HID13 and HID26 isolates) (Figure 5-19).

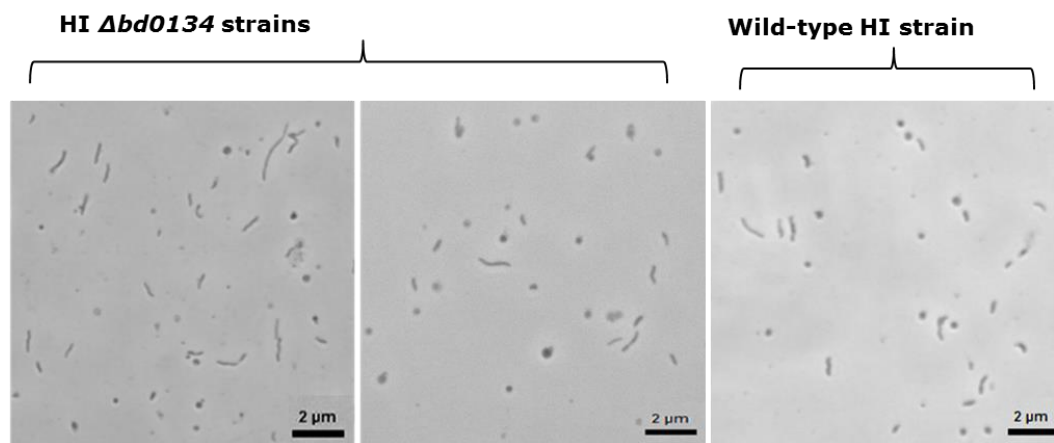


Figure 5. 19: HI *B. bacteriovorus* $\Delta bd0134$ and wild-type HI strains isolates under phase contrast microscopy. HI $\Delta bd0134$ isolates show similar cell shapes of the wild-type Host-independent and deletion of *bd0134* led to a reduction in the round cells that were found in HI *bd0134-mcherry* strain.

The results showed that HI $\Delta bd0134$ isolates did not differ morphologically from the wild-type (HID13 and HID26 isolates) HI cells with a reduction in the round cells that were found in HI *bd0134-mcherry* strain (Figure 5-19).

Furthermore, a growth curve (monitoring OD₆₀₀ nm) for HI *B. bacteriovorus* $\Delta bd0134$ strain was performed by selecting three isolates from HI *B. bacteriovorus* $\Delta bd0134$ and three from the wild-type HI (HID13, HID22 and HID26) isolates used as controls (Figure 5-20). Each culture of HI was back-diluted in PY broth to OD₆₀₀ 0.6 and the growth of HI strains were measured for 72 hr every 30 mins using BMG Fluostar plate reader. The results showed that all HI *B. bacteriovorus* $\Delta bd0134$ isolates did not show any significant differences in growth rate to the wild-type HI isolates. It is known that WT HI strains show variable growth rates but the mutant rate was within this range (Chang et al. 2011).

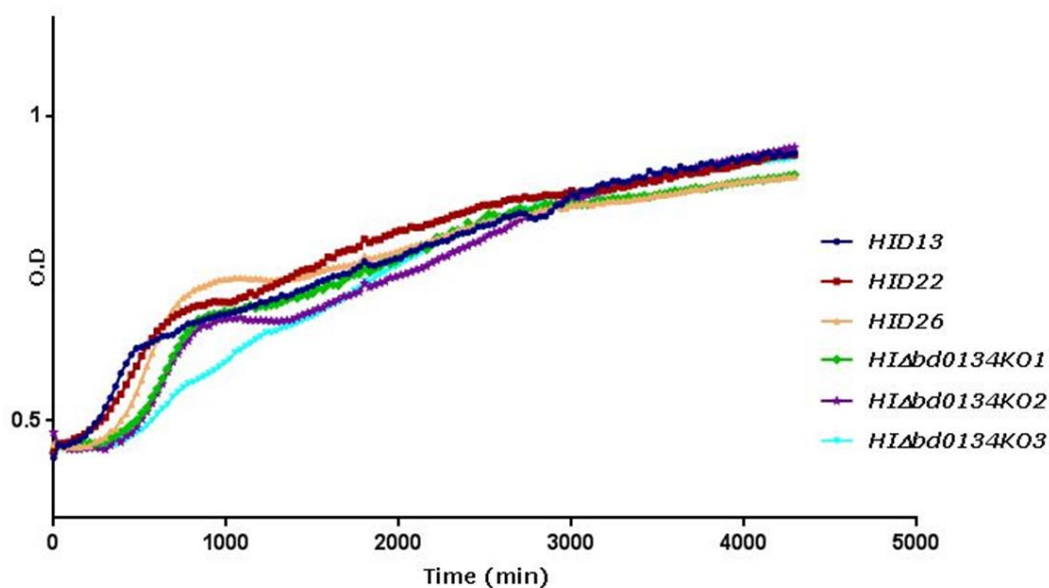


Figure 5.20: The growth curve for HI *B. bacteriovorus* $\Delta bd0134$ mutant isolates and the wild-type HI (HID13, HID22 and HID26) isolates. Data represented average of three HI $\Delta bd0134$ and wild-type HI (HID13, HID22 and HID26) isolates with 12 replicates for all isolates. Error bar shows 95% confidence intervals.

In summary, the data indicated that deletion of the *bd0134* gene did not lead to any abnormal growth defects or changes in cell morphology compared to *B. bacteriovorus* HI wild-type so the Bd0134-mCherry effect was due to abnormal activity/targeting of the tagged enzyme. These results support the proposal that *bd0134* gene product has a role in both the predatory life cycle and Host-independent lifestyle of *Bdellovibrio* which are also supported by the RT-PCR results.

5.4.11 Testing if the point mutation Bd0134-T355I in *bd0134* has an effect on predation and plaque formation ability

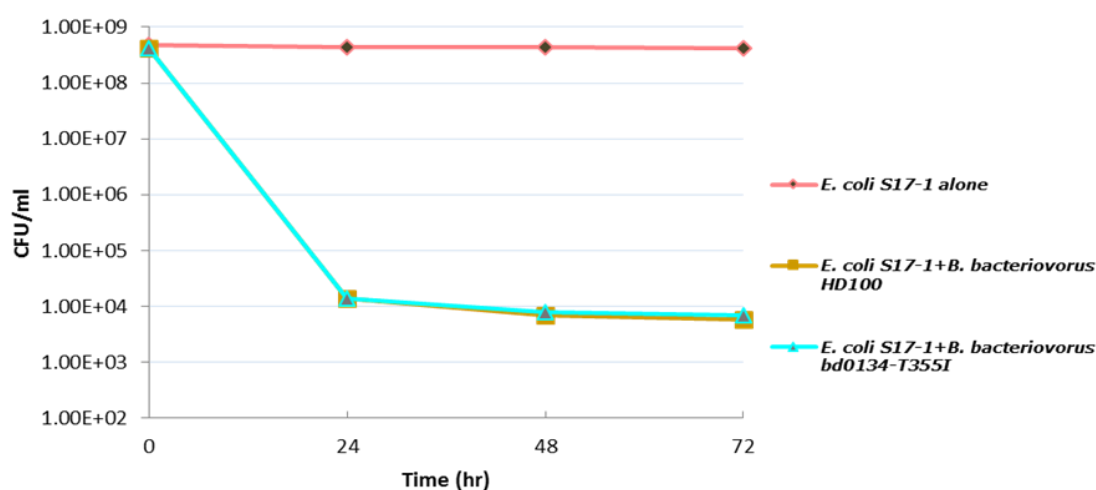
Genome sequencing data for some $\Delta bd0742$ suppressor strains showed that there was point mutation in *bd0134* (T355I). To identify if this point mutation had a role in the predation ability of the $\Delta bd0742$ suppressor strains or play a role in loss of plaque formation, it was necessary to introduce this point mutation to *B. bacteriovorus* HD100.

The point mutation *bd0134-T355I* was obtained using the method described in (see Section 2.10. 2) but with using the genomic DNA containing the point mutation *bd0134-T355I*. A PCR fragment of 2458 bp was amplified from the genomic DNA of candidate *bd0134-T355I* strain was sent for sequencing across the gene to detect the point mutation and confirm that the DNA sequence was identical to wild type DNA of *B. bacteriovorus* HD100 with only the difference in *bd0134-T355I* point mutation present. The *B. bacteriovorus bd0134-T355I* strain was isolated as a host-dependent culture (HD).

5.4.11.1 Testing the ability of *B. bacteriovorus bd0134-T355I* to prey on *E. coli* S17-1 and *A. baumannii* in comparison with wild type control strain *B. bacteriovorus* HD100

To study the ability of *B. bacteriovorus bd0134-T355I* to prey on *E. coli* and on *A. baumannii*, predation assays were conducted using a starting *E. coli* S17-1 or *A. baumannii* stationary phase inoculum of OD₆₀₀ 1.0 (10 ml containing 4.76x10⁸ and 7.57x10⁸ per ml for *E. coli* and *A. baumannii* respectively) with *B. bacteriovorus bd0134-T355I* and *B. bacteriovorus* HD100 strains matched by Lowry protein assay (500 mg protein added per strain). The assays were performed for 72 hrs for both strains in triplicate. It is revealed from Figure (5-21) that *B. bacteriovorus bd0134-T355I* can reduce the number of *E. coli* S17-1 (~ four log drops in CFU of *E. coli* S17-1) within 24 hrs similarly compared with *B. bacteriovorus* HD100

strain (which is a positive predatory control) which decreased the number of *E. coli* S17-1 within 24 hrs (~ four log drop in CFUs of *E. coli* S17-1) (Figure 5-21).

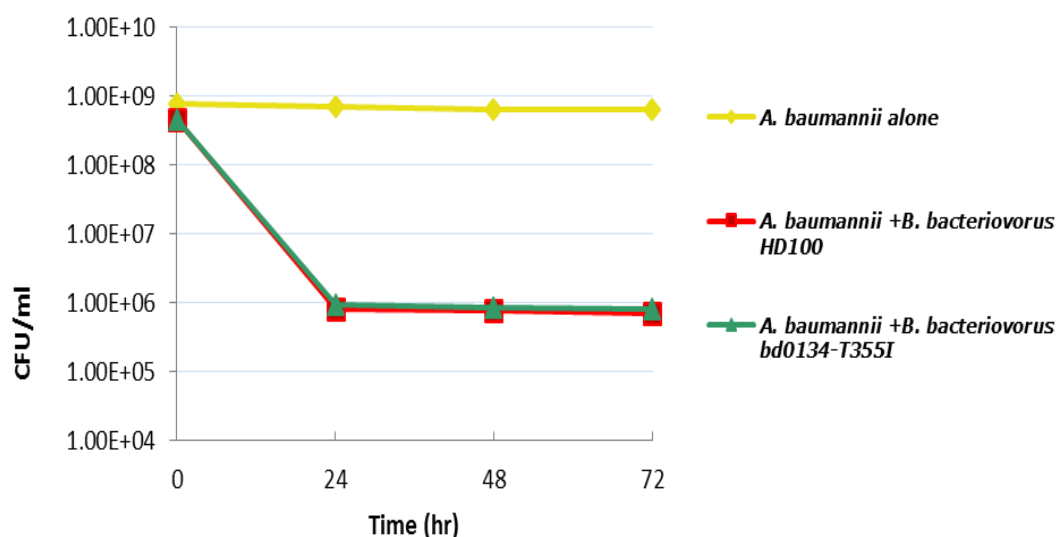


stdev	0	24	48	72
<i>E. coli</i> S17-1 alone	1.92E+06	3.85E+06	4.19E+06	8.82E+06
<i>E. coli</i> S17-1+ <i>B. bacteriovorus</i> HD100	1.68E+07	5.36E+02	1.76E+03	9.28E+02
<i>E. coli</i> S17-1+ <i>B. bacteriovorus</i> bd0134-T355I	1.02E+07	6.31E+02	2.89E+02	7.70E+02

Figure 5.21: Dynamics of predation of *B. bacteriovorus* bd0134-T355I was tested against wild-type *B. bacteriovorus* HD100 strain on *E. coli* S17-1 prey. The error bars represent 1 SD from the mean. Predation of *B. bacteriovorus* bd0134-T355I strain on *E. coli* S17-1 prey was examined by drop in number of *E. coli* S17-1 over 72 hrs. In liquid cultures, *B. bacteriovorus* bd0134-T355I strain started to reduce the number of *E. coli* S17-1 within 24 hrs which was similar to *B. bacteriovorus* HD100 strain that showed a drop in the number of *E. coli* S17-1 within 24 hrs. These results are from 3 biological repeats in separate weeks) that provided with table of standard division.

With regard of the predation ability of *B. bacteriovorus* bd0134-T355I to prey on *A. baumannii* compared to wild-type control *B. bacteriovorus* HD100, *B. bacteriovorus* bd0134-T355I had the ability to reduce the number of *A. baumannii* (~ three log drop in CFU of *A. baumannii*) within 24 hrs for three biological repeat (Figure 6-22) compared to *B. bacteriovorus* HD100 strain (a positive predatory control) which rapidly decreased the number of *A. baumannii* within 24 hrs (~ three log drop in CFUs of *A. baumannii*) for all three biological repeats (Figure 5-

22). This indicates that the point mutation *bd0134-T355I* does not affect the predation ability of $\Delta bd0742$ suppressor strains on *E. coli* and on *A. baumannii* prey.



stdev	0	24	48	72
<i>A. baumannii</i> alone	1.92E+07	9.62E+06	9.62E+06	1.92E+07
<i>A. baumannii</i> + <i>B. bacteriovorus</i> HD100	1.02E+08	9.62E+03	3.47E+04	8.39E+04
<i>A. baumannii</i> + <i>B. bacteriovorus</i> <i>bd0134-T355I</i>	1.45E+08	4.41E+04	8.22E+04	8.66E+04

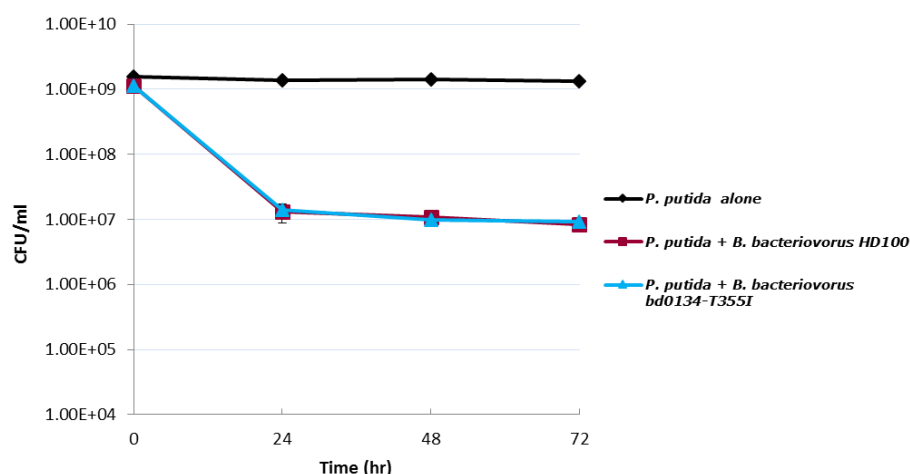
Figure 5. 22: Dynamics of predation of *B. bacteriovorus* *bd0134-T355I* was tested against wild-type *B. bacteriovorus* HD100 strain on *A. baumannii* prey. The error bars represent 1 SD from the mean. Predation of *B. bacteriovorus* *bd0134-T355I* strain on *A. baumannii* prey was examined by drop in number of *A. baumannii* over 72 hrs. In liquid cultures, *B. bacteriovorus* *bd0134-T355I* strain started to reduce the number of *A. baumannii* within 24 hrs which was similar to *B. bacteriovorus* HD100 strain that showed drop in the number of *A. baumannii* within 24 hrs. These results are from 3 biological repeats in separate weeks) that provided with table of standard division.

5.4.11.2 Testing the ability of *B. bacteriovorus* *bd0134-T355I* to prey on *P. putida*, *S. marcescens* and *S. typhimurum* in comparison with wild-type control strain *B. bacteriovorus* HD100

The predation ability of *B. bacteriovorus* *bd0134-T355I* to prey on other prey: *P. putida*, *S. marcescens* and *S. typhimurum* were tested using a starting *P. putida*, *S. marcescens* and *S. typhimurum* inoculum of OD₆₀₀ 1.0 (10 ml containing 1.58

$\times 10^9$, 2×10^9 , 1.65×10^9 for *P. putida*, *S. marcescens* and *S. typhimurum* respectively) with *B. bacteriovorus* *bd0134-T355I* and *B. bacteriovorus* HD100 strains matched by Lowry protein assay (500 mg protein per strain). The predation assay was performed for 72 hrs for all strains in triplicate.

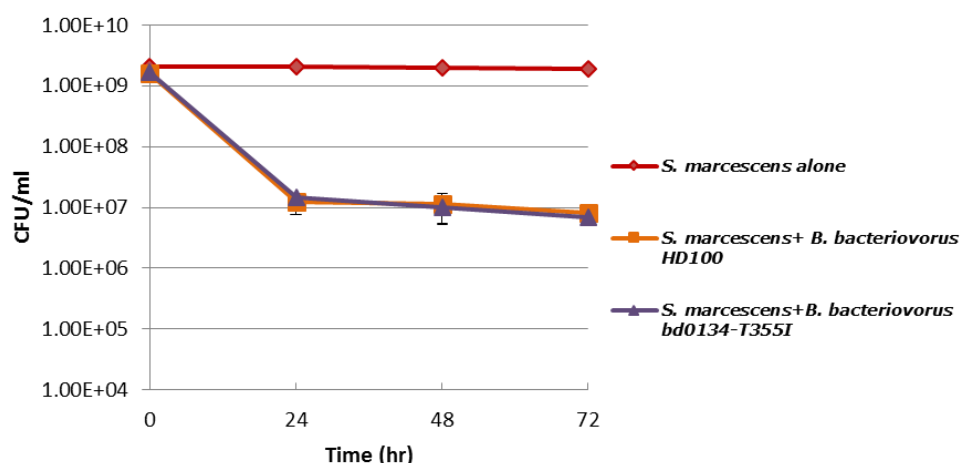
As it is obvious from Figure (5-23) that *B. bacteriovorus* *bd0134-T355I* was able to decrease the number of *P. putida* after 24 hrs (~ 2 log drop in CFU of *P. putida*) in comparison with *B. bacteriovorus* HD100 (Figure 5-23) which was able to drop the number of *P. putida* after the same time period 24 hrs (~ 2 log drop in CFUs of *P. putida*).



stdev	0	24	48	72
<i>P. putida</i> alone	8.82E+07	6.01E+07	1.44E+07	5.77E+07
<i>P. putida</i> + <i>B. bacteriovorus</i> HD100	9.18E+07	4.19E+06	2.56E+06	1.87E+06
<i>P. putida</i> + <i>B. bacteriovorus</i> <i>bd0134-T355I</i>	4.19E+07	3.91E+06	1.08E+06	8.82E+05

Figure 5.23: Dynamics of predation of *B. bacteriovorus* *bd0134-T355I* was tested against wild-type *B. bacteriovorus* HD100 strain on *P. putida* prey. The error bars represent 1 SD from the mean. Predation of *B. bacteriovorus* *bd0134-T355I* strain on *P. putida* prey was examined by drop in number of *P. putida* over 72 hrs. In liquid cultures, *B. bacteriovorus* *bd0134-T355I* strain able reduce the number of *P. putida* within 24 hrs which was similar to *B. bacteriovorus* HD100 strain that showed drop in the number of *P. putida* within 24 hrs. These results are from 3 biological repeats in separate weeks) that provided with table of standard division.

With respect to *S. marcescens* as a prey, *B. bacteriovorus* *bd0134-T355I* was able to kill *S. marcescens* after 24 hrs (~ two log drop in CFUs of *S. marcescens*) (Figure 5-24) compared with *B. bacteriovorus* HD100 (a positive predatory control) which was able to decrease the number of *S. marcescens* after 24 hrs (~ two log drop in CFU of *S. marcescens*) (Figure -24).

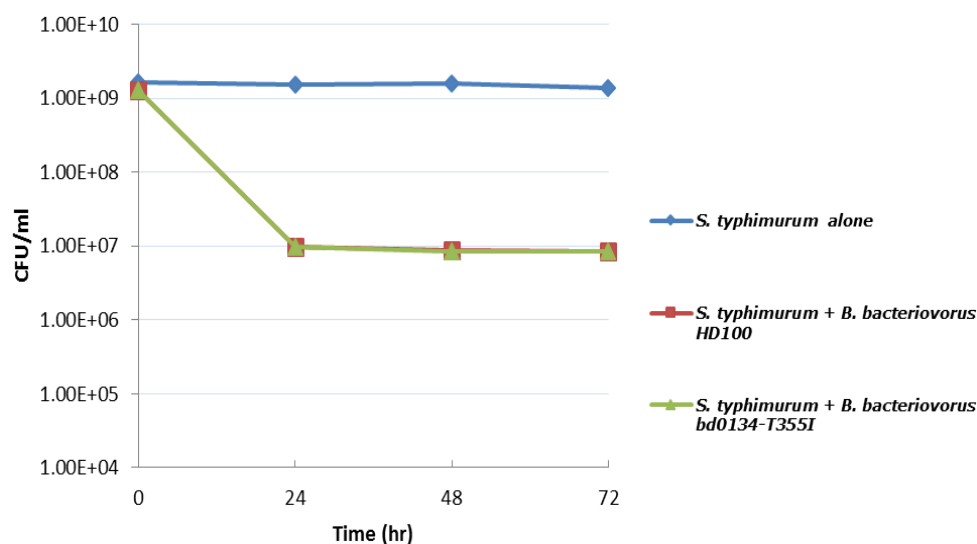


stdev	0	24	48	72
<i>S. marcescens</i> alone	1.67E+07	1.13E+08	1.92E+07	1.11E+08
<i>S. marcescens</i> + <i>B. bacteriovorus</i> HD100	6.74E+07	4.53E+06	5.83E+06	1.00E+06
<i>S. marcescens</i> + <i>B. bacteriovorus</i> <i>bd0134-T355I</i>	5.85E+07	1.78E+06	4.72E+06	9.18E+05

Figure 5.24: Dynamics of predation of *B. bacteriovorus* *bd0134-T355I* was tested against wild-type *B. bacteriovorus* HD100 strain on *S. marcescens* prey. The error bars represent 1 SD from the mean. Predation of *B. bacteriovorus* *bd0134-T355I* strain on *S. marcescens* prey was examined by drop in number of *S. marcescens* over 72 hrs. In liquid cultures, *B. bacteriovorus* *bd0134-T355I* strain decreased the number of *S. marcescens* after 24 hrs which was similar to *B. bacteriovorus* *hd100* strain that dropped the number of *S. marcescens* after 24 hrs. These results are from 3 biological repeats in separate weeks) that provided with table of standard division.

Concerning *S. typhimurum* as prey, *B. bacteriovorus* *bd0134-T355I* reduced the number of *S. typhimurum* after 24 hrs (~ two log drop in CFUs of *S. typhimurum*) (Figure 5-24) as compared to positive predatory control *B. bacteriovorus* HD100 which decreased the number of *S. typhimurum* after 24 hrs (~ two log drop in CFU of *S. typhimurum*) (Figure 5-25). This means that *B. bacteriovorus* *bd0134-*

T355I strain was able to lyse *P. putida*, *S. marcescens*, *S. typhimurum* and the point mutation *bd0134-T355I* does not affect the predation ability as it has the same rate as *B. bacteriovorus* HD100.



stdev	0	24	48	72
<i>S. typhimurum</i> alone	8.82E+07	3.47E+07	8.55E+07	1.67E+07
<i>S. typhimurum</i> + <i>B. bacteriovorus</i> HD100	6.74E+07	8.22E+05	5.09E+05	1.55E+06
<i>S. typhimurum</i> + <i>B. bacteriovorus</i> <i>bd0134-T355I</i>	6.67E+07	1.61E+06	1.67E+05	1.29E+06

Figure 5. 25: Dynamics of predation of *B. bacteriovorus* *bd0134-T355I* was tested against wild-type *B. bacteriovorus* HD100 strain on *S. typhimurum* prey. The error bars represent 1 SD from the mean. Predation of *B. bacteriovorus* *bd0134-T355I* strain on *S. typhimurum* prey was examined by drop in number of *S. typhimurum* over 72 hrs. In liquid cultures, *B. bacteriovorus* *bd0134-T355I* strain started to reduce the number of *S. typhimurum* within 24 hrs which was similar to *B. bacteriovorus* HD100 strain that showed drop in the number of *S. typhimurum* within 24 hrs. These results from 3 biological repeats (in separate weeks) that provided with table of standard division.

5.4.11.3 Testing the effect of point mutation in *B. bacteriovorus* *bd0134-T355I* on the ability of plaque formation

In order to determine if the point mutation T355I in *bd0134* in the genome of some $\Delta bd0742$ suppressor strains has an effect on the plaque formation ability of $\Delta bd0742$ suppressors strains, *B. bacteriovorus* *bd0134-T355I* strain was plated out on double layer YPSC overlay plates containing prey *E. coli* S17-1 in the top

layer. *B. bacteriovorus* *bd0134-T355I* strain is still able to produce plaques like *B. bacteriovorus* HD100 (Figure 5-26) within the 5 days. This result confirms that $\Delta bd0742$ suppressor strains have lost their ability to produce plaques from deletion of *bd0742* gene and not from the point mutation in *bd0134* gene that was identified in some of $\Delta bd0742$ suppressor strains.

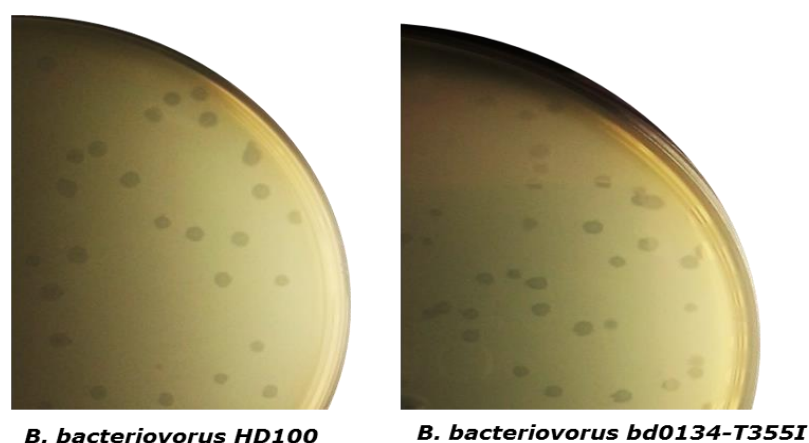


Figure 5. 26: The plaque plates of predatory *B. bacteriovorus* HD100 and *B. bacteriovorus* *bd0134-T355I* strains on double layer YPSC overlay plates containing prey *E. coli* S17-1 in the top layer. The point mutation T355I in *bd0134* gene does not affect the ability of *B. bacteriovorus* strain to produce plaques.

5.4.12 Testing the effect of addition extra copy of *WTbd0134* to HI *B. bacteriovorus* $\Delta bd0742$ mutant and $\Delta bd0742$ suppressor strains

As previously mentioned, HI *B. bacteriovorus* $\Delta bd0742$ mutant strain and $\Delta bd0742$ suppressor strains were unable to form plaques (see Chapter 3 and 7 section 3.6.4 and 7.4.5), therefore in this section I will examine the effect of an additional extra copy of wild-type *bd0134* on the ability of plaque formation for HI *B. bacteriovorus* $\Delta bd0742$ mutant and $\Delta bd0742$ suppressor strains. A pK18mobsacB vector containing wild-type *bd0134* was generated and then transformed into *E. coli* S17-1 and conjugated from donor *E. coli* strain S17-1 into the original HI *B. bacteriovorus* $\Delta bd0742$ mutant and $\Delta bd0742$ suppressor strains by single crossover with maintenance of plasmid by kanamycin selection.

Surprisingly, the addition of an extra copy of wild-type *bd0134* into the original HI *B. bacteriovorus* Δ *bd0742* mutant and Δ *bd0742* suppressor strains resulted in restoring the ability of these strains to produce plaques within 12 days for original *B. bacteriovorus* Δ *bd0742* deletion mutant strain and 7 days for Δ *bd0742* suppressor strain of incubation at 29 °C (Figure 5-27).

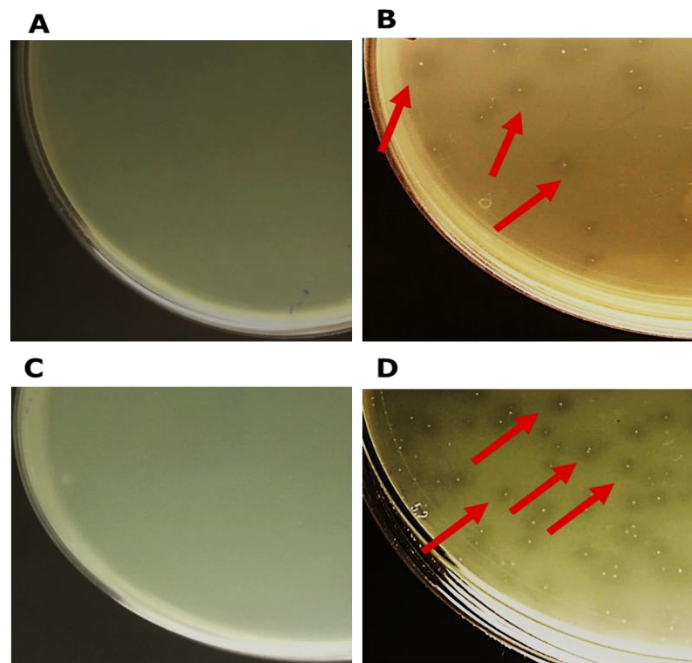


Figure 5.27: The plaques plates of HI *B. bacteriovorus* Δ *bd0742* mutant (+WT *bd0134*) and Δ *bd0742* suppressor (+WT *bd0134*) strains on double layer YPSC overlay plates containing the prey *E. coli* (pZMR100) in the top layer. Addition of extra copy of wild-type *bd0134* restores the ability of HI *B. bacteriovorus* Δ *bd0742* mutant and Δ *bd0742* suppressor strains to produce plaques (the red arrows) that was lost by deletion of *bd0742* gene. The plaques formed during 12 days for original *B. HI bacteriovorus* Δ *bd0742* mutant (+WT *bd0134*) strain and 7 days for Δ *bd0742* suppressor strain (+WT *bd0134*). (A: *B. bacteriovorus* Δ *bd0742* strain, B: *B. bacteriovorus* Δ *bd0742* (+WT *bd0134*) strain, C: Δ *bd0742* suppressor strain and D: Δ *bd0742* suppressor (+WT *bd0134*) strain.

5.4.13 Transcription of *bd0528* (*ask β*) throughout the predatory lifecycle of *Bdellovibrio* and HI lifestyle

RT-PCR was performed to examine expression of *bd0528* gene (*ask β*) using RNA samples taken from points across a single round of a synchronous *B. bacteriovorus* HD100 WT and $\Delta bd0134$ strain predatory cycles. Figure (5-28) showed that *bd0528* was constitutively expressed in *B. bacteriovorus* HD100 WT, but expression of *bd0528* dropped in attack phase and 4hr for RNA isolated from *B. bacteriovorus* $\Delta bd0134$ strain. This pattern was somewhat similar to the expression pattern of *bd0134* itself in wild type predatory cycle (Figure 5-7). The overall result of *bd0528* expression indicated that this gene has an important role in the predatory lifecycle of *Bdellovibrio* and it is a housekeeping gene.

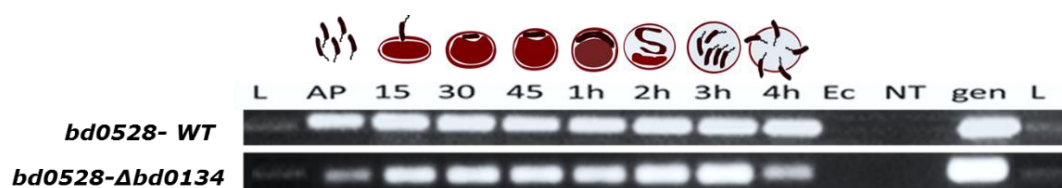


Figure 5.28: Reverse-transcriptase PCR showing relative transcriptional expression of *ask bd0528* (*ask β*) throughout predatory life cycle. RNA was isolated at the indicated time points during one round of synchronous *Bdellovibrio* HD100 and $\Delta bd0134$ infection of *E. coli* host cells, and primers specific to the gene was used to amplify (RT-PCR, 30 cycles) an approximately 110 bp internal fragment from each transcript if present. AP = Attack phase (free-swimming *Bdellovibrio*); 15, 30, 45 = minutes since start of infection (attachment to and rounding of *E. coli* host cell into a bdelloplast structure); 1h, 2h, 3h, 4h = hours since start of infection (host cell resources being degraded and used for *Bdellovibrio* growth into a filament, followed by septation into multiple progeny and eventually lysis from host cell); Ec = *E. coli* S17-1 RNA (no *Bdellovibrio* control); gen = *B. bacteriovorus* HD100 genomic DNA (positive control); NT = No template control; L = NEB 100 bp DNA ladder.

To examine if *bd0528* is expressed in HI lifestyle, RT-PCR was performed on total RNA (samples matched 10 ng/μl) isolated from the attack phase and different HI strains (HID2, HID13, HID22 and HID26). Figure (5-29) showed that *bd0528* expression is higher in all HI strains than the attack phase.

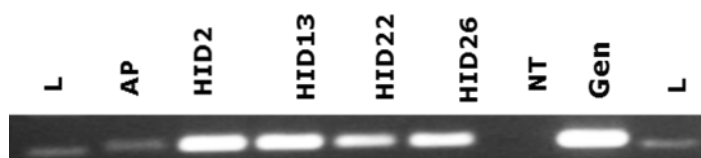
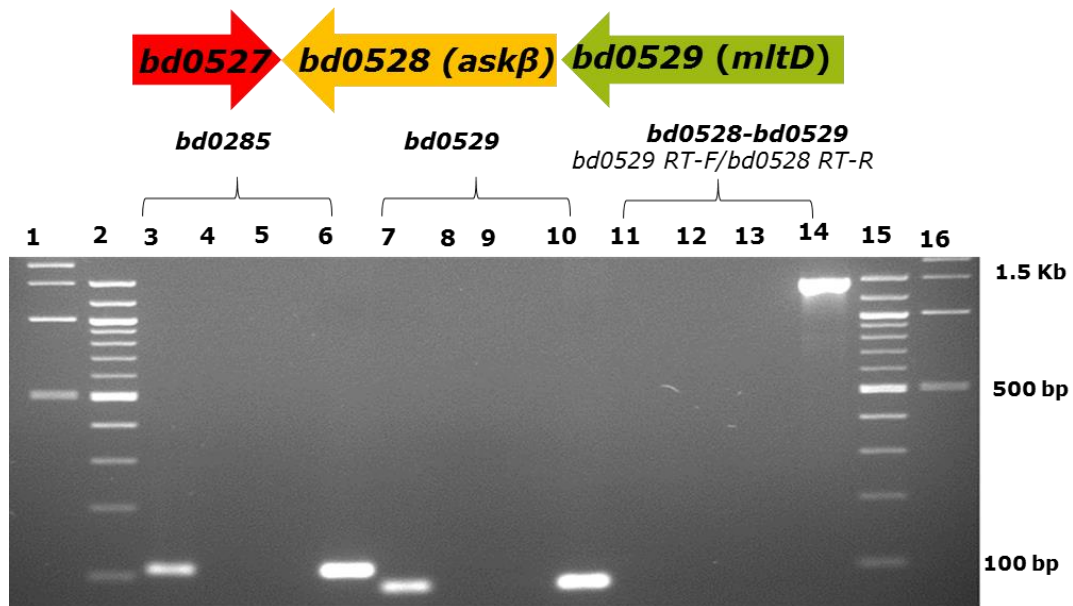


Figure 5. 29: Reverse-transcriptase PCR showing relative transcription expression of *B. bacteriovorus* HD100 *bd0528* during attack phase and host-independent growth. RNA was isolated from *B. bacteriovorus* HD100 attack phase (AP) cells and four host-independent strains (HID2, HID13, HID22, HID26) at OD₆₀₀ 0.6 and the RNA concentration was matched to 10 ng μl⁻¹. Primers specific to the gene were used to amplify (RT-PCR, 30 cycles) a 110 bp internal fragment from each transcript if present. Gen = *B. bacteriovorus* HD100 genomic DNA (positive control); NT = no template control; L = NEB 100 bp DNA ladder.

5.4.14 The *bd0528* gene is not in an operon with *bd0529*

To identify whether *bd0528* and *bd0529* of *B. bacteriovorus* genes were co-transcribed, RT-PCR was performed for joining space between them on RNA isolates from *B. bacteriovorus* HD100 strain during the predatory cycle (2 hrs). The result showed that there is no observable RT-PCR product between *bd0528* and *bd0529* genes (Figure 5-30). This means that there is no shared operon between them.



5. 30: RT-PCR shows that the *B. bacteriovorus* genes *bd0528* and *bd0529* are not co-transcribed. Lanes 1 and 16: NEB 100 bp ladder; Lanes 2 and 15: NEB 100 bp ladder; Lanes 3, 7 and 11: *B. bacteriovorus* RNA from 3 hrs; Lanes 4, 8 and 12: *E. coli* S17-1 RNA; Lanes 5, 9 and 13: No template; Lanes 6, 10 and 14: *B. bacteriovorus* HD100 genomic DNA. No band found in lane 11 indicates that there are no co-transcription between *bd0528* and *bd0529* genes.

5.4.15 Initial location of Bd0528-mCherry heterologously expressed in *E. coli* DH5a

To assess the cellular location Bd0528-mCherry (Askβ) during predatory lifecycle, I generated a C-terminal fluorescently tagged *B. bacteriovorus* *bd0528* (*askβ*) strain using the method described in (see Section 2.10.1). The location of Bd0528-mCherry in *E. coli* DH5a gave an initial picture about the possible location of Bd0528 inside *E. coli* and ensured that the construct of *bd0528-mCherry* was made correctly. It was found that Bd0528-mCherry was found near the poles as shown in (Figure 5-31).

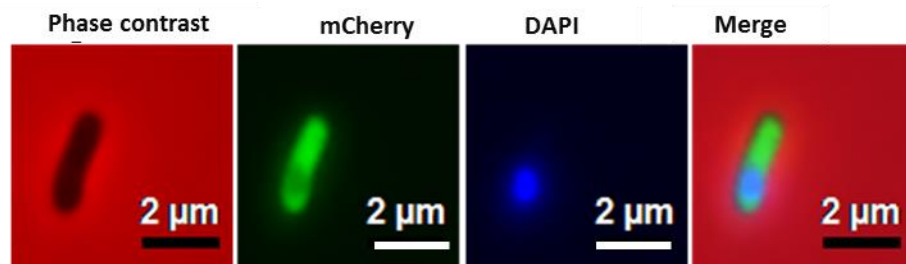


Figure 5. 31: Heterologous expression of pAKF56 Bd0528-mCherry in *E. coli* DH5a in stationary phase (grown in YT broth for 16 hours with appropriate antibiotic). The fluorescence is visible and located near poles.

5.4.16 Fluorescently tagged Bd0528 (ASK β) showed diverse cellular location in the attack phase *Bdellovibrio*

The location of Bd0528-mCherry in *Bdellovibrio* was assessed in the attack phase (non- replicating phase) which shows vast majority of the fluorescence in the attack phase localised at both poles ($\sim 50.7\%$) with others showing diffuse fluorescence throughout the cell ($\sim 28.6\%$), at one pole ($\sim 19.8\%$), and with some *Bdellovibrio* not fluorescent ($\sim 0.9\%$) (Figure 5-32 A and B).

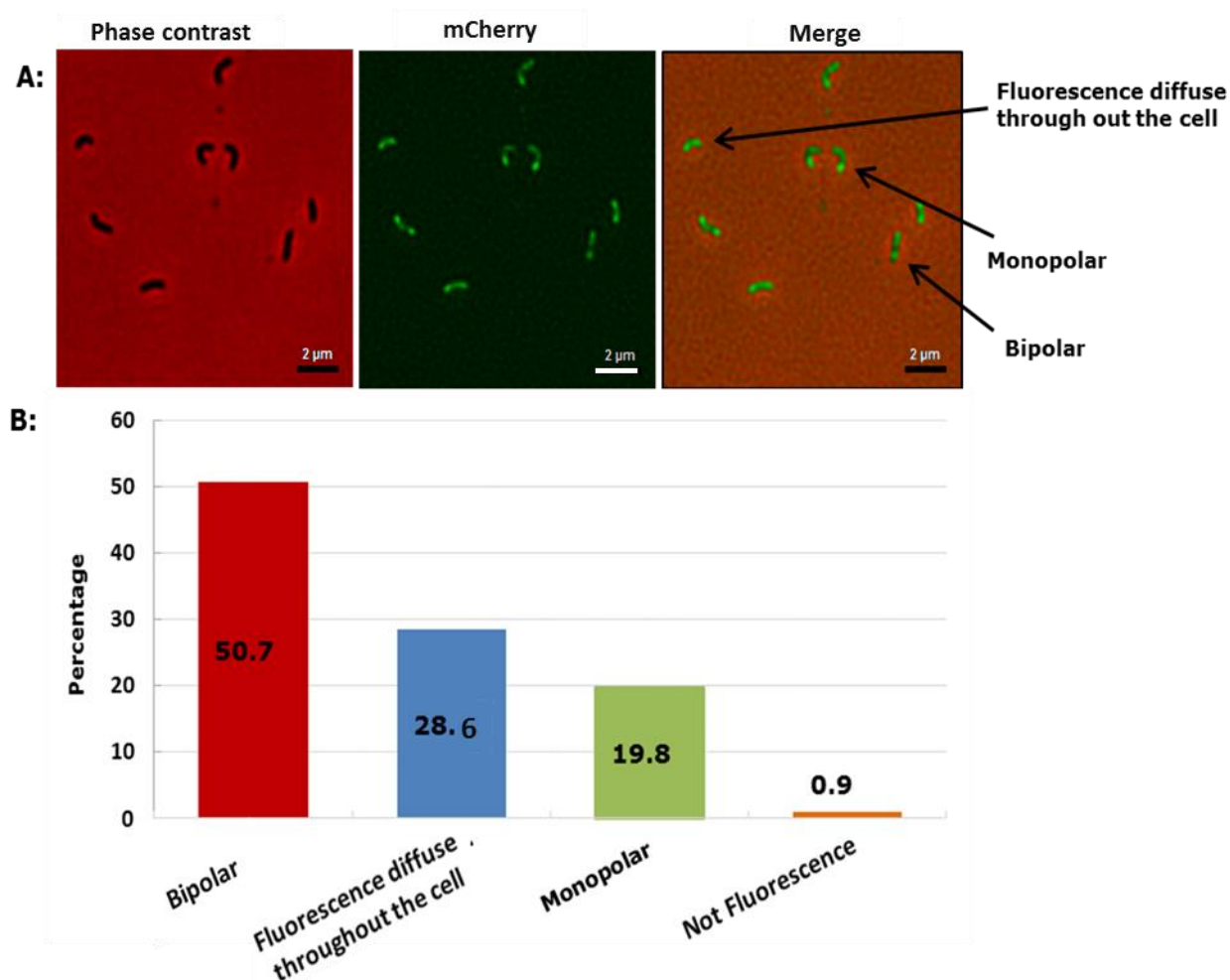


Figure 5. 32: Examples of localisation of Bd0528-mCherry in attack phase *B. bacteriovorus* HD100 cells. (A) Image of *B. bacteriovorus* HD100 *bd0528-mcherry* cells (scale bar = 2 µm). (B) Overall distribution of Bd0528-mCherry fluorescence in *B. bacteriovorus* attack phase cells population (no of cells=424) from three biological repeats.

Whereas, during the attachment of *Bdellovibrio* to *E. coli* (at 15 min) the fluorescence was shown to be mostly diffuse through the cell and the rest at both poles and at one of the poles of *Bdellovibrio* (Figure 5-33).

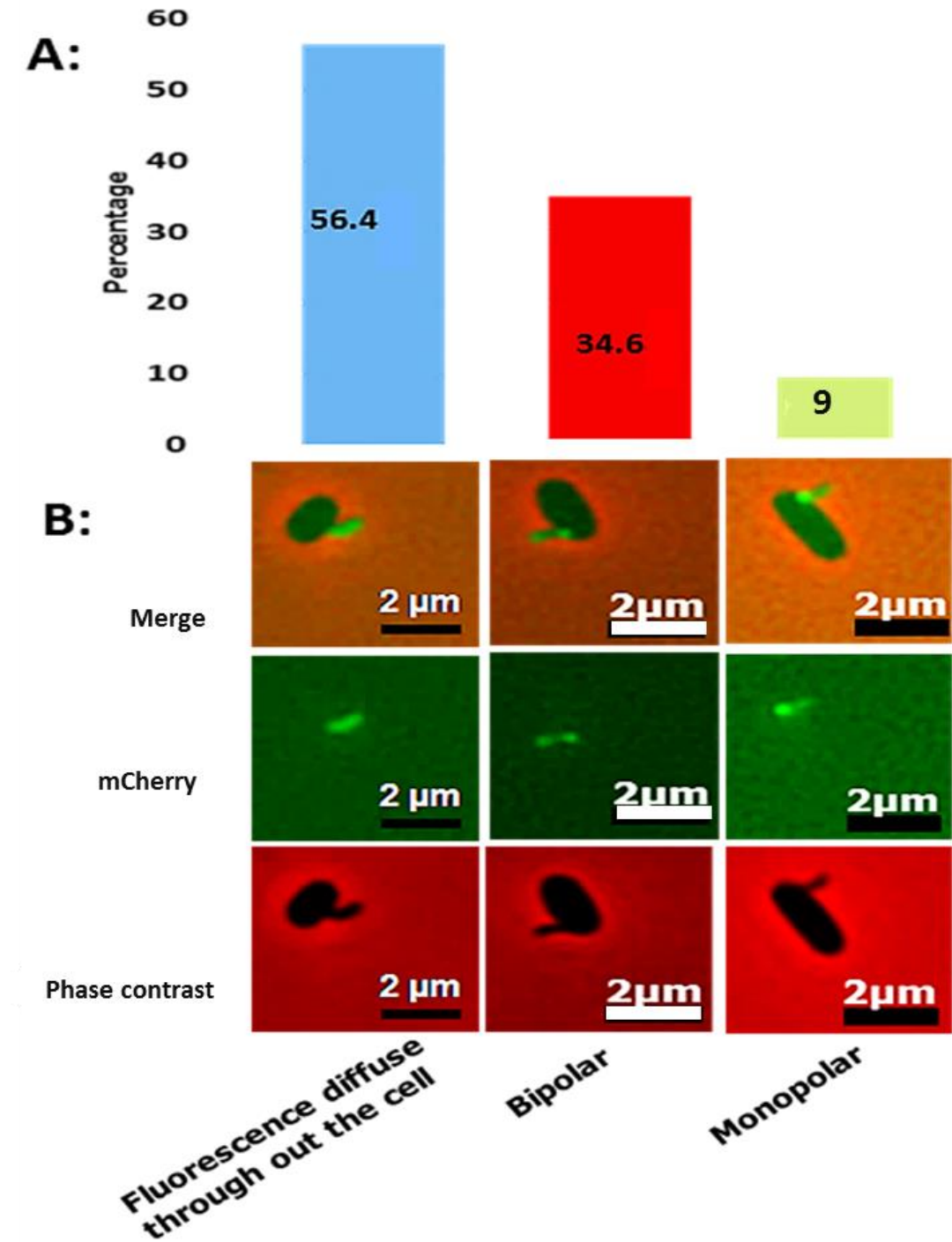


Figure 5. 33: Examples of localisation of Bd0528-mCherry when the *B. bacteriovorus* HD100 cells are attached to *E. coli*. (A): Distribution of Bd0528-mCherry fluorescence in *B. bacteriovorus* HD100 cells during attachment to *E. coli* (no of cells=248) from three biological repeats. (B): Images of *B. bacteriovorus* HD100 *bd0528-mcherry* attached to *E. coli* (scale bar = 2 μ m).

With regard to the rest of the stages of predatory life cycle (30min, 45min, 1 hr, 2hr, 3hr) the fluorescence pattern of Bd0528-mCherry in *Bdellovibrio* was diffuse and at 4hr of the life cycle, the pattern of fluorescence was the same as that in attack phase (mostly bipolar with others monopolar and diffuse) (Figure 5-34).

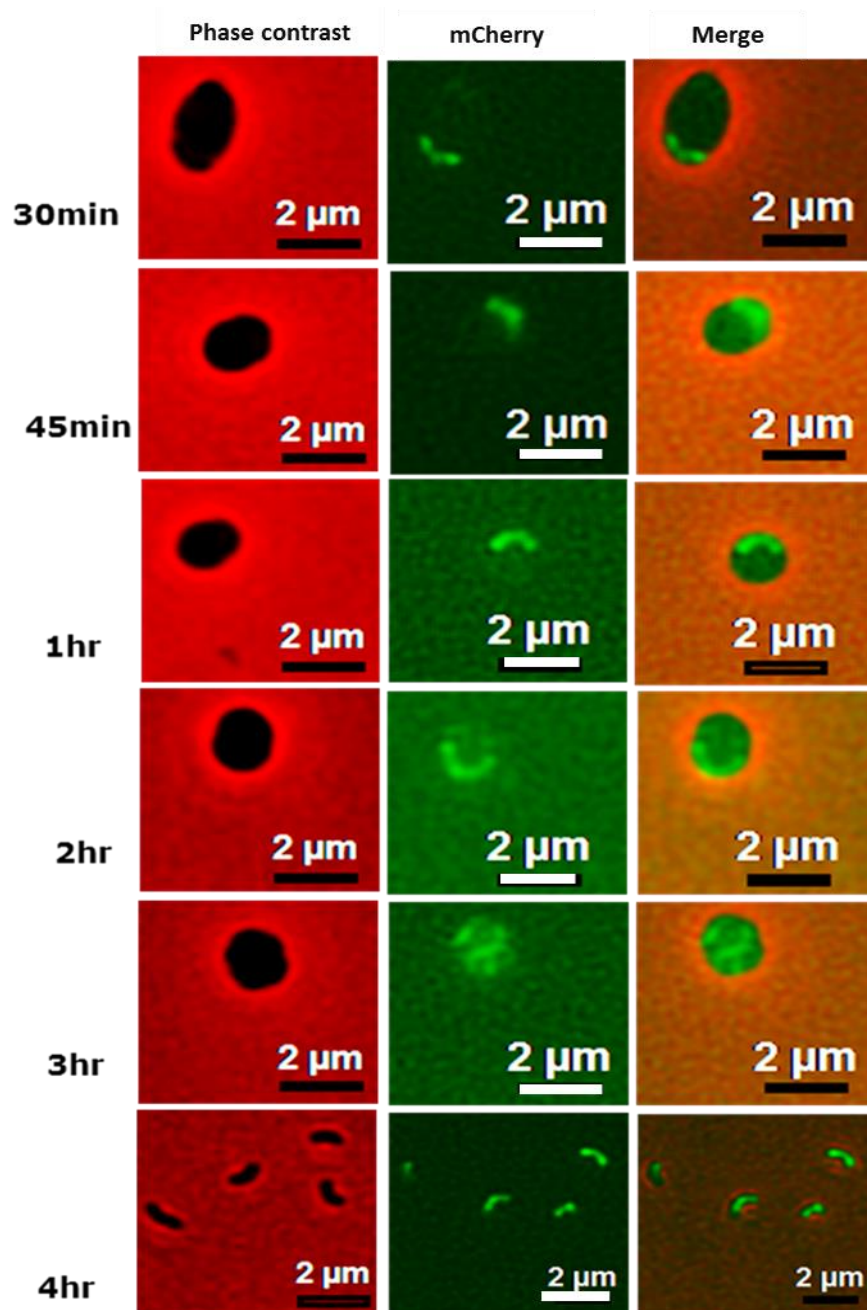


Figure 5. 34: The fluorescence pattern of *B. bacteriovorus* HD100 *bd0528-mCherry* during the whole predatory lifecycle on *E. coli* (pZMR100). Fluorescence was acquired with 1.5 second exposure with maximum gain.

5.4.17 Bd0528-mCherry fluorescence was visualized in growing HI cells of *Bdellovibrio*

To examine if Bd0528-mCherry exhibited any fluorescent signal in host-independent growing cells and their location in HI cells, isolates of the *B. bacteriovorus* HD100 *bd0528-mCherry* strain were generated and grown as HI. The HI *B. bacteriovorus bd0528-mCherry* is visualized in all cell shapes (Figure 5-35). This may mean that *bd0528* plays a role in the host independent growth of *Bdellovibrio*. This can be further investigated by generating a deletion strain of this gene.

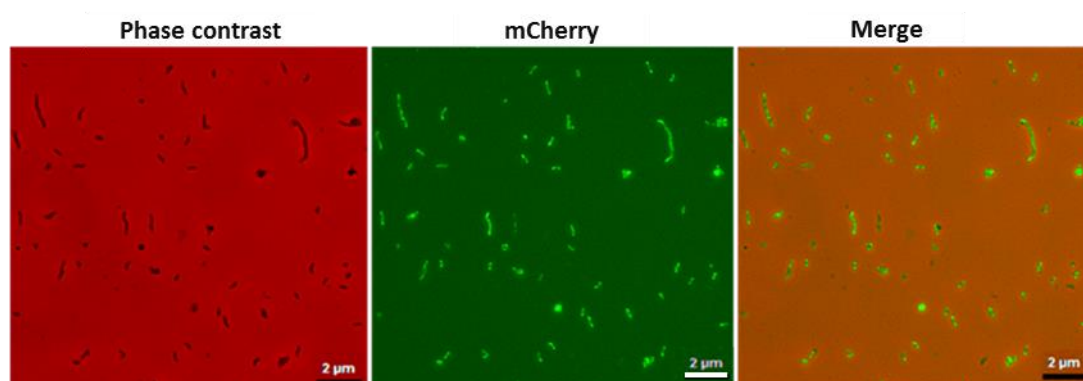


Figure 5. 35: HI *B. bacteriovorus* Bd0528-mCherry strain. Bd0528-mCherry expressed in Host- Independent growth phase in all cell types. The images were taken with 0.6 second fluorescence exposure and maximum gain.

The DAPI stain of the HI *B. bacteriovorus bd0528-mCherry* strain showed that the fluorescence was not in same place as the DNA in the HI cells (Figure 5-36).

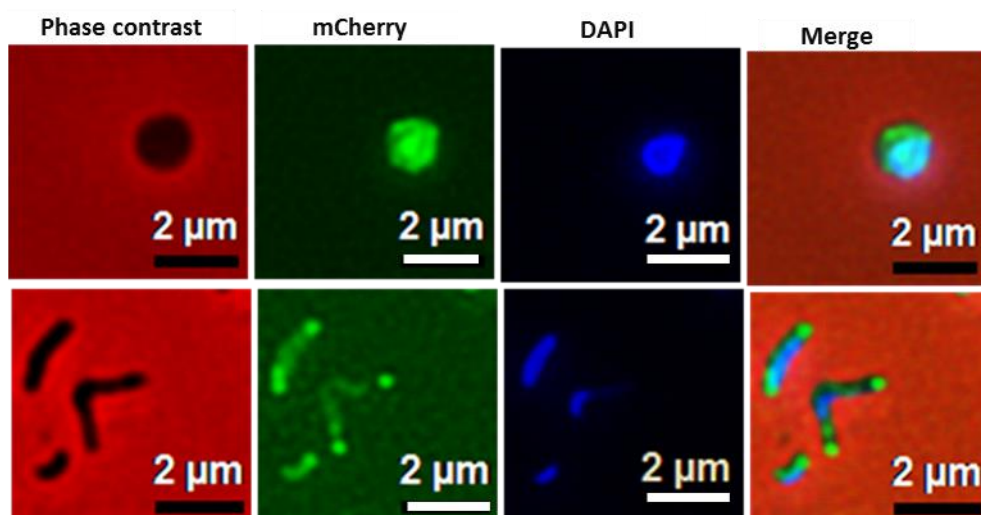


Figure 5. 36: HI *B. bacteriovorus* *bd0528-mCherry* cells were stained with DAPI stain. HI cells were stained with DAPI and imaged with 0.6 seconds fluorescence exposure for mCherry imaging and 0.04 seconds for DAPI images.

5.4.18 Deletion of *bd0528* (*askβ*)

To discover the role of the *bd0528* gene in both *B. bacteriovorus* lifestyles and its function, a silent knockout deletion strain of *bd0528* was required. A deletion plasmid for *bd0528* was constructed by amplifying 599 bp upstream and 932 bp downstream of the gene, this DNA was then joined together by using phusion polymerase (NEB). After that $\Delta bd0528$ construct was cloned and transformed in the plasmid pK18mobsacB using *E. coli* S17-1 as a host and conjugated into *B. bacteriovorus*, recombining by single crossover and maintained by kanamycin selection.

The resultant *Bdellovibrio* plaques from conjugation were grown as host-dependent and host-independent cultures, and then screened for gene replacement and plasmid loss using 2.5% sucrose. 60 exconjugants for HD and 55 from HI were screened for isolating $\Delta bd0528$ strain. All exconjugants were sensitive to kanamycin and PCR was used for initial screening for deletion mutants (Figure 5-36 A and B). All exconjugants gave a band of wild HD100 size which

meant that they were revertants to wild-type (Figure 5-37 A and B). This suggests that the deletion of *bd0528* is impossible in both HD and HI.

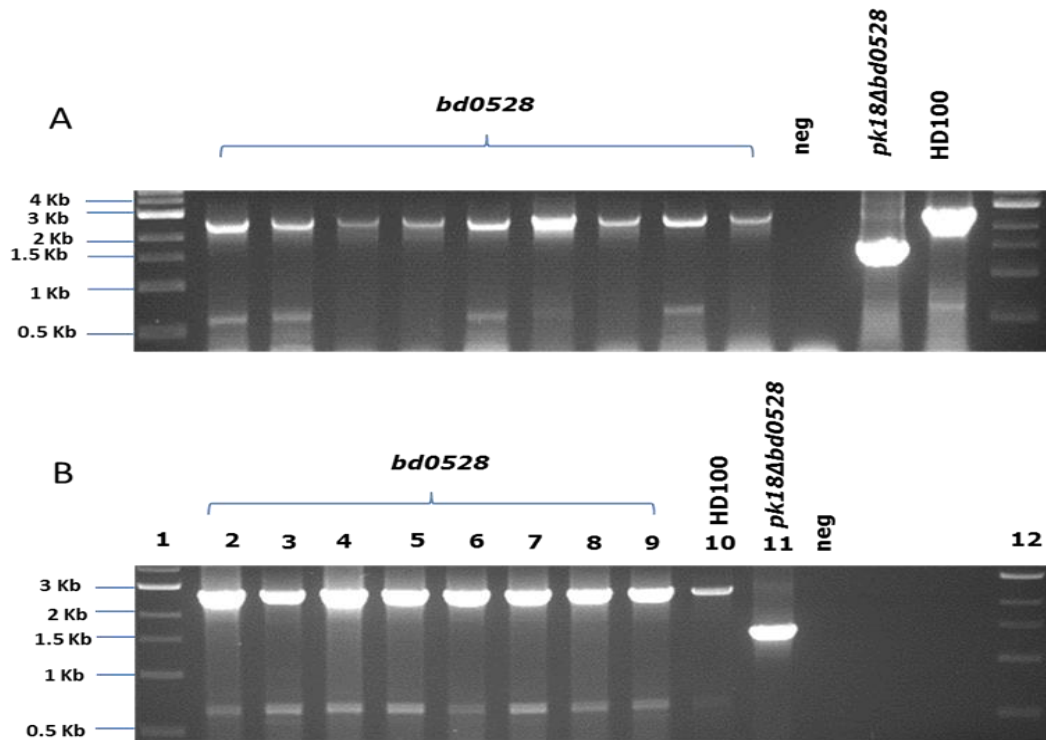


Figure 5. 37: Agarose gel showing amplified products from initial PCR screen of genomic DNA from potential HD and HI *bd0528* knockout mutants. A: PCR of potential knockouts of HD *bd0528* using Taq polymerase PCR and primers *bd0528*-F and *bd0528*-R. Lanes 1, 14 are 1 Kb ladder. Lanes 2-10 are potential knockout showing bands of wild-type HD100 seen at 2,706 KB. Lanes 11,12 and 13 are negative control and HD100 positive control and *pk18Δbd0528*. **B:** PCR of potential knockouts of HI *bd0528* using Taq polymerase PCR and primers *bd0528*-F and *bd0528*-R. Lanes 1, 12 are 1 Kb ladder. Lanes 2-9 are potential knockout showing bands of wild-type HD100 seen at 2,706 KB. Lanes 10, 11 and 13 are HD100 control, *pk18Δbd0528* and negative control.

5.5 Discussion

B. bacteriovorus have two aspartokinase enzymes ASK α and ASK β . ASK α is encoded by *bd0134*, considered to be acquired by lateral gene transfer from Gamma proteobacteria, which in turn acquired the gene from *Bacteroidetes*. ASK β is encoded by *bd0528* that is the ancestral gene (Lo et al. 2009). ASK α and ASK β are key enzymes in DAP synthesis pathway that may be important for predatory bacteria like *Bdellovibrio* for both prey wall and self-wall modification.

With regard to Bd0134 (Ask α) results, the transcription profile of *bd0134* (*ask α*) occurred across the predatory cycle but the expression of *bd0134* reduced when the predator cells were outside the prey (attack phase and 4 hrs) (Figure 5- 8). The fluorescent microscopy confirmed that *bd0134* was expressed during all stages of predatory life cycle and this matched with the RT-PCR results (Figure 5- 13). On the other hand, the data from Figures (5-14 and 5-15) revealed that fluorescence was seen within all of the HI cell types and with an increase in percentage of rounded cells which indicated that the mCherry tag negatively affected *bd0134* function. The expression of Bd0134-mCherry in HI growth fitted with the results of HI RT-PCR profile and microarray data that showed that the level is upregulated in HI growing cells (Figure 5-9).

Deletion of *bd0134* was possible in Host-dependent *B. bacteriovorus* and its deletion affected the early stages (attachment and invasion time) of the predatory lifecycle of *B. bacteriovorus*. Figure (5- 17) revealed that deletion of *bd0134* significantly increased the attachment and the invasion time of *Bdellovibrio* passing into the prey periplasm for the $\Delta bd0134$ mutant strain in comparison with *B. bacteriovorus* HD100 control strain. These results suggest that this gene plays a role in invasion of *Bdellovibrio* into the prey. The complementation of $\Delta bd0134:pK18bd0134$ strain resulted in a significant decrease of the attachment time but partially and invasion time decreased but not significantly (Figure 5- 18).

Furthermore, the deletion of *bd0134* gene did not lead to any abnormal growth defects or changes in cell morphology or growth in HI cells (Figure 5- 19 and 5-20).

With regard to the point mutation T355I in *bd0134* results, the predation ability of *B. bacteriovorus bd0134-T355I* was tested preying on *E. coli* S17-1, *A. baumannii*, *P. putida*, *S. marcescens* and *S. typhimurum* compared with *B. bacteriovorus* HD100 control strain (Figure 5-21, 5-22, 5-23, 5-24 and 5-25). The predation of *B. bacteriovorus bd0134-T355I* strain findings corroborate that the predation ability not affected, no delay in predation or inability to prey on some types of prey was observed as a result of the T355I point mutation in *bd0134* in comparison with *B. bacteriovorus* HD100 and the *B. bacteriovorus bd0134-T355I* strain and has the same predation rate as *B. bacteriovorus* HD100.

The plaque formation of *B. bacteriovorus bd0134-T355I* strain was also tested and it was revealed that T355I in *bd0134* did not affect the plaque formation (Figure 5- 26) in comparison with *B. bacteriovorus* HD100. It is possible therefore, that there was no connection between T355I point mutation and the delay or selectivity of killing the prey cells and loss of plaques formation ability on prey lawn for $\Delta bd0742$ suppressor strains.

In connection with adding extra copy of wild-type *bd0134* to the original HI *B. bacteriovorus* $\Delta bd0742$ mutant and $\Delta bd0742$ suppressor strains, the addition of an extra copy of wild type *bd0134* led to a surprising restoring of plaque formation for original HI *B. bacteriovorus* $\Delta bd0742$ mutant and $\Delta bd0742$ suppressor strains (Figure 5- 27). This may be due to both the wild type copy and extra copy working together at the same time which result in the ability to form plaques.

With reference to the results of Bd0528, the RT-PCR result showed that this gene is expressed in a continuously throughout the predatory life cycle when using the wild type strain *B. bacteriovorus* HD100 RNA and *bd0528* expression is effected

by deletion of *bd0134* and drops in attack phase and 4 hr when the predators have exited the prey (Figure 5- 28) when using RNA prepared from $\Delta bd0134$ deletion strain. The fluorescent microscopy throughout a predatory time course showed that Bd0528-mCherry was expressed during all stages of the life cycle (Figure 5-32, 5-33 and 5-34) which fitted with RT PCR results showing *bd0528* continuous expression in the wild type. In addition, Bd0528-mCherry fluorescence was exhibited in the HI growth mode in all cell types (Figure 5-35 and 5-36) and this fitted also with HI RT-PCR (Figure 5-29).

The deletion of *bd0528* was not achieved, with only strains that had reverted to the wild-type being isolated, suggesting that this may be lethal and not possible in either HD or HI lifestyle (Figure 5-36). This suggests that *bd0528* may be essential for *Bdellovibrio* viability in HD and HI growth pattern and its deletion is lethal for *Bdellovibrio* (as is the case in other bacteria). The possible reason for being unable to delete *bd0528* is that the protein product of this gene plays an important role in DAP biosynthesis pathway; an essential component in crosslinking of cell wall (or possibly in synthesis of a different amino acid). This contrasts with the *bd0134* which although it had a similar expression pattern and also encodes a partly homologous aspartokinase enzyme, was dispensable for viability in both HI and HD lifecycles, although does participate in the predatory invasion process as the $\Delta bd0134$ mutant had an extended period attached to prey prior to invasion. This may mean that Bd0528 and Bd0134 take part in the synthesis of different end compounds in the possible pathways from aspartate (such as other amino acids or DAP) or that the ratio of compounds produced is different depending on whether Bd0134 or Bd0528 makes precursors.

5.6 Conclusions

The results of this chapter clearly showed the following:

1. *bd0134* is a true *Bdellovibrio* gene, even though laterally transferred and it is expressed in both predatory life cycle and HI growth mode.
2. A deletion strain of *bd0134* was obtained and *bd0134* deletion significantly increased the attachment time of *Bdellovibrio* on the outside of the prey, prior to invasion. Complementation reduced the attachment time towards the *B. bacteriovorus* HD100 value.
3. Deletion of *bd0134* did not affect the cell morphology or growth of HI which means that *bd0134* is not essential in HI growth.
4. The T355I point mutation in *bd0134* did not affect the predation ability or plaques formation ability of *B. bacteriovorus*. This does not give evidence about any possible positive suppressive effect of Bd134-T355I in the *Δbd0742* suppressor. It is however a robust proof that the T355I point mutation in *bd0134* did not play role in delaying the predation or causing inability to prey on some prey types that occurred in *Δ bd0742* suppressor strains.
5. So by testing the mutant version of Bd0134 (T355I originally found in only some of the *Δbd0742* suppressor strains) by putting it into *B. bacteriovorus* HD100 artificially I found that the point version of *bd0134* never produce bigger or more plaques in comparison to *B. bacteriovorus* HD100 plaques were normal on plates and the mutant version of Bd0134 never affected the predation ability in comparison to *B. bacteriovorus* HD100 were normal. This suggests that the T355I version of Bd0134 is not a more active enzyme than the original WT Bd0134.

6. Addition of an extra copy of wild-type *bd0134* to the original HI *B. bacteriovorus* $\Delta bd0742$ mutant and $\Delta bd0742$ suppressor strains result in restored plaque formation ability. This might be due to extra DAP production by adding additional WT copy of *bd0134* to these strains resulted in starting to form the plaques on plates.
7. *bd0528* is expressed in both HD and HI lifestyles and it likely is a housekeeping gene.
8. *Bd0528* plays a vital role in both HD and HI lifestyle, as its deletion appears to be lethal and this is expected for house-keeping gene required for amino-acid and mDAP synthesis.

6 Chapter Six: Testing the role of some DAP/Lysine synthesis pathway genes in *Bdellovibrio* invasion

6.1 Introduction

To further understand the demands on the DAP synthesis pathway in a predatory bacterium, the function of *bd0047* (*dapB*) and *bd0129* (*dapE*) of *Bdellovibrio* were investigated in this chapter. In the DAP synthesis pathway, DapE is near the end of DAP production. Ruby and Rittenberg (1984) found that DAP was added to prey cells during predation of *Bdellovibrio* from an unknown source. Testing DapE which is located in the pathway close to the DAP product, unlike Bd0134 and Bd0528 which are at the top of the general pathway that leads eventually to DAP. DapB at an earlier stage of DAP synthesis was used to see any possible role of this gene in predation. RT-PCR transcription, fluorescence tagging and deletion-based phenotyping approaches were used. If DAP synthesis is important for predation, *dapB* and *dapE* with a mCherry tag and *dapE* mutant may show predatory phenotypes (see Figure 6-1).

The product of the *dapB* gene is responsible for conversion of L-2, 3-dihydrodipicolinate to L-tetrahydrodipicolinate in the DAP/Lysine synthesis pathway (McCoy, et al. 2006). The deletion of *dapB* in *S. flexneri* led to bursting of cells when they were grown on medium not provided with DAP, due to formation of defective peptidoglycan during their proliferation (Cersini, et al. 1998).

The *dapE* gene is one of the genes in the DAP/lysine pathway that participates in the conversion of N-succinyl-L, L -6-diaminopimelate to L,L-diaminopimelate (McCoy, et al. 2006, Nocek, et al. 2014). In *H. pylori* and *M. segmatis*, when *dapE* was deleted it was fatal for the cells which indicated that DapE plays an important role in growth of those bacteria (Nocek, et al. 2010). *H. pylori* mutants grew only when media was supplied with DAP but not when supplied with lysine. This gave strong evidence that DapE plays a vital role in the DAP synthesis pathway in those bacteria (Karita, et al. 1997, Nocek, et al. 2010).

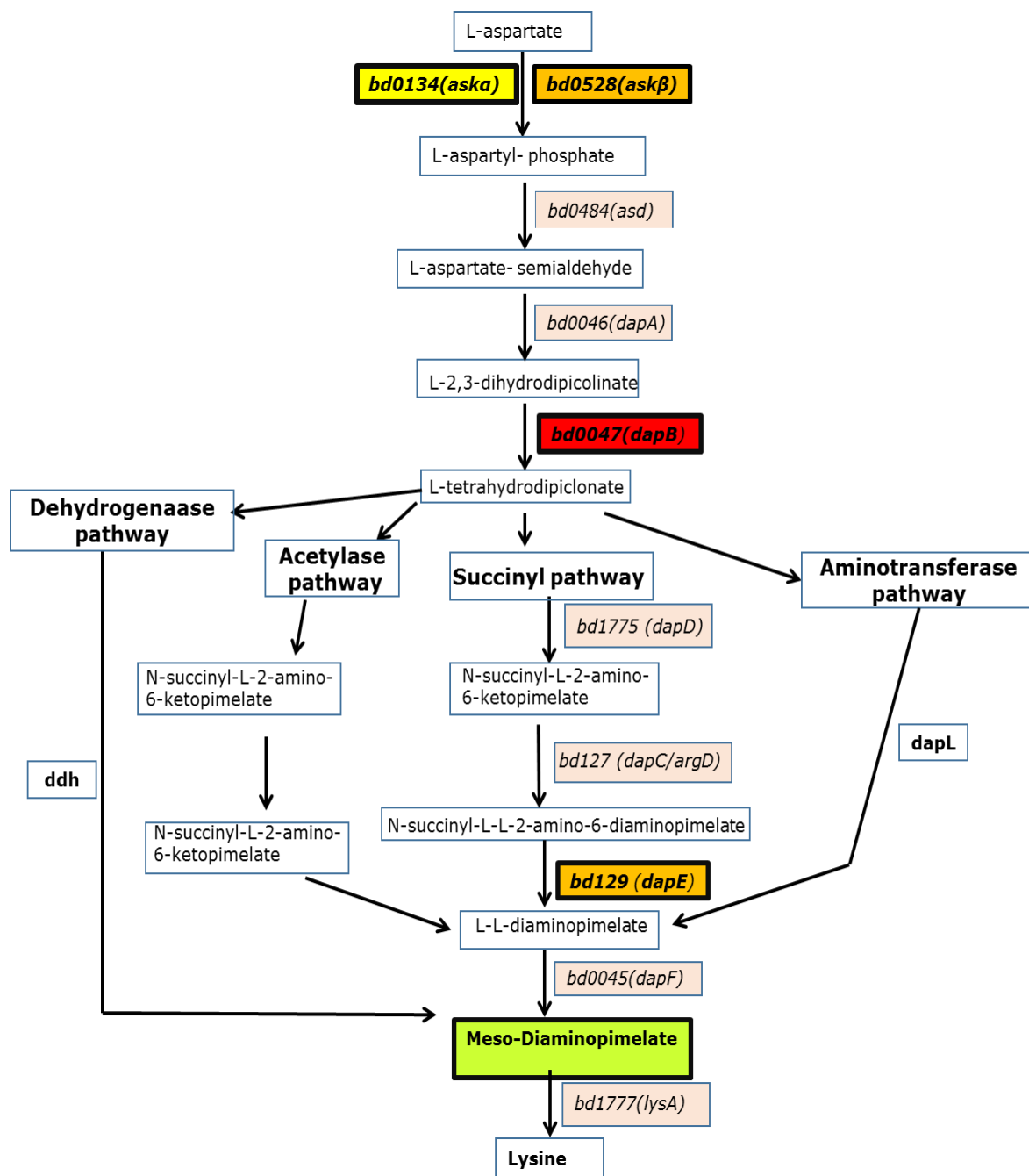


Figure 6. 1: Pathway of DAP/lysine synthesis in plants and bacteria. The pathway adapted from (McCoy et al. 2006) with my addition of *Bdellovibrio* enzymes and deletion of the Met and Thr pathways which are not present in *Bdellovibrio* (Rendulic et al 2004).

Bd0047 (DapB) is a synthesis protein that is annotated as a 4-hydroxy-tetrahydrodipicolinate reductase protein. The two adjacent genes to *bd0047* are in same orientation; they are *bd0046* (*dapA*) and *bd0048*; *bd0046* (*dapA*) encodes 4-hydroxy-tetrahydrodipicolinate synthase, an enzyme that plays a role

in DAP/Lysine synthesis pathway by catalysing the condensation of (S)-aspartate-beta-semialdehyde and pyruvate to 4-hydroxy-tetrahydrodipicolinate (HTPA) while *bd0048*, encodes a hypothetical protein (Figure 6-2).



Figure 6. 2: The genomic context of *B. bacteriovorus* *bd0047* gene (<http://hactar.shef.ac.uk/xbase/>).

Bd0129 (DapE) is an amino acid synthesis protein that is annotated as a Succinyl-diaminopimelate desuccinylase protein (SDDS). The two adjacent genes to *bd0129* are in same orientation; they are *bd0127* and *bd0130*; *bd0127* (*dapC/argD*) encodes Acetylornithine/succinyldiaminopimelate aminotransferase, an enzyme that plays a role in DAP/Lysine synthesis pathway whereas *bd0130* (*astA*), encodes the arginine N-succinyltransferase that plays a role in arginine metabolism pathway Figure (6-3).

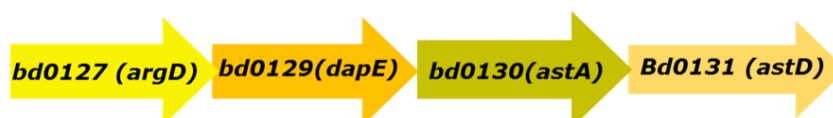


Figure 6. 3: The genomic context of *B. bacteriovorus* *bd0129* gene (<http://hactar.shef.ac.uk/xbase/>).

Bd0047 is predicted to be encoded within an operon with Bd0046 (DapA) encoded upstream and Bd0048 encoded downstream. Also, Bd0129 is predicted to be encoded within an operon with Bd127 (ArgD) encoded upstream and Bd0130 (AstA) encoded downstream (<http://operons.ibt.unam.mx/OperonPredictor/>). These prediction were investigated further in this chapter.

BLAST analysis shows that Bd0047 and Bd0046 are conserved in *B. exovorus* and *B. bacteriovorus Tiberius* as expected from their roles in basic cell wall function but Bd0048 is in *B. bacteriovorus Tiberius* but not in *B. exovorus*. Bd0047 has a conserved DapB-N and DapB-C domains as shown in (Figure 6-4) and it belongs to a NADB-Rossmann and DapB-C superfamilies (Figure 6-4).

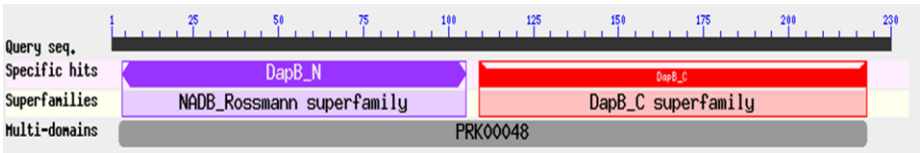


Figure 6. 4: Conserved domains of Bd0047 (DapB) (<https://blast.ncbi.nlm.nih.gov/>).

Additional to that, BLAST analysis also showed that Bd0129 is conserved in *B.bacteriovorus exovorus* and *B.bacteriovorus Tiberius*. The adjacent genes for *dapE*, *dapC/argD* and *astA* are also conserved in *B. exovorus* and *B.bacteriovorus Tiberius*. Bd0129 has a conserved M20-ArgE-DapE like domain as shown in Figure (7-5) and it belongs to a zinc-peptidase like superfamily. Bd0129 have a gap in the specific and superfamily homologies from amino acid 268 to 326 (Figure 6-5) (<https://blast.ncbi.nlm.nih.gov/>).

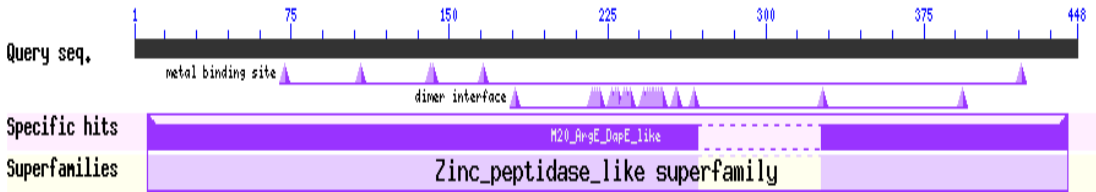


Figure 6. 5: Conserved domains of Bd0129 (DapE) (<https://blast.ncbi.nlm.nih.gov/>).

The CELLO Prediction revealed for Bd0047 and Bd0129 proteins location is cytoplasmic with 3.516 and 4.686 scores respectively (<http://cello.life.nctu.edu.tw/>). The occurrence of *bd0047 (dapB)* and *bd0129 (dapB)* in delta proteobacteria was tested using STRING neighbourhood studies

(Figure 7-6 A and B respectively). The presence of *bd0047* (*dapB*) and *bd0129* (*dapB*) are usual in the *delta proteobacteria* and found in *Bdellovibrio* but what is unusual is presence of *bd0048* in *delta proteobacteria* and found in *Bdellovibrio* only (Figure 6-6 A).

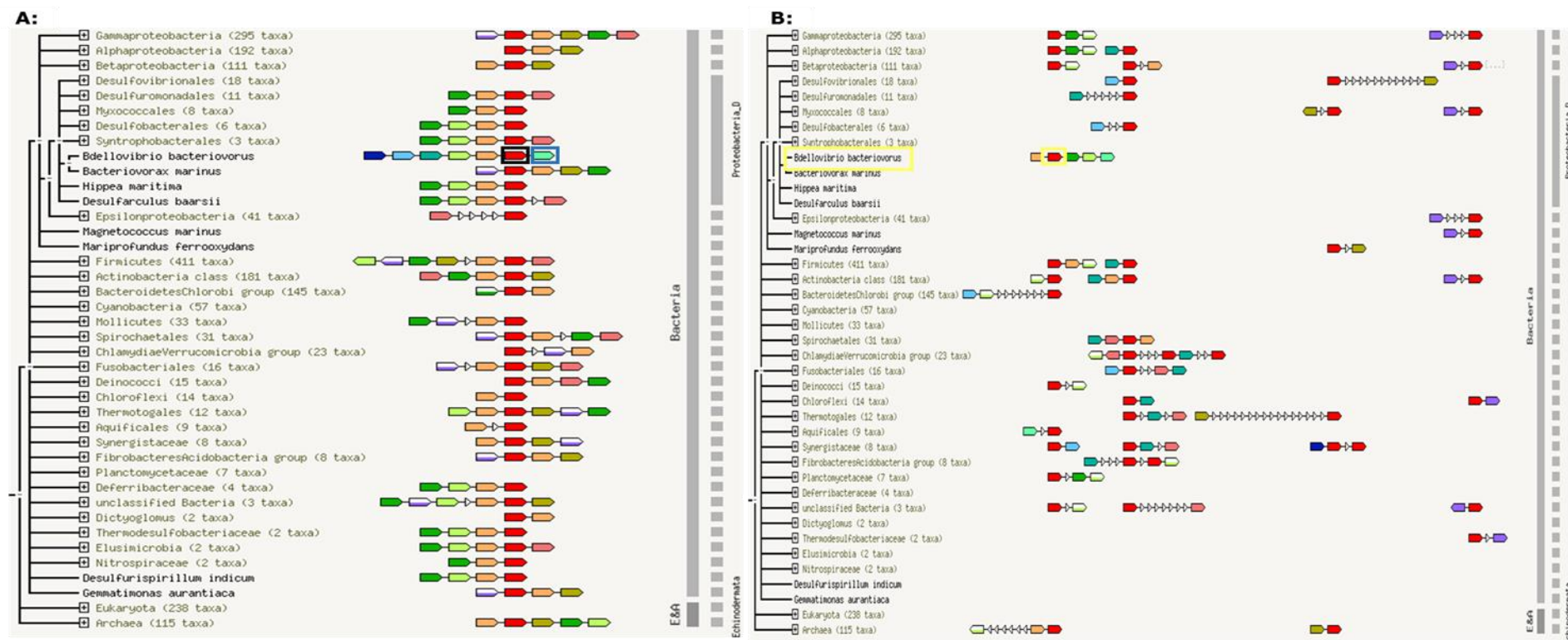


Figure 6. 6: The Occurrence of *bd0047* (*dapB*) and *bd0129* (*dapE*) in all bacteria phyla. A: *dapB* gene is conserved (red arrow with empty black box) in *B. bacteriovorus* and in all bacteria phyla. In all bacteria phyla, the *dapA* gene (orange arrow) is typically adjacent, either upstream or downstream of *dapB*, the *dapF* gene (pale green arrow) is typically adjacent, either upstream or downstream of *dapA*. For *Bdellovibrio* *dapF* and *dapA* is upstream of *dapB* and *bd0048* (Pale purple arrow with empty blue box) downstream of *dapB* which is unique for *Bdellovibrio*. B: *bd0129* (yellow arrow with empty light blue box) is usual in deltaproteobacteria group versus typical housekeeping arrangement of other bacteria. Image generated using STRING (<http://string-db.org/cgi/neighborhood>).

6.2 Specific Research Aims

1. To investigate transcription of *bd0047* (*dapB*) and *bd0129* (*dapE*) of *B. bacteriovorus* throughout the predatory lifecycle (in wild type RNA and RNA from Δ *bd0134*) and in host-independent lifestyle.
2. To investigate if there are any shared operons between *bd0047* and *bd0046* and between *bd0047* and novel *bd0048* gene. Also, investigating if there are any shared operons between *bd0129* and *bd0127* and between *bd0129* and *bd0130* gene.
3. To assess the cellular localisation of DapB and DapE heterologously expressed in *E. coli*.
4. To assess the cellular localisation of Bd0047 and Bd0129 in *B. bacteriovorus* during the predatory lifecycle of *B. bacteriovorus* in case DAP is made by *Bdellovibrio* during prey- peptidoglycan access.
5. To assess the cellular localisation of Bd0047 and Bd0129 in *B. bacteriovorus* during host- independent growth.
6. To isolate an in-frame deletion strain of *bd0129* in *B. bacteriovorus* HD100 and assay the phenotypes of the mutant strain in the presence and absence of added DAP.

6.3 Expected outcome

1. Transcription of *bd0047* increases in later stages of *B. bacteriovorus* predatory cycle, associated with predator cell wall synthesis.
2. The cellular location of Bd0047-DapB-mCherry in *E. coli* will be similar to location of B0031-DapB-mCherry in *E. coli* indicating similar sites of peptidoglycan synthesis in binary fission growth and filament septation growth.

3. C-terminal fluorescent tagging of *bd0047* changes its function and the shape of attack phase cells and increases the length of the predatory lifecycle.
4. Fluorescently tagged Bd0047 might be visible in attack phase, inside the bdelloplast and in host-Independent cells if they participate in *Bdellovibrio* cell wall maintenance.
5. Fluorescently tagged Bd0047 might be visible secreted from invading attack phase, and in prey cell but not in host-Independent cells if they participate in *Bdellovibrio* predation and prey modification.
6. Transcription of *bd0129* increases in later stages of *B. bacteriovorus* predatory cycle, associated with predator cell wall synthesis.
7. The cellular location of Bd0129-DapE-mCherry in *E. coli* will be similar to location of B4024-DapE-mCherry in *E. coli*.
8. C-terminal fluorescent tagging of *bd0129* affects attack phase shapes and elongates the predatory lifecycle.
9. Fluorescently tagged Bd0129 might be visible in attack phase, inside bdelloplast and in host-Independent cells if they participate in *Bdellovibrio* cell wall maintenance.
10. Fluorescently tagged Bd0129 might be visible secreted from invading attack phase, and in prey cell but not in host-Independent cells if they participate in *Bdellovibrio* predation and prey modification.
11. Deletion of *bd0129* in *B. bacteriovorus* is possible or it may need the presence of an external source of DAP like in other bacteria?

6.4 Results:

6.4.1 Transcription of *bd0047* in host- dependent (HD) and host-independent (HI) lifestyle

In order to look at the gene expression of the *Bd0047* genes during the predatory life cycles of *B. bacteriovorus* HD100, RT-PCR was carried out using RNA samples isolated at indicated time points of the predatory life cycle of *B. bacteriovorus* and $\Delta bd0134$ strains during one round of synchronous *Bdellovibrio* infection of *E. coli* host cells (Figure 6-7). For both WT and $\Delta bd0134$ RNA, the expression of *bd0047* clearly showed peaks at 2 - 4 hours post-infection when the *Bdellovibrio* begin to become elongated and later division stage of the *Bdellovibrio* predatory lifecycle. This expression pattern suggests that this gene involved in growth phase of *Bdellovibrio* during predatory lifecycle as expected for cell wall components (Figure 6-7).

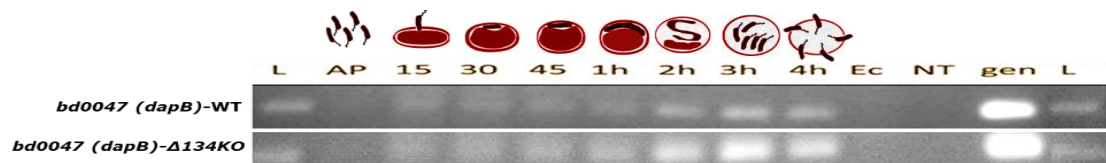


Figure 6. 7: Reverse-transcriptase PCR showing transcriptional expression of *bd0047* throughout the predatory cycle. RNA was isolated at the indicated time points during one round of synchronous *Bdellovibrio* HD100 and $\Delta bd0134$ infection of *E. coli* host cells, and primers specific to the gene were used to amplify (RT-PCR, 28 cycles) a 102 bp internal fragment from each transcript if present. AP = Attack phase (free-swimming *Bdellovibrio*); 15, 30, 45 = minutes since start of infection (attachment to and rounding of *E. coli* host cell into a bdelloplast structure); 1h, 2h, 3h, 4h = hours since start of infection (host cell resources being degraded and used for *Bdellovibrio* growth into a filament, followed by septation into multiple progeny and eventually lysis from host cell); Ec = *E. coli* S17-1 RNA (no *Bdellovibrio* control); gen = *B. bacteriovorus* HD100 genomic DNA (positive control); NT = No template control; L = NEB 100 bp DNA ladder.

Because the microarray data (using matched concentrations of total RNA) (Lambert et al. 2010) stated that this gene was expressed during HI growth and compared to attack phase was upregulated 12.64 fold for *bd0047*, I therefore

performed RT-PCR on total RNA matched at 10 ng/μL isolated from the attack phase and different wild-type HI strains (HID2, HID13, HID22 and HID26) to confirm this result. Figure (6-8) showed that *bd0047* expression is higher in all growing HI strains than the non-growing attack phase and this agreed with Microarray data.

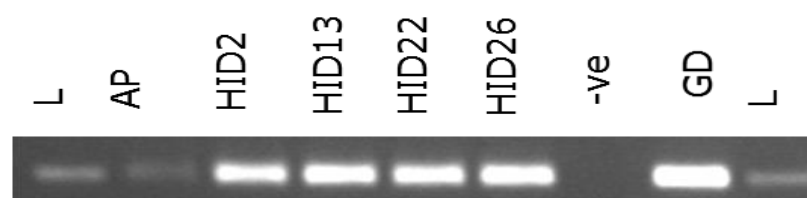


Figure 6. 8: Reverse-transcriptase PCR showing relative transcription expression of the *B. bacteriovorus* HD100 *bd0047* during attack phase and host-independent growth. RNA was isolated from *B. bacteriovorus* HD100 attack phase (AP) cells and four host-independent strains (HID2, HID13, HID22, HID26) at OD₆₀₀ 0.6 and the RNA concentration was matched to 10 ng μl⁻¹. Primers specific to gene were used to amplify (RT-PCR, 30 cycles) a 102 bp internal fragment from each transcript if present. Gen = *B. bacteriovorus* HD100 genomic DNA (positive control); NT = no template control; L = NEB 100 bp DNA ladder.

6.4.2 The *bd0046* (*dapA*) gene is in an operon with *bd0047* (*dapB*)

RT-PCR was performed in order to identify if whether *bd0046* and *bd0047* are co-transcribed in an operon using matched amounts of RNA (10 ng/μL) isolated from *B. bacteriovorus* HD100 strain at one point during the predatory lifecycle (3 hrs) this was chosen as the time point when self-wall elongation of predator was maximal around septation. A 1275 bp product (the faint band due to the big size of the cotranscribed product in RT-PCR system optimise for small product) indicated that *bd0046* and *bd0047* are all in an operon which might suggest associated function as might be expected as *dapA* has a role in DAP synthesis (Figure 6-9).

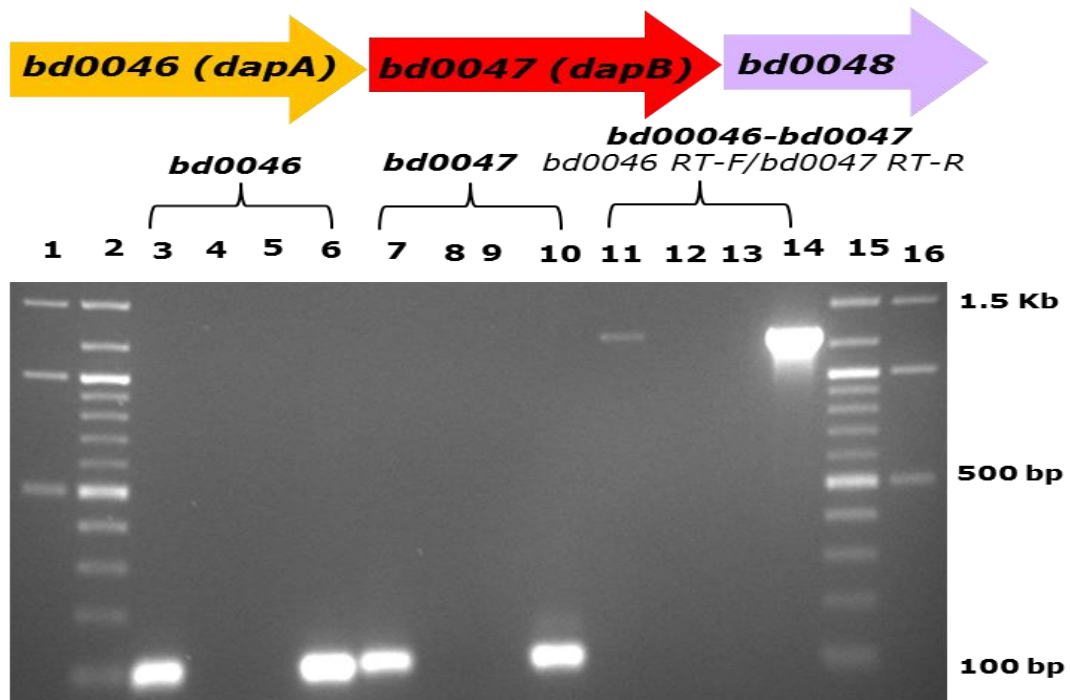


Figure 6. 9: RT-PCR shows that the *B. bacteriovorus* genes *bd0046* (*dapA*) and *bd0047* (*dapB*) are co-transcribed. Lanes 1 and 16: NEB 1000 bp ladder; Lanes 2 and 15: NEB 100 bp ladder; Lanes 3, 7 and 11: predator and prey total RNA at 3 hr; Lanes 4, 8 and 12: *E. coli* S17-1 RNA; Lanes 5, 9 and 13: No template; Lanes 6, 10 and 14: *B. bacteriovorus* HD100 genomic DNA. 1275 bp band found in lane 11 indicates that there is co-transcription between *bd0046* and *bd0047* genes.

6.4.3 The *bd0047* (*dapB*) and novel *bd0048* genes are co-transcribed

In order to confirm that the *bd0047* and *bd0048* genes are co-transcribed in an operon as predicted by (<http://operons.ibt.unam.mx/OperonPredictor/>), RT-PCR were performed in order to assess if whether *bd0047* and *bd0048* are co-transcribed in an operon using matched amount of RNA (10 ng/μL) isolated from *B. bacteriovorus* HD100 strain at one point during the predatory lifecycle (3 hrs) this was also chosen as the time point when self-wall elongation of predator was maximal around septation and so potentially maximum use of DAP. The result showed that there is 509 bp band product that could be produced only if there was a continuous transcript indicating that the *bd0047* and *bd0048* genes were co-transcribed (Figure 6-10).

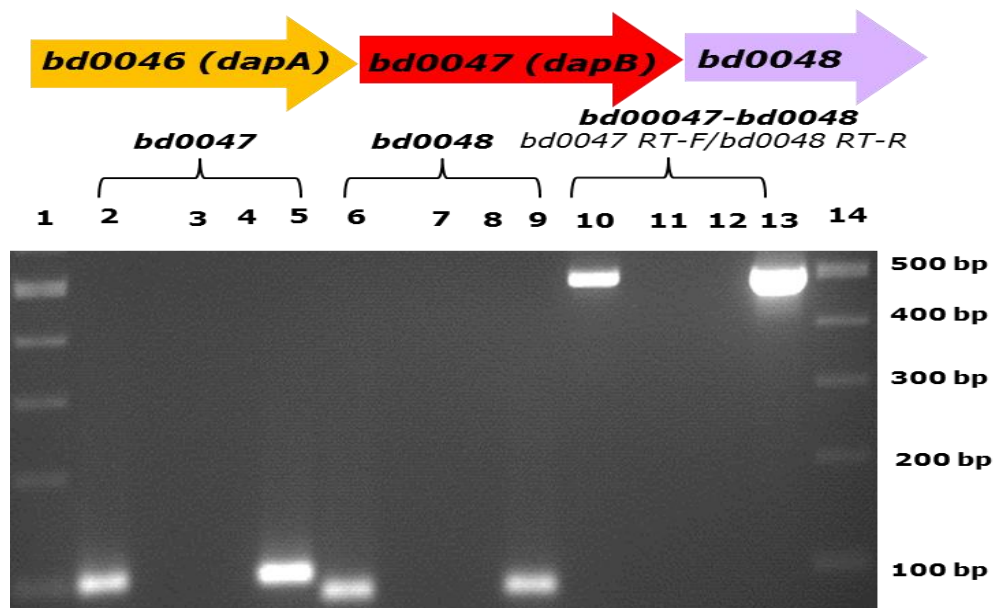


Figure 6. 10: RT-PCR shows that the *B. bacteriovorus* genes *bd0047* (*dapB*) and *bd0048* are co-transcribed. Lanes 1 and 16: NEB 1000 bp ladder; Lanes 2 and 15: NEB 100 bp ladder; Lanes 3, 7 and 11: predator and prey total RNA at 3 hr; Lanes 4, 8 and 12: *E. coli* S17-1 RNA; Lanes 5, 9 and 13: No template; Lanes 6, 10 and 14: *B. bacteriovorus* HD100 genomic DNA. 509 bp band found in lane 10 indicates that there are co-transcription between *bd0047* and *bd0048* genes.

The occurrences of Bd0047 in deltaproteobacteria as well as this co-transcription revealed very interesting results that *bd0048* extra gene found only in *Bdellovibrio* (Figure 6-6 A Pale purple arrow with empty blue box) that could be act as novel DAP synthesis regulator or signaller of DAP gene transcription and BLAST analysis shows that Bd0048 is conserved in invasive *B. bacteriovorus* Tiberius but not in epidiotic *B. exovorus* and it is belongs to a hydroxymethylpterin-pyrophosphokinase (HPPK) superfamily (Figure 6-11) (<https://blast.ncbi.nlm.nih.gov/>).



Figure 6. 11: Conserved domains of Bd0048 (<https://blast.ncbi.nlm.nih.gov/>).

6.4.4 Localisation studies for *E. coli* DapB

As a control for the study of DapB in *B. bacteriovorus*, *E. coli* *dapB* gene (*b0031*) was fused with mCherry gene and expressed it in *E. coli* DH5 α as *E. coli* also elongates its cell wall by binary fission not filamentously. The cellular location of *dapB* (*bd0031*) in *E. coli* was assessed by preparing a B0031-mcherry construct by fusion of a 719 bp ORF fragment of *dapB* without stop codon in frame with mCherry and expressing it in *E. coli* DH5 α . The fluorescence was visible in *E. coli* DH5 α DapB-mCherry (Bd0031) strain and it localised near the poles (avoiding the middle of the cell) (Figure 6-12). The DAPI stain revealed that the Bd0031-mCherry avoided the location of the DNA with inhibited region (nucleoid occlusion) where the peptidoglycan is synthesised for new wall.

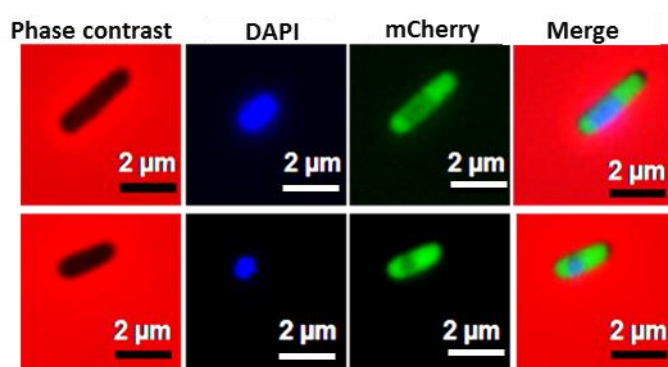


Figure 6. 12: The fluorescence of *E. coli* DH5 α expressing pAKF56 Bd0031-mCherry (DapB) of *E. coli* in stationary phase that grown in YT broth for 16 hours with appropriate antibiotic. The fluorescent is visible avoid the middle of the cell and located near the poles. The cells were stained with DAPI and imaged taken with 0.7 seconds fluorescent exposure for mCherry imaging and 0.03 seconds for DAPI images.

6.4.5 Native localisation of Bd0047-mCherry (DapB) assessed by fluorescent microscopy in *B. bacteriovorus*

To assess the cellular location of Bd0047- mCherry (DapB) during the predatory lifecycle, a C-terminal fluorescently tagged *B. bacteriovorus* *bd0047* (*dapB*) strain was generated (Figure 6-13).

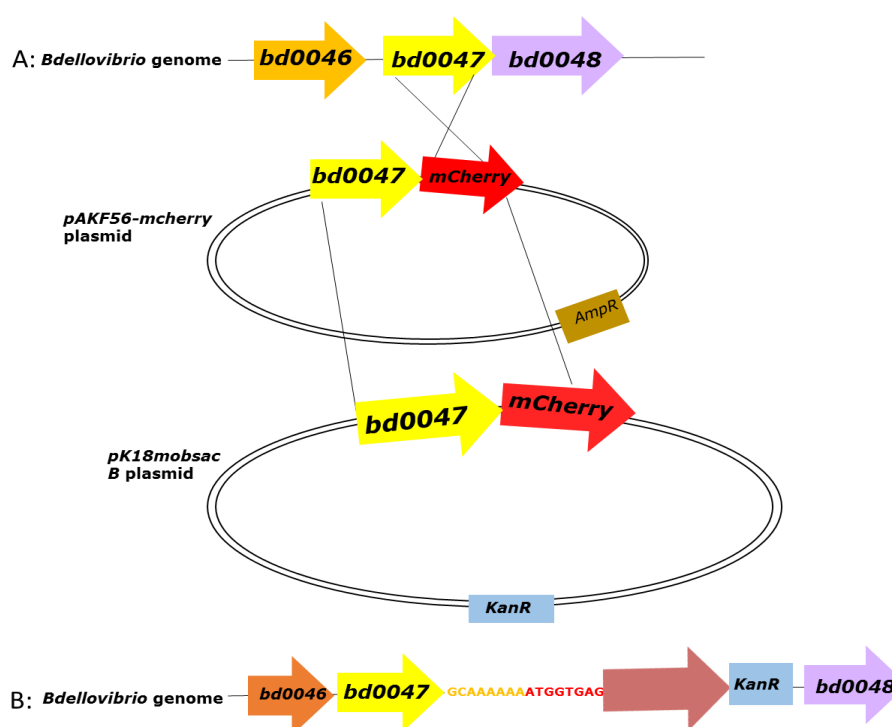


Figure 6. 13: Strategy of *bd0047*- *mCherry* tagging in *B. bacteriovorus*. The *bd0047* ORF was cloned in frame with *mCherry* in the pAKF56 and after that transformed in pK18mobsacB vector (A) and conjugated in *B. bacteriovorus*. *bd0047*- *mCherry* is a copy of the *bd0047* gene with the stop codon removed (B).

Bd0047-mCherry was constructed by fusion of a 687-bp ORF fragment of *bd0047* without the stop codon, cloned in frame with *mCherry* in the plasmid pAKF56 using *E. coli* DH5α as a host. pAKF56::Bd0047- mCherry plasmid tagged protein was florescent (Figure 6-14) and localized in a central cytoplasmic pattern with an obvious dark edge.

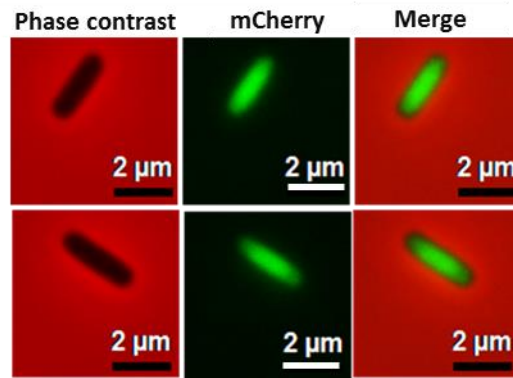


Figure 6. 14: Heterologous expressing of pAKF56 Bd0047-mCherry (DapB) in *E. coli* DH5α in stationary phase that grown in YT broth for 16 hours with appropriate antibiotic. The fluorescent is visible spread cytoplasmically with dark edge. The image taken with 0.2 second fluorescent exposure with maximum gain.

The fluorescent microscopy of attack phase *B. bacteriovorus* cells showed that the fluorescent pattern of the resultant strain of *B. bacteriovorus* Bd0047-mCherry attack phase mostly was central (did not reach the poles of the cell) (Similar to that found in DH5α pAKF56::Bd00047-mCherry (Figure 6-14). The shape of attack phase also slightly altered to “S shaped cells” this might be caused by the fluorescent tag that affected on *Bdellovibrio* own self wall (Figure 6-15).

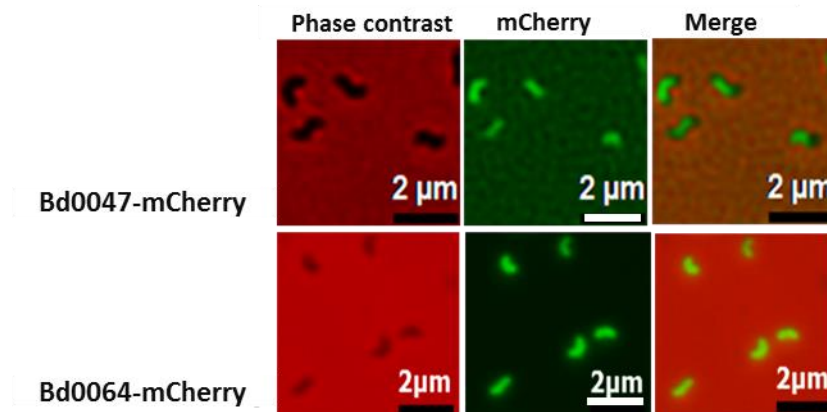


Figure 6. 15: The attack phase of *B. bacteriovorus* bd0047-mCherry expressed fluorescence in central location with dark edge for the most attack phase cells. The shape of attack phase affected by mCherry tag in comparison with normal attack phase of *B. bacteriovorus* bd0064-mCherry that not affected by mCherry tag. The image taken with 1.8 Second exposure for mCherry with maximum gain.

The location of Bd0047– mCherry in attack phase of *Bdellovibrio* (non- replicating phase) was assessed and it was displayed various cellular cytoplasmic locations with the vast majority of the fluorescence in the attack phase had central location (~54%) with others having fluorescence bipolar (~26%), diffuse through the cell (~13.2%), monopolar (~5.5%) and with some *Bdellovibrio* not fluorescent (~1.3%) (Figure 6-16 A and B).

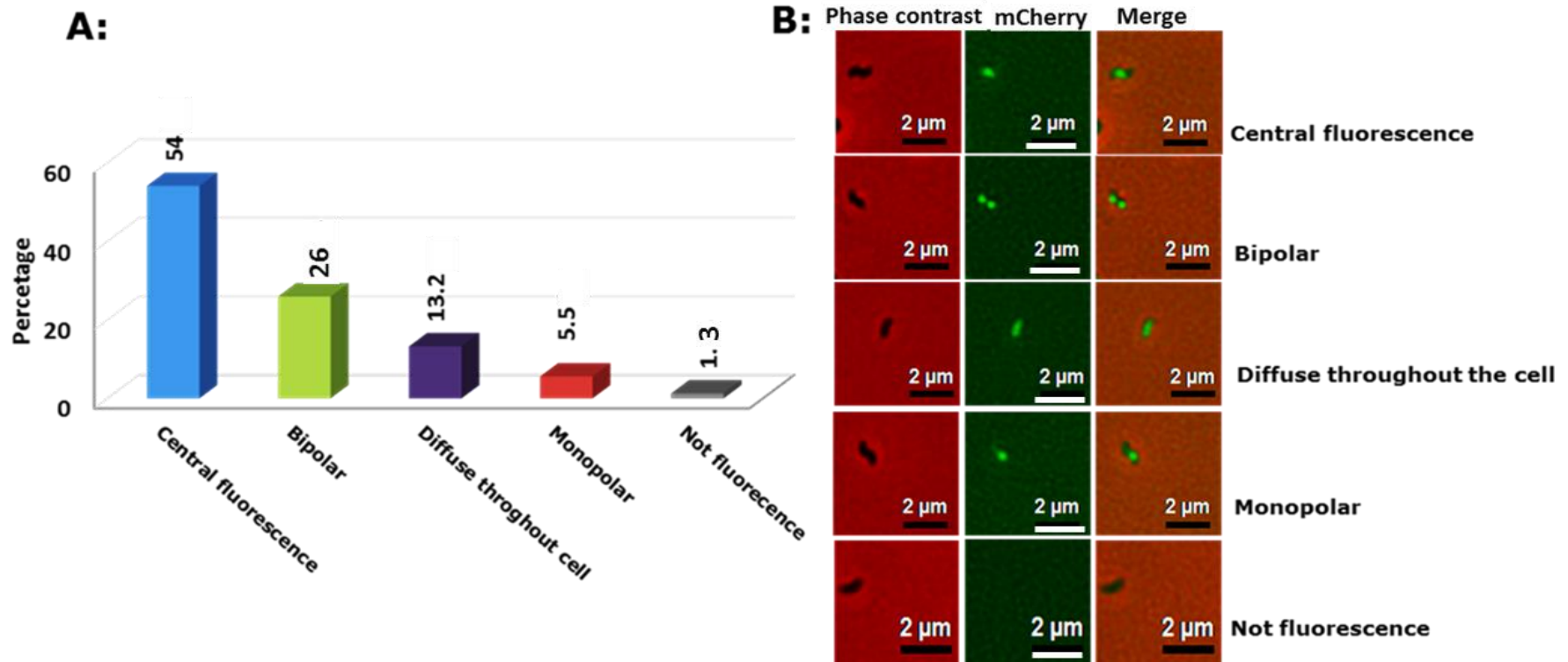


Figure 6. 16: Examples of localisation of Bd0047- mCherry in the attack phase of *B. bacteriovorus* cells. (A) Overall distribution of Bd0047- mCherry fluorescence in *B. bacteriovorus* attack phase cells population (no of cells=718) from three biological repeats (B) Image of *B. bacteriovorus* HD100 bd0047- mCherry cells (scale bar = 2 μ m). The image taken with 1.8 Second exposure for mCherry with maximum gain.

Also, during the attachment of *Bdellovibrio* to the prey *E. coli* (at 15 min) the fluorescence was shown to have a similar pattern mostly central (~50%) and the rest bipolar (~26%), diffuse throughout the cell (~19%) and monopolar *Bdellovibrio* (~5%) (Figure 6-17).

In contrast, after *Bdellovibrio* invade the prey and during intracellular growth (30 mins, 45 mins, 1hr, 2hrs and 3hrs), Bd0047-mCherry diffused through the filamentous growing cell inside the bdelloplast and then return to qualitatively similar pattern that attack phase had when *Bdellovibrio* escape prey. (Figure 6-18).

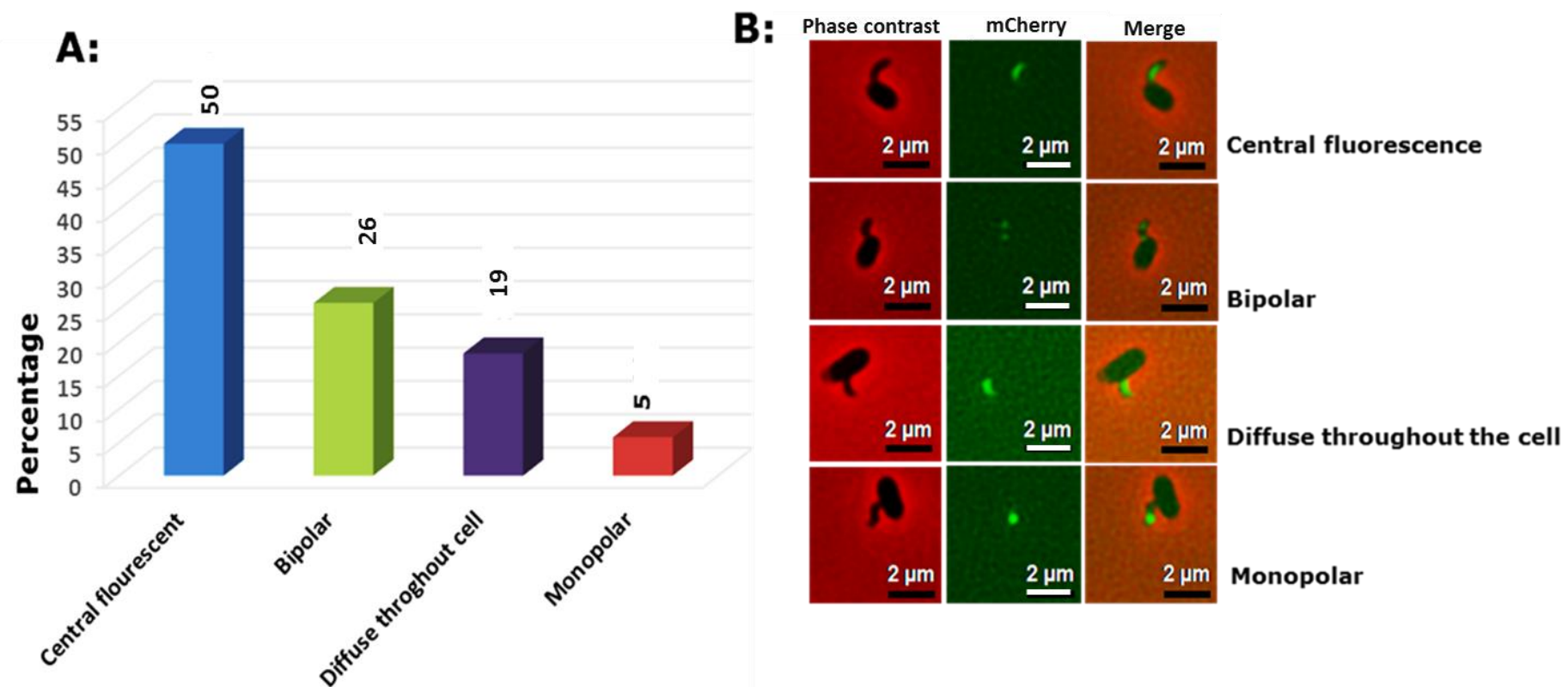


Figure 6. 17: Examples of Location of Bd0047-mCherry during the attachment to the prey. (A) Overall distribution of Bd0047- mCherry fluorescence in *B. bacteriovorus* attack phase cells population (The cells no =740) from three biological repeats. (B) Image of *B. bacteriovorus* HD100 *bd0047- mCherry* cells during attachment (scale bar = 2 μ m). The image taken with 1.8 Second exposure for mCherry with maximum gain.

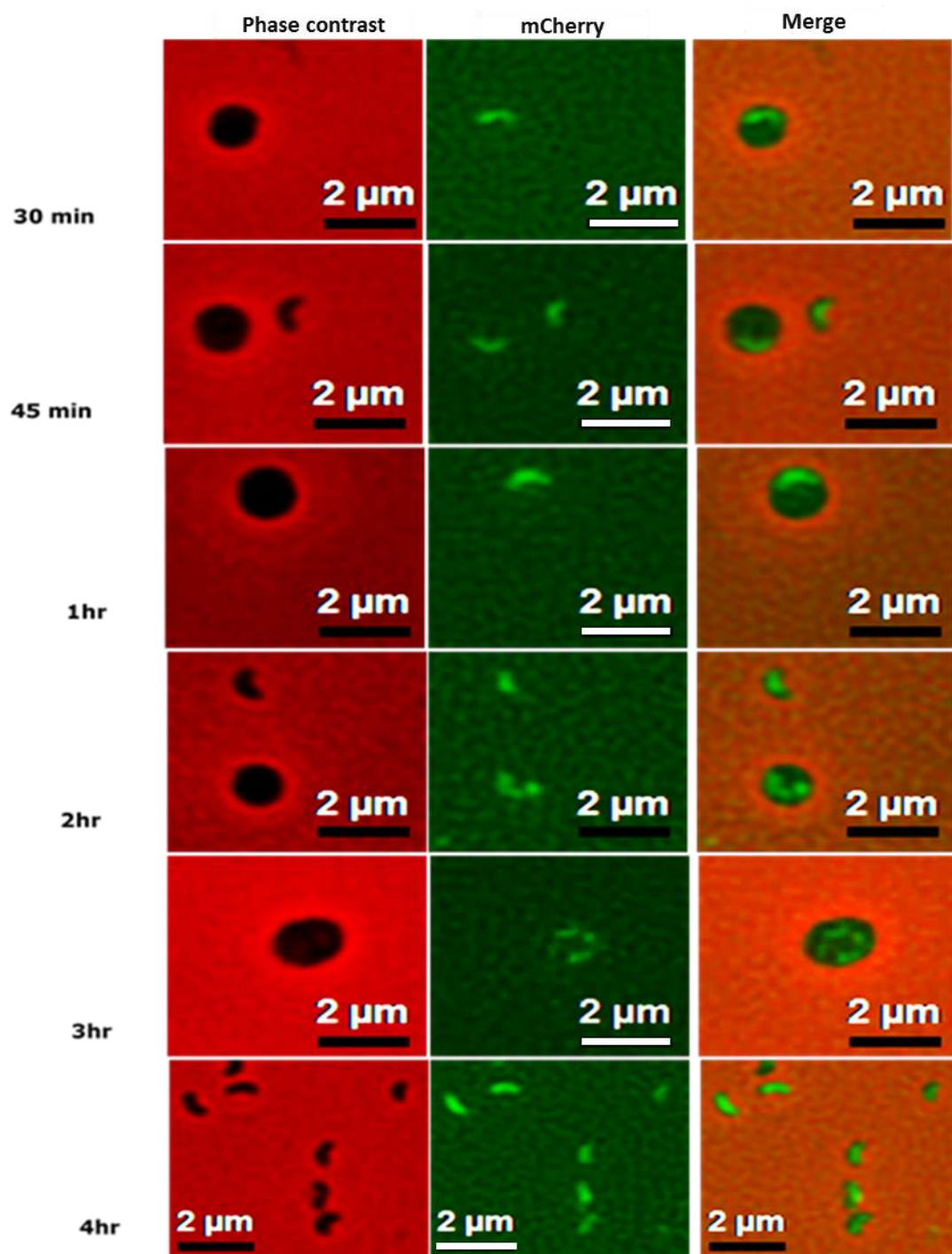


Figure 6. 18: The fluorescence pattern of *B. bacteriovorus* HD100 *bd0047-mCherry* during the whole predatory lifecycle on prey *E. coli* pZMR100. The image taken with 1.8 Second exposure for mCherry with maximum gain.

6.4.6 *Bd0047-mCherry* expressed in host-independent cells and had central location in growing HI cells

To examine if the *Bd0047-mCherry* is expressed in host-independent growth, the HI *B. bacteriovorus bd0047-mCherry* strains were generated from host dependent attack phase cells by growing on PY media. The fluorescently tagged *Bd0047* protein was visible in various cell shapes (figure 6- 19) and this fitted with host-independent RT-PCR results of this gene (figure 6-8). Interestingly this included spherical cell shapes; these had previously been little studied.

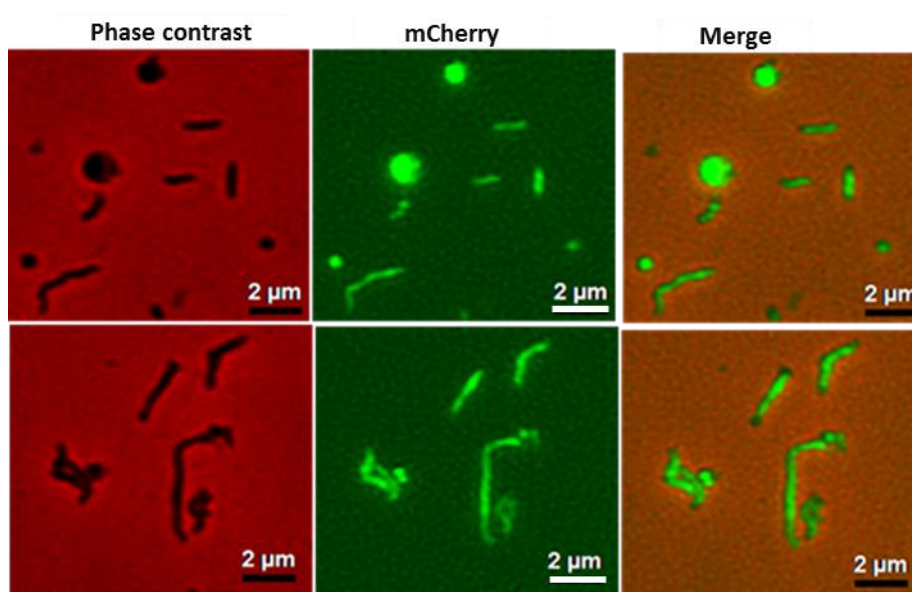


Figure 6. 19: HI *B. bacteriovorus bd0047-mCherry* strain. *Bd0047-mCherry* expressed in Host- Independent growth phase. The image was taken with 0.2 second fluorescent exposure with maximum gain.

The fluorescent *Bd0047-mCherry* explicit in all cells shapes and it had a central localization with dark edge in growing HI cells as can be seen in Figure (6-14). This location is expected from non-predatory bacteria that this gene product could has an essential role for cell wall synthesis of *Bdellovibrio*. The DAPI stain presented that the location of mCherry is the same location of the DNA proved that DapB is active and making the cross link raw materials for cell wall especially

in HI growing cells especially in self-bdelloplast cells (Rounded cell) that means that they are not dead/dying structures (6-20).

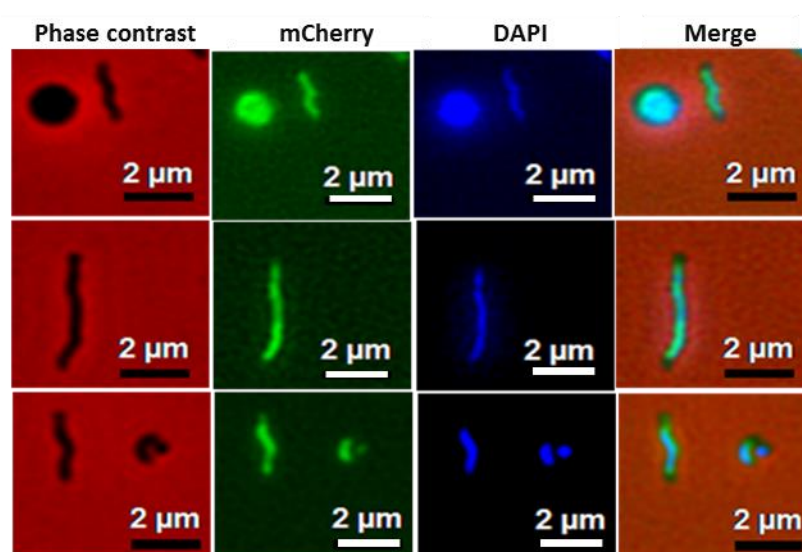


Figure 6. 20: HI *B. bacteriovorus* Bd0047-mCherry cells were stained with DAPI strain. HI cells were stained with DAPI and imaged taken with 0.9 seconds fluorescent exposure for mCherry imaging and 0.03 seconds for DAPI images with maximum gain.

6.4.7 Bioinformatic analysis of succinyl-diaminopimelate descuccinylase (DapE) *B. bacteriovorus* HD100 homologue

There is very little published information about the *B. bacteriovorus* DAP synthesis gene-product Bd0129 (DapE). It is predicted to participate in cell wall synthesis. Therefore, bioinformatic analysis was used to identify this gene product.

Previous studies have shown the essential nature of *dapE* in Gram-negative bacteria and Mycobacteria. To assess the important residues that are conserved, the protein sequence of DapE (Bd0129) encoded from the Gram-negative *B. bacteriovorus* HD100 genome was aligned with the DapE sequences from the Gram-negative *Haemophilus influenzae* (HI0102). Figure (6-23) has been annotated using protein structural information obtained from the *H. influenzae* crystal structure (Nocek et al. 2010).

It is clear from this alignment that both DapE of *H. influenzae* and *B. bacteriovorus* share sequence similarity (22.3% sequence identity and 35.7 similarity, NEEDLE global alignment). *H. influenzae* DapE exists as a dimer and each monomer consists of a catalytic domain and a dimerization domain (Gillner et al. 2009). The catalytic domain is located within the residues 1-179 and 293-376 residues (that contains the active site residues) and the dimerization domain composed of 114 residues (180-292) (provides direct contact between the two monomers) (Figure 6-21) (Dutta and Mishra 2016).

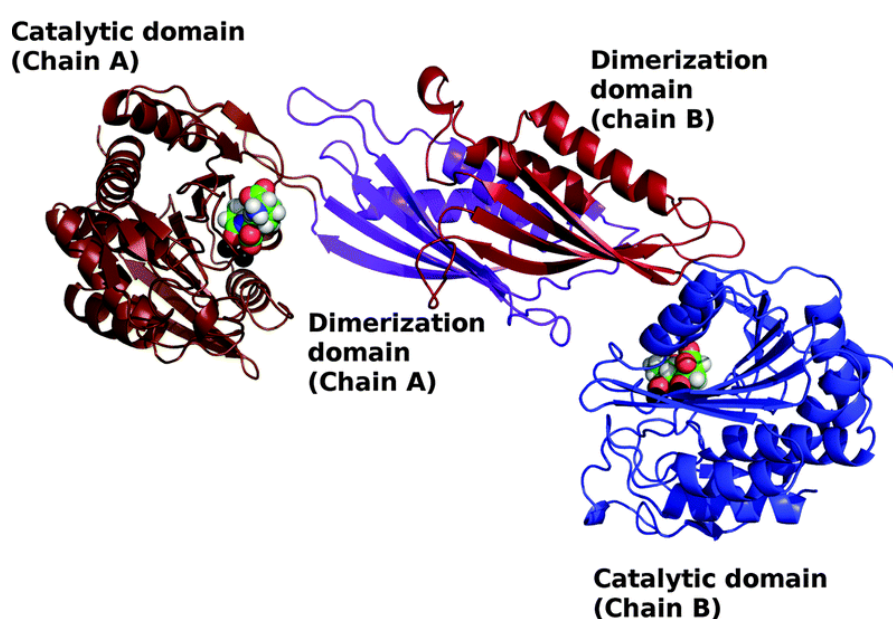


Figure 6. 21: The dimeric structure of *Haemophilus influenzae* DapE protein that consists of both catalytic and dimerization domains in each chain of the protein. The image reproduced from (Dutta and Mishra 2016).

The active site (Zn-Zn interaction) in the catalytic domain consists of the following residues in *H. influenzae*: H67, D100, E135, E163 and H349 (Gillner et al. 2013). DapE of *B. bacteriovorus* shares the same conserved amino acids of the *H. influenzae* active site (Zn-Zn interaction) residues which are in *Bdellovibrio* H72, D108, E141, E166 and H421 (Figure 6-22 and 6-23).

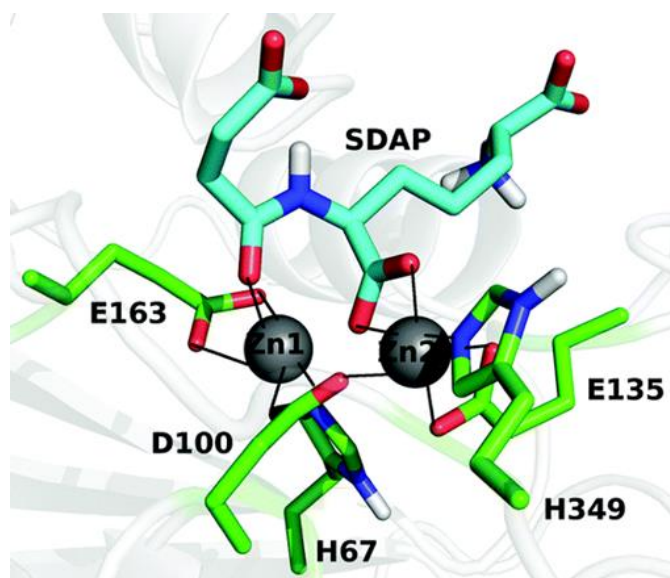


Figure 6. 22: The cartoon picture of the active sites found in catalytic domain of the *Haemophilus influenzae* DapE enzyme, taken from (Dutta and Mishra 2016).

Two of the interesting *H. influenzae* residues in the alignment (Figure 6-23) are H67 and H349. When H67 was mutated to H67A, this reduced catalytic efficiency of this enzyme by 180 fold, while when H349 was mutated to H349A, this abolished catalytic activity of DapE compared to wild type DapE (Gillner et al. 2009, Gillner et al. 2013).

Nocek et al. (2014) found that replacement of T325 with a cysteine results in depleted DapE activity in *H. influenzae* and deletion of the dimerization domain of DapE results in an inactive enzyme under standard conditions (in vitro enzymatic assay). Nocek et al. (2014) pointed that H179, H194, R258 and T325 play an essential role in substrate recognition and binding.

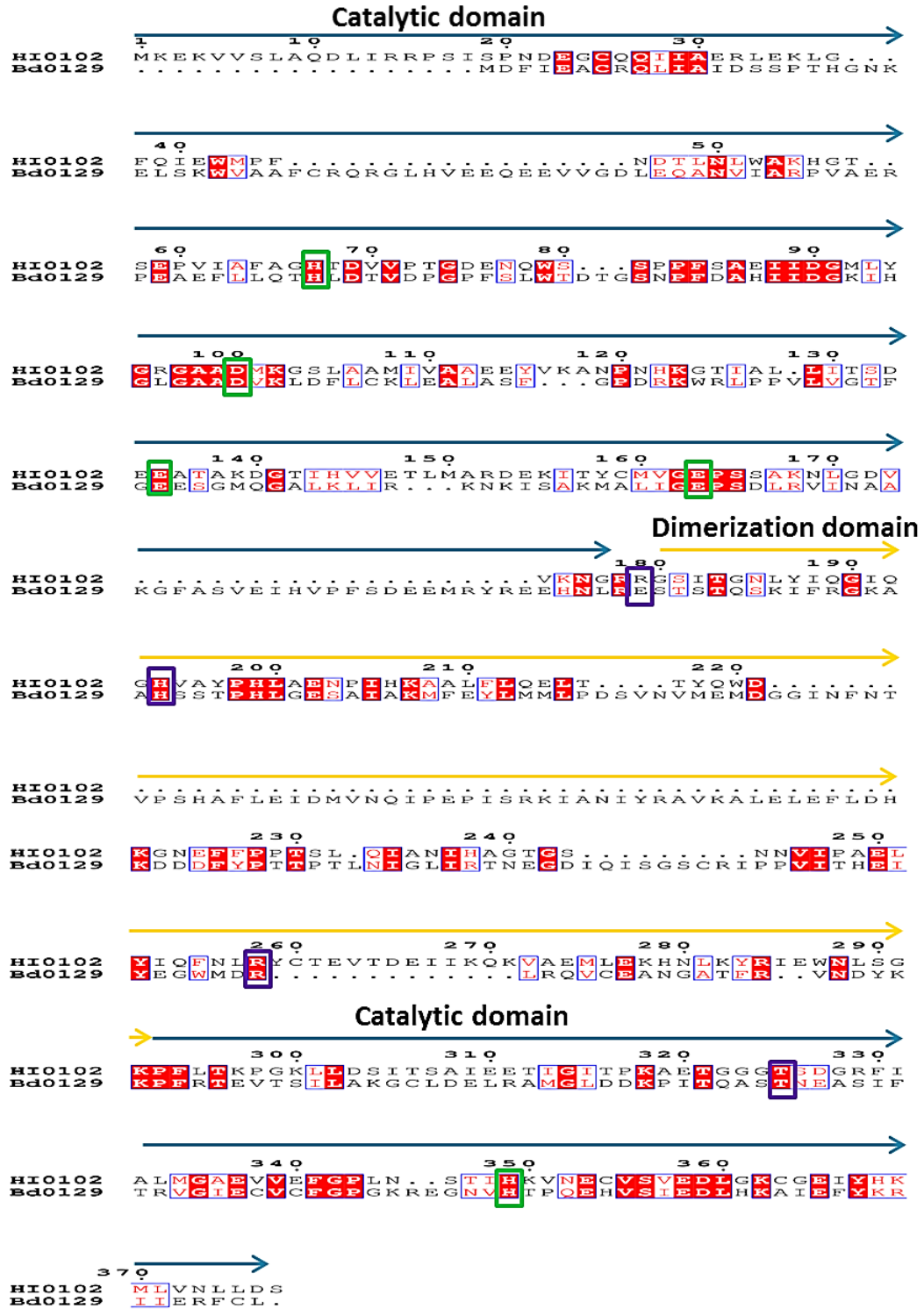


Figure 6. 23: ClustalW alignment between DapE (Bd0129) in *B. bacteriovorus* and known DapE (HI0102) protein in *H. influenzae*. The active site residues are surrounded by the green empty boxes and the substrate recognition and binding residues are the purple empty boxes.

6.4.8 Transcription of *bdo129* in host- dependent (HD) and host-independent (HI) lifestyles

The expression of *bd0129* *dapE* during the predatory life cycles of *Bdellovibrio* was examined using RNA samples isolated at indicated time points of the predatory life cycle of *B. bacteriovorus* and $\Delta bd0134$ strains during one round of synchronous *Bdellovibrio* infection of *E. coli* host cells (Figure 6-24). The expression of *b0129* remained at same level throughout the predatory lifecycle in both WT and $\Delta bd0134$ KO RNA. This expression pattern suggests that this gene product may have an important role in each stage of HD lifestyle and this indicated that this gene has a housekeeping role in *B. bacteriovorus* lifecycle (Figure 6-24) which is not altered by deletion of *bd0134* (see overall discussion).

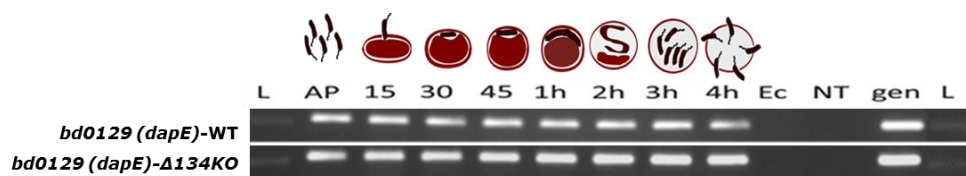


Figure 6. 24: Reverse-transcriptase PCR showing relative transcriptional expression of *bd0129* throughout the predatory cycle. RNA was isolated at the indicated time points during one round of synchronous *Bdellovibrio* HD100 and *Δbd0134* infection of *E. coli* host cells, and primers specific to the gene were used to amplify (RT-PCR, 28 cycles) a 114 bp internal fragment from each transcript if present. AP = Attack phase (free-swimming *Bdellovibrio*); 15, 30, 45 = minutes since start of infection (attachment to and rounding of *E. coli* host cell into a bdelloplast structure); 1h, 2h, 3h, 4h = hours since start of infection (host cell resources being degraded and used for *Bdellovibrio* growth into a filament, followed by septation into multiple progeny and eventually lysis from host cell); Ec = *E. coli* S17-1 RNA (no *Bdellovibrio* control); gen = *B. bacteriovorus* HD100 genomic DNA (positive control); NT = No template control; L = NEB 100 bp DNA ladder.

Microarray data (using matched concentration of total RNA) (Lambert et al. 2010) found that *bd0129* expressed during HI growth was 2.25 fold higher than in attack phase. Therefore, RT-PCR was performed on total RNA matched 10 ng/μL isolated from the attack phase and different wild type HI strains (HID2, HID13, HID22 and

HID26). Figure (6-25) showed that *bd0129* expression is higher in all HI strains than the attack phase and this agreed qualitatively with microarray data.

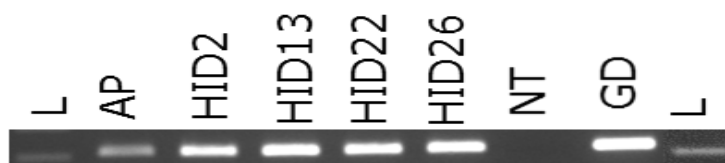


Figure 6. 25: Reverse-transcriptase PCR showing relative transcription expression of the *B. bacteriovorus* HD100 *bd0129* during attack phase and host-independent growth. RNA was isolated from *B. bacteriovorus* HD100 attack phase (AP) cells and four host-independent strains (HID2, HID13, HID22, HID26) at OD600 0.6 and the RNA concentration was matched to 10 ng μl^{-1} . Primers specific to gene were used to amplify (RT-PCR, 30 cycles). a 114 bp internal fragment from each transcript if present. Gen = *B. bacteriovorus* HD100 genomic DNA (positive control); NT = no template control; L = NEB 100 bp DNA ladder.

6.4.9 The *bd0127* gene is in an operon with *bd0129* (*dapE*)

RT-PCR was performed in order to identify if whether *bd0127* and *bd0129* are co-transcribed in an operon using matched amount of RNA (10 ng/ μL) isolated from *B. bacteriovorus* HD100 strain at one point during the predatory lifecycle (3hr was chosen as the time when self wall elongation of the predator was maximal around the septation). A 1200 bp product is indicated that *bd0127* and *bd0129* are all in an operon which might suggest associated functions and that arginine amino acid synthesis not just DAP synthesis is relevant in *Bdellovibrio* (Figure 6-26).

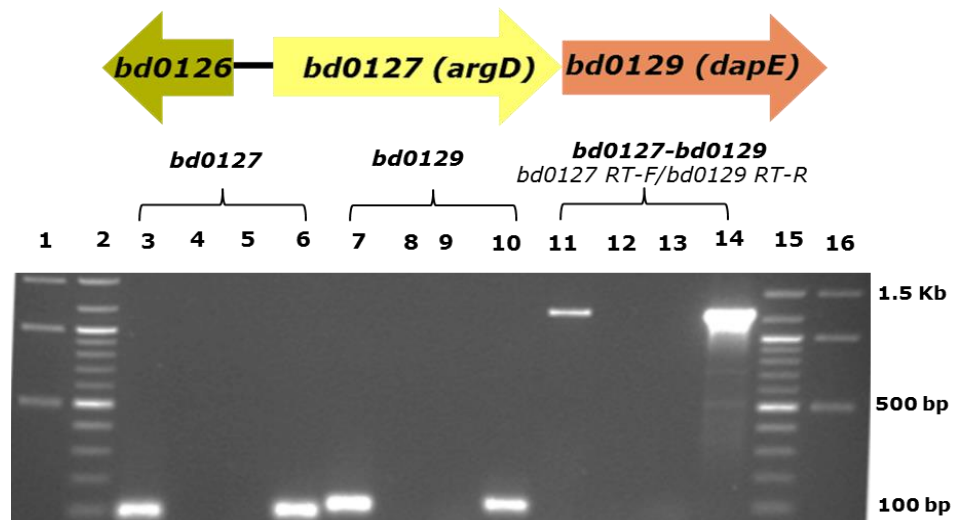


Figure 6. 26: RT-PCR shows that the *B. bacteriovorus* genes *bd0127* (*argD*) and *bd0129* (*dapE*) are co-transcribed. Lanes 1 and 16: NEB 1000 bp ladder; Lanes 2 and 15: NEB 100 bp ladder; Lanes 3, 7 and 11: predator and prey total RNA at 3 hr; Lanes 4, 8 and 12: *E. coli* S17-1 RNA; Lanes 5, 9 and 13: No template; Lanes 6, 10 and 14: *B. bacteriovorus* HD100 genomic DNA. A 1200 bp band found in lane 11 indicates that there was co-transcription between *bd0127* and *bd0129* genes.

6.4.10 The *bd0129* (*dapE*) and *bd0130* (*astA*) genes are not co-transcribed

RT-PCR were performed in order to assess if whether *bd0129* and *bd0130* are co-transcribed in an operon using matched amount of RNA (10 ng/μL) isolated from *B. bacteriovorus* HD100 strain during predatory lifecycle (3hr). The result showed that there is no observable concerted operon between *bd0129* and *bd0130* genes (Figure 6-27), meaning that there is no shared transcription between them.

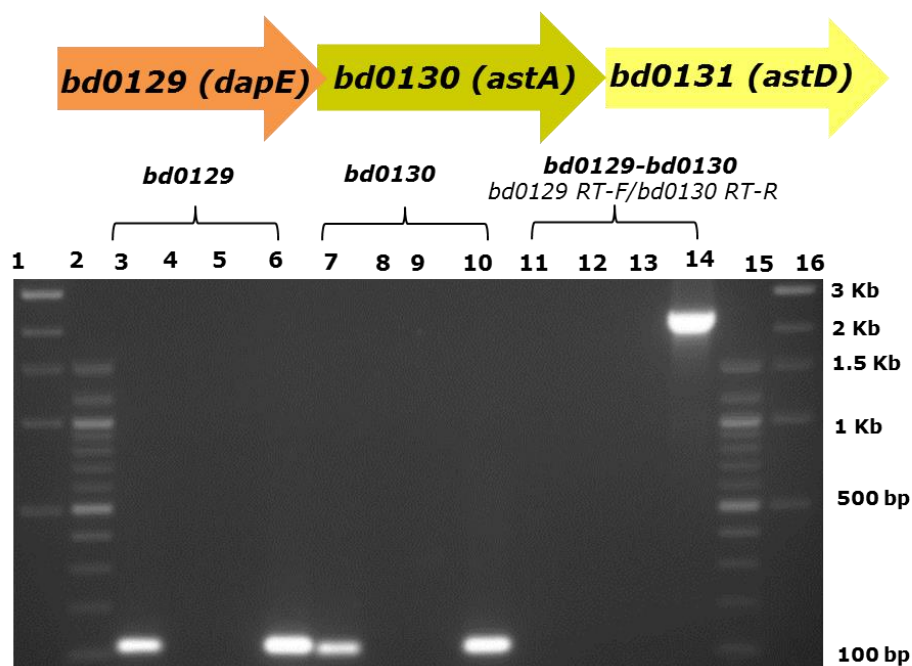


Figure 6. 27: RT-PCR shows that the *B. bacteriovorus* genes *bd0129* (*dapE*) and *bd0130* (*astA*) are not co-transcribed. Lanes 1 and 16: NEB 1000 bp ladder; Lanes 2 and 15: NEB 100 bp ladder; Lanes 3, 7 and 11: predator and prey total RNA at 3 hr; Lanes 4, 8 and 12: *E. coli* S17-1 RNA; Lanes 5, 9 and 13: No template; Lanes 6, 10 and 14: *B. bacteriovorus* HD100 genomic DNA. No band found in lane 11 indicates that there was no co-transcription between *bd0129* and *bd0130* genes.

6.4.11 Location of *E. coli* DapE-mCherry (B2472) in *E. coli*

As a control for the study of DapE in *B. bacteriovorus*, *E. coli* *dapE* gene (*b2472*) was fused with mCherry gene and expressed it in *E. coli* DH5α as *E. coli* elongates its cell wall by binary fission not filamentously.

Therefore, to assess the cellular location of *dapE* in *E. coli*, a strain with C-terminal fluorescently tagged *bd2472* (*dapE*) *E. coli* was generated. B2472-mCherry was constructed by fusion of a 1125 bp ORF fragment of *dapE* without stop codon in frame with mCherry. The fluorescence was visible in *E. coli* DH5α DapE-mCherry (Bd2472) strain and ~ 46% localised near poles (like avoiding the middle of the cell) and ~ 54% of the fluorescence diffuse throughout the cytoplasm (Figure 6-28). This results contrast with recent by Du and Lutkenhaus (2017) found that

DapE in *E. coli* tagged with GFP after growth overnight was distributed near the poles.

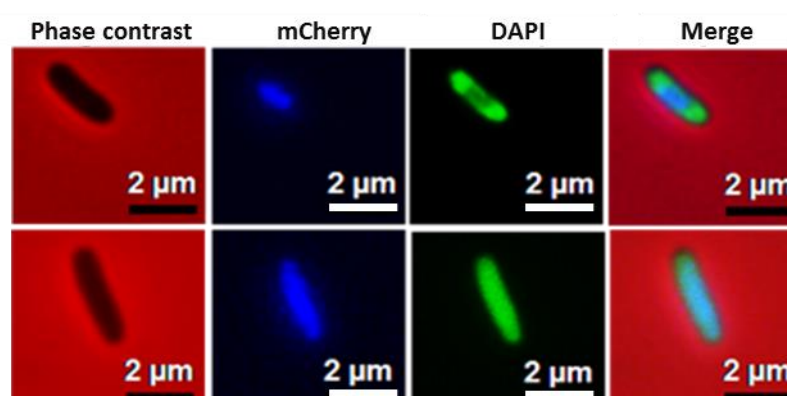


Figure 6. 28: Fluorescence of *E. coli* DH5α expressing pAKF56 B2472-mCherry (DapE) of *E.coli* in stationary phase that grown in YT broth for 16 hours with appropriate antibiotic. The cells were stained with DAPI and images were taken with 0.6 seconds fluorescent exposure for mCherry imaging and 0.04 seconds for DAPI images.

6.4.12 Native localisation of Bd0129-mCherry (dapE) assessed by fluorescently tagged mCherry *B. bacteriovorus*

To assess the cellular location of Bd0129- mCherry (dapE) during predatory lifecycle, C-terminal fluorescently tagged *B. bacteriovorus* bd0129 (*dapE*) strain was generated (as for *dapB* see section 6.13 in this chapter). Bd0129-mCherry construction was cloned in frame with mCherry in the plasmid pAKF56 using *E. coli* DH5α as a host. pAKF56::Bd0129- mCherry plasmid tagged protein was fluorescent and localized in pattern different from the location of Bd2472- mCherry of *E. coli*. Bd0129-mCherry was near the poles in an unusual subcellular space which has never been seen in Sockett laboratory when they did previous tagging for any protein (Figure 6-29). A short video was done to see if this protein is movable and it was found that it was not.

Bd0129- mCherry construct was transformed into pK18*mobsacB* plasmid and conjugated from donor *E. coli* strain S17-1 in *B. bacteriovorus* by single crossover with maintenance by kanamycin selection.

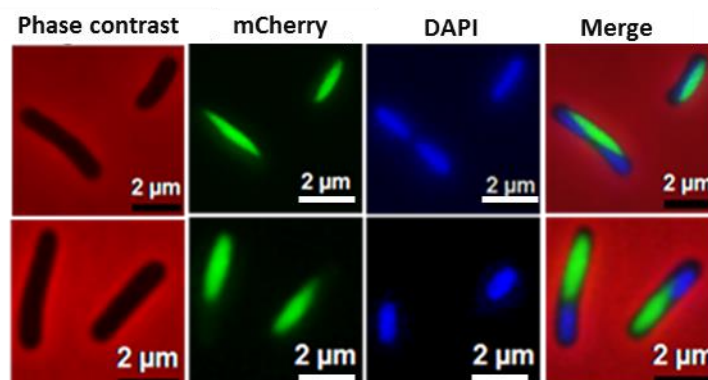


Figure 6. 29: Fluorescence of *E. coli* DH5α heterologously expressing pAKF56 Bd0129-mCherry of *B. bacteriovorus* in stationary phase that grown in YT broth for 16 hours with appropriate antibiotic. The fluorescent is visible and it is unusually places that not seen previously in any previous tagging protein in Sockett laboratory. The image was taken with 0.2 second fluorescent exposure with maximum gain.

The resultant strain of *B. bacteriovorus* *bd0129-mCherry* did grow predatorily and showed fluorescent pattern but the mCherry tag affected slightly the population distribution of cell shapes that resulted in some long attack phase and elongated the time course to 7 hr instead of 4hrs (Figure 6-30).

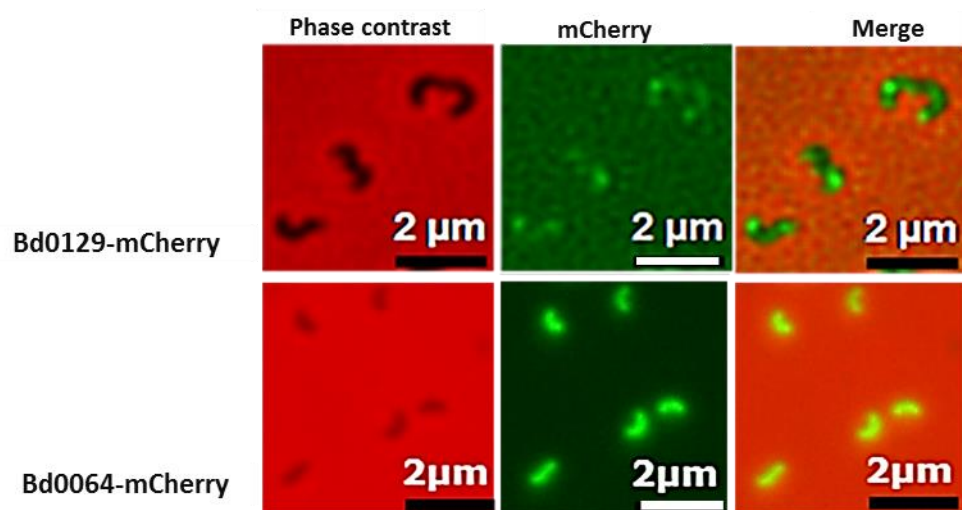


Figure 6. 30: The attack phase of *B. bacteriovorus* *bd0129-mCherry* (*dapE*) shapes affected by mCherry tag in comparison with normal attack phase of *B. bacteriovorus* *bd0064-mcherry* that not affected by mCherry tag.

The location of Bd0129– mCherry in *Bdellovibrio* was assessed in the attack phase (non- replicating phase) and showed diverse cellular locations and the vast majority of the fluorescence in the attack phase localised at both poles (~51%) with others fluorescence localised at one pole (~30%), diffuse through the cell (~10%), more than two foci (~8%) and with some *Bdellovibrio* not fluorescent (~1%) (Figure 6-31 A and B).

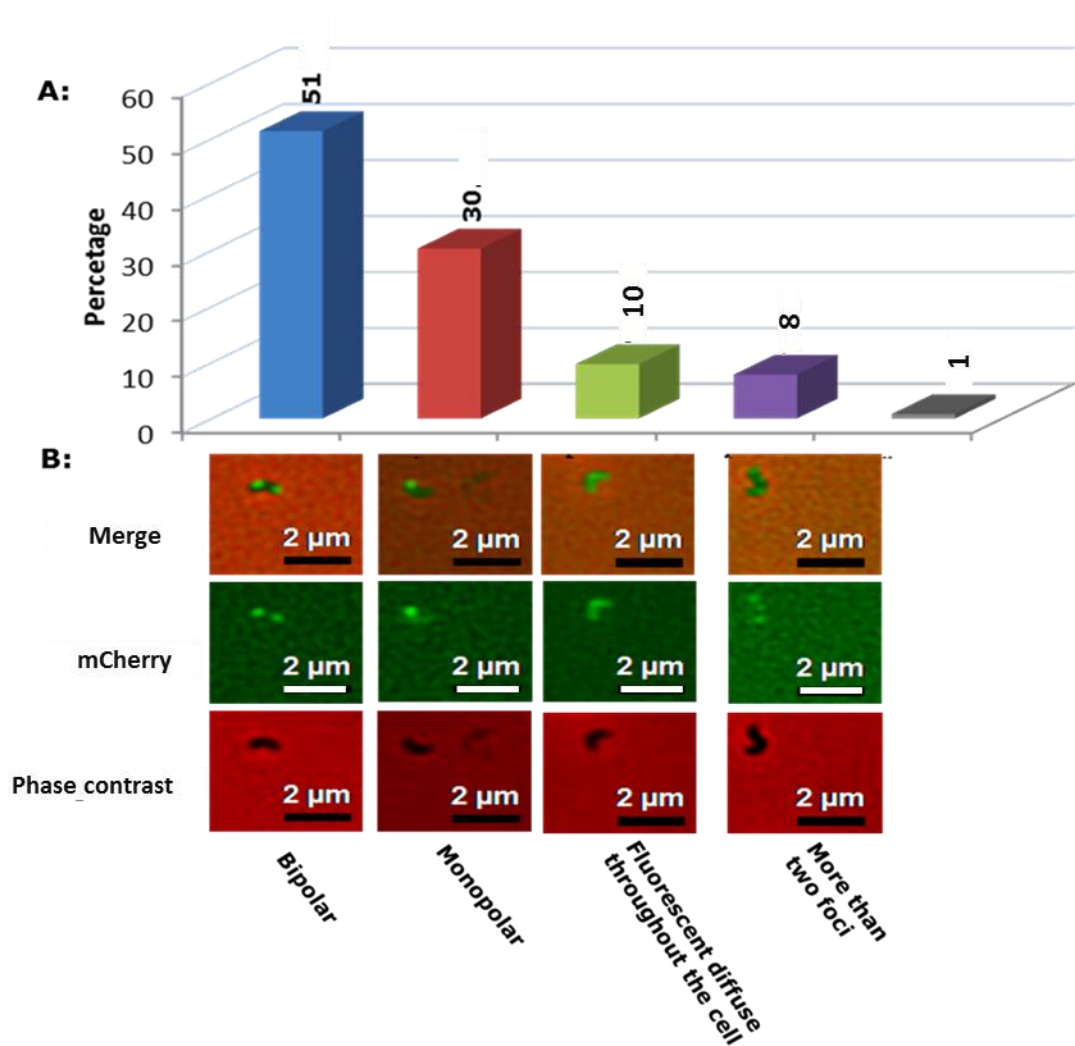


Figure 6.31: Examples of localisation of Bd0129- mCherry in the attack phase of *B. bacteriovorus* cells. (A) Overall distribution of Bd0129- mCherry fluorescence in *B. bacteriovorus* attack phase cells population (no of cells=639) from three biological repeats. (B) Image of *B. bacteriovorus* HD100 *bd0129- mCherry* cells (scale bar = 2 μ m).

Whereas, during the attachment of *Bdellovibrio* to *E. coli* (at 15 min) the fluorescence was shown to be mostly bipolar (~58%) and the rest at one of the poles (~19%), diffuse throughout the cell (~16%) and more than two foci *Bdellovibrio* (~7%) (Figure 6-32) so there is a little change in overall distribution of location of Bd0129- mCherry during attachment on prey but not a huge difference than what was found in attack phase and this meant that Bd0129 is not participate in the invasion process of the *Bdellovibrio*.

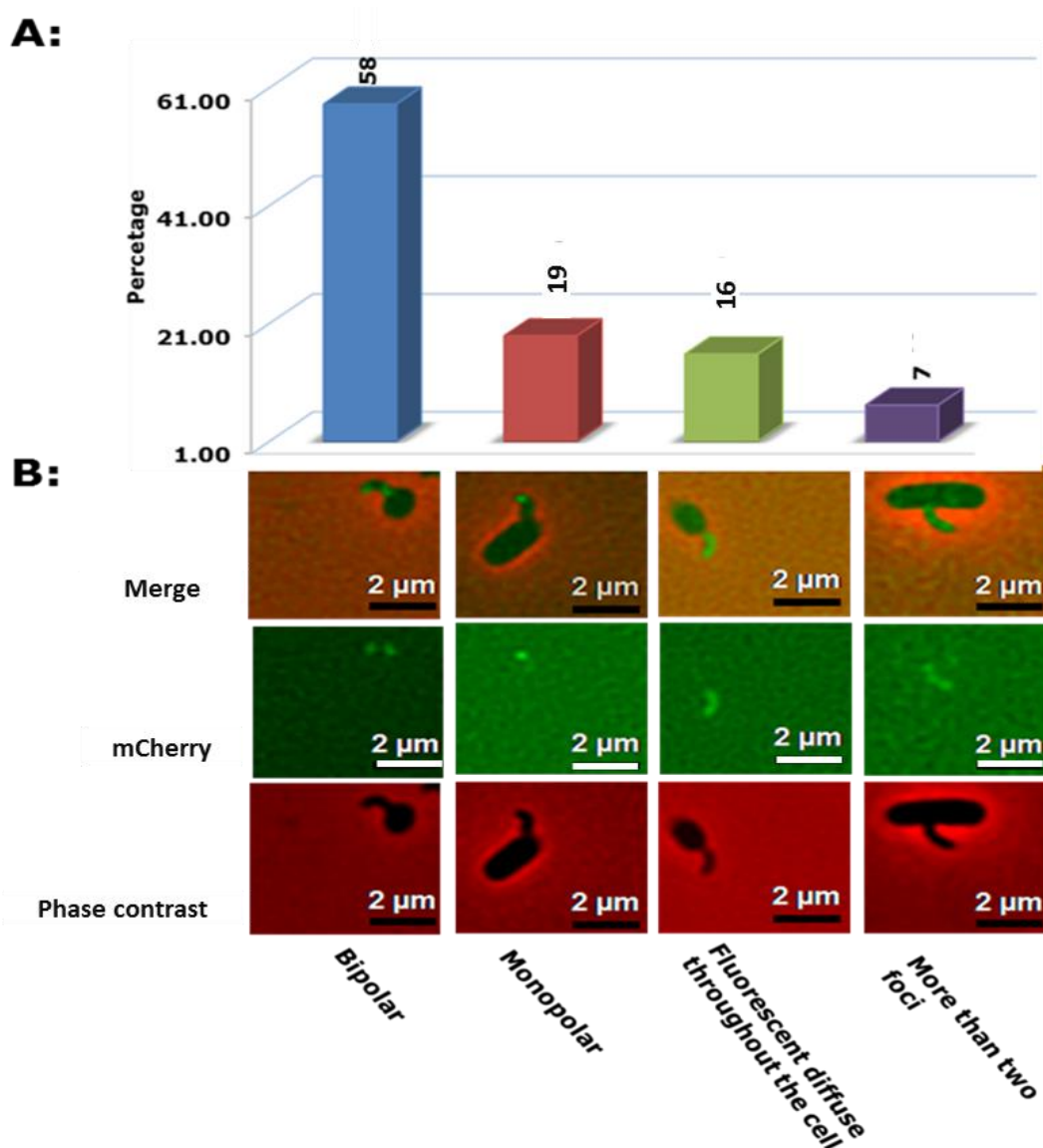


Figure 6. 32: Examples of Location of *Bdellovibrio bd0129-mCherry* during the attachment to the prey. (A) Overall distribution of Bd0129- mCherry fluorescence in *B. bacteriovorus* attack phase cells population (The cells no =639) from three biological repeats. (B) Image of *B. bacteriovorus* HD100 Bd0129- mCherry cells during attachment (scale bar = 2 μ m).

With regard to the rest of the stages of predatory life cycle, after early invasion (30min, 45min) fluorescence was shown to be bipolar and from (1hr- 6hr) fluorescence was diffuse all over elongating growing filaments of *Bdellovibrio* inside prey and then return to bipolarity as they escaped prey (7 hrs). (Figure 6-33).

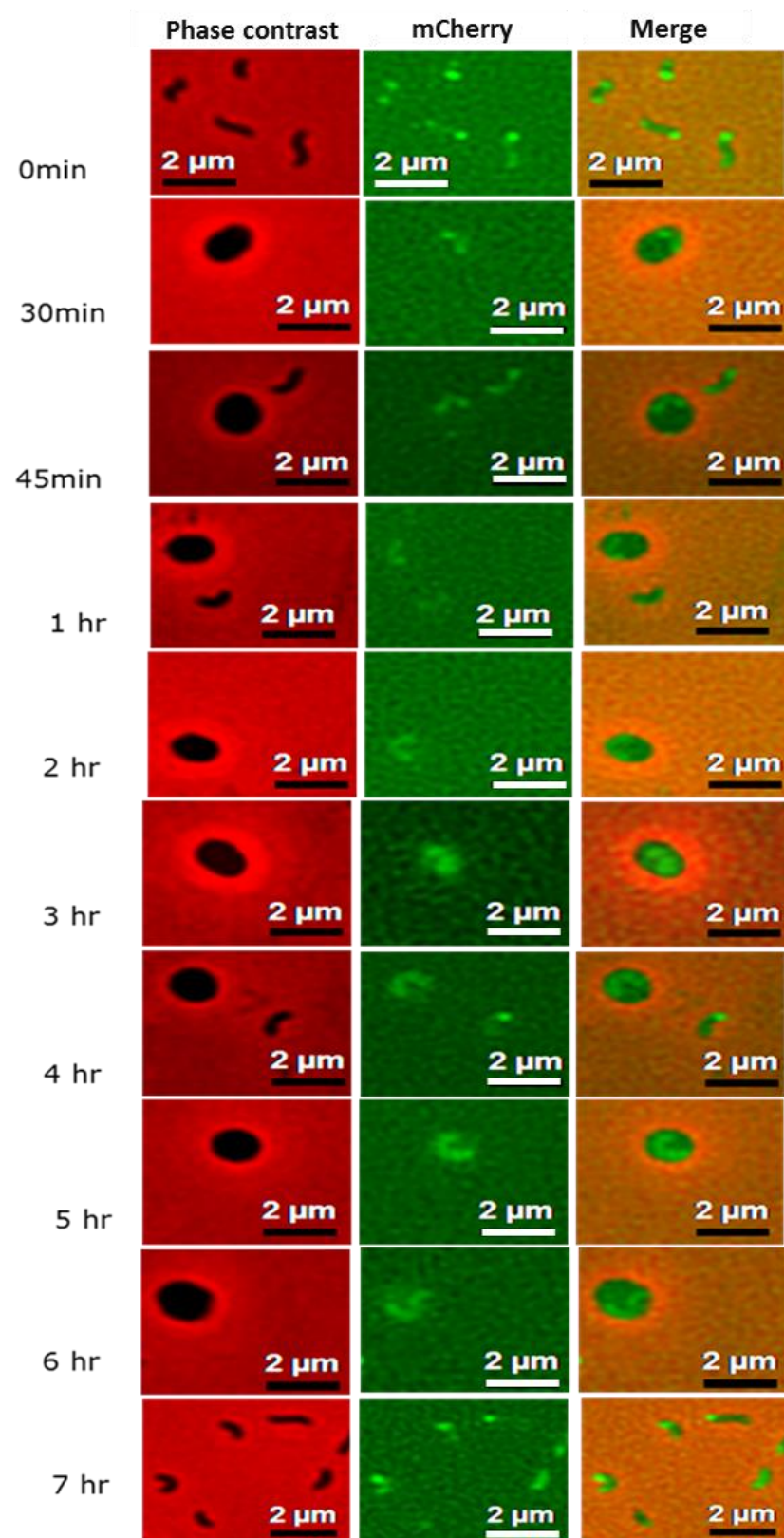


Figure 6. 33: The fluorescence pattern of *B. bacteriovorus* HD100 *bd0129-mCherry* during the whole predatory lifecycle.

6.4.13 Bd0129-mCherry expressed in growing host-independent cells

To investigate if Bd0129-mCherry are expressed in HI growth, *B. bacteriovorus* *bd0129-mcherry* strain was grown as host-independently (HI). The colonies of the generated HI *B. bacteriovorus* *bd0129-mcherry* take 20 days to grow on SPY media that was provided with Kanamycin ($50 \mu\text{g ml}^{-1}$) and the colonies were small in size. The colonies when picked up in PY broth, for this strain grew normally. Due to this reason, I prepared the genomic DNA from this strain and sent for genomic sequencing that will be explained in the next sections. The growing HI *B. bacteriovorus* Bd0129-mCherry strain expressed the fluorescence within all various cell types and shapes (Figure 6-34), and this is matched with RT-PCR results.

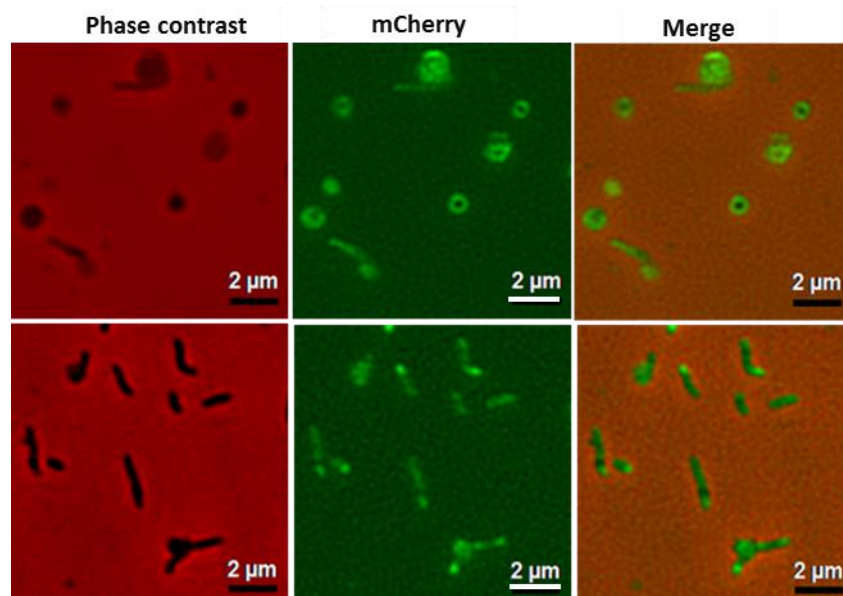


Figure 6. 34: HI *B. bacteriovorus* *bd0129-mCherry* strain. Bd0129-mcherry expressed in Host- Independent growth phase. The image was taken with 0.3 second fluorescent exposure and maximum gain.

Bd0129-mCherry exhibited fluorescence in all types of cells and location of the fluorescent mostly near poles avoid the septal places in long cells and in rounded cells was peripheral illuminating a *Bdellovibrio*-like vibroid like shape inside an “auto/self-bdelloplast” as can be seen in Figure (6-35).

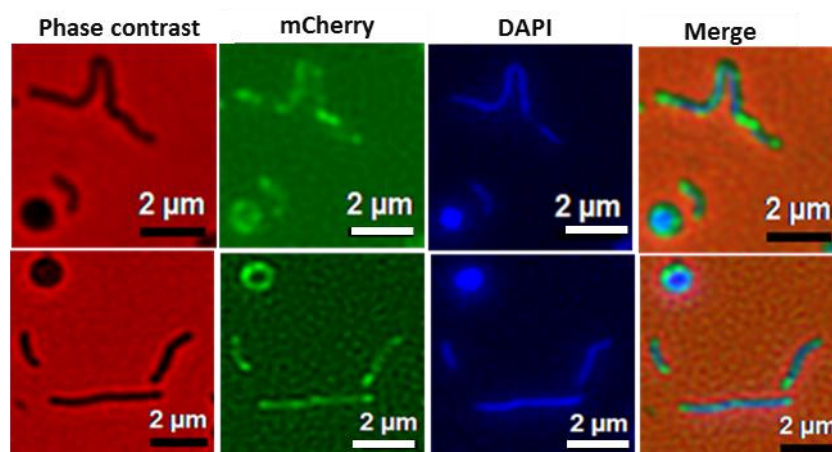


Figure 6. 35: HI *B. bacteriovorus* bd0129-mCherry cells were stained with DAPI strain. HI cells were stained with DAPI and imaged with 0.6 seconds fluorescent exposure for mCherry imaging and 0.05 seconds for DAPI images.

6.4.14 Genome sequencing showed surprise point mutations in the *Bdellovibrio* genomes of “HI bd0129-mCherry strain”

Visualising colonies of the HI *bd0129-mCherry* strain on PY media required approximately 20 days growth, when compared to most HI strains which require 7-12 days. Genomic DNA from the HI culture of the HI *bd0129-mCherry* strain was prepared and sent for genome sequencing in order to discover the possible cause(s) of delay of growth of this strain (Table 6-1).

The result of genome sequencing showed that the HI *B. bacteriovorus* *bd0129-mcherry* strain had two point mutations. In *bd1493* the amino acid change is (N347T), and in *bd2167* the amino acid change is (A42D). The results of this genome sequencing are shown in the Table (6-1). Bd1493 is the BamA outer membrane transport protein located in the outer membrane of bacteria for

assembly and export of outer membrane proteins (<http://smart.embl-heidelberg.de/>, <http://www.uniprot.org/uniprot>). The point mutation N347T that occurred in the POTRA (polypeptide translocation associated) of BamA that might be affected on function of all Bam complex proteins. In contrast, Bd2167 is a regulator of pilus synthesis protein and the region where the point mutation occurred was in a signalling region that might be switch *pill* gene on and off for doing its role in the phosphorelay signal transduction system (<http://smart.embl-heidelberg.de/>, <http://www.uniprot.org/uniprot>). Both these proteins are still not studied till now in details.

Table 6-1: The results of genome sequencing of HI *B. bacteriovorus* *bd0129-mcherry* strain. The result illustrating the gene that has the point mutation, what amino acid and nucleotide change in the genome of this strain.

Name of strain	Gene with point mutation	Amino acid changed	Nucleotide changed
HI <i>B. bacteriovorus</i> <i>bd0129-mcherry</i>	<i>Bd1493</i>	N347T	aAc/aCc
	<i>Bd2167</i>	A42D	gCt/gAt

6.4.15 Deletion of *bd0129* (*dapE*)

To determine the function of *bd0129* gene in *B. bacteriovorus*, a silent knockout deletion strain of *bd0129* would be required. A deletion plasmid for *bd0129* was constructed as mention in (see Section 2.10.2). After that, $\Delta bd0129$ construct was cloned and transformed in the plasmid pK18*mobsacB* using *E. coli* DH5 α as a host and conjugated in *B. bacteriovorus*, recombining by single crossover initially and maintained initially by kanamycin selection. The resultant *Bdellovibrio* plaques from conjugation were grown as host- dependent cultures, and then initial kanamycin resistant selection was removed and strains were screened for gene replacement and plasmid loss due to sucrose suicide selection using 5% sucrose in both presence and absence of DAP supply for their growth. 64 and 63

exconjugants for HD in both presence and absence of added DAP supplied (100 µg/ml) for their growth were screened for isolating $\Delta bd0129$ strain. All exconjugants were sensitive to kanamycin, and dream Taq PCRs was used for initial conformation of strain genotype. All exconjugants gave amplified 3100 bp band of wild *dapE* HD100 which mean that they were revertants (Figure 6-35 A and B). This indicates that the deletion of *bd0129* is impossible in HD even adding DAP or not to their cultures.

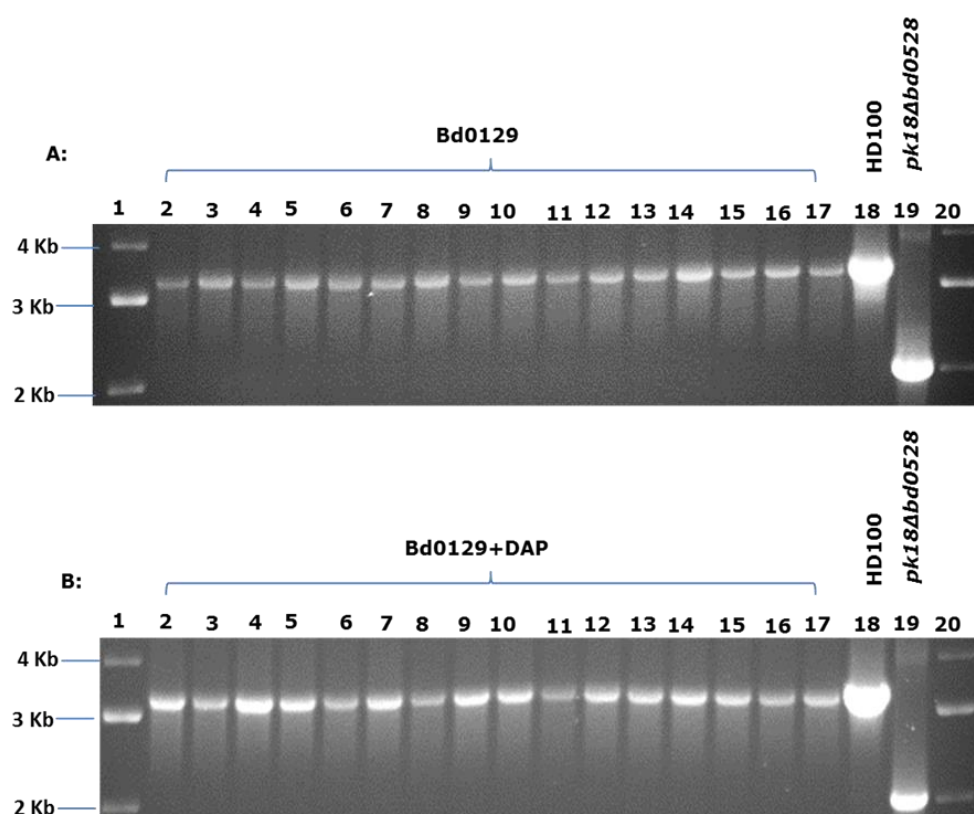


Figure 6. 36: Agarose gel showing amplified products from initial PCR screen of genomic DNA from potential HD *bd0129* knockout strain. A: PCR of potential knockouts of HD *bd0129* using Taq polymerase PCR and primers *bd0129-F* and *bd0129-R*. Lane 1, 20 are 1 Kb ladder. Lane 2-17 are potential knockout show band of wild HD100 that seen at 3280 KB. Lane 18, 19 are HD100 control and *pk18Δbd0129*. B: PCR of potential knockouts of HD *bd0528* using Taq polymerase PCR and primers *bd0129-F* and *bd0129-R*. Lane 1, 20 are 1 Kb ladder. Lane 2-17 are potential knockout show band of wild HD100 that seen at 3280 KB. Lane 18 and 19 are HD100 control and *pk18Δbd0129*.

6.4.16 Discussion

Synthesis of the cell wall of *Bdellovibrio* inside the bdelloplast and maintaining the prey cell wall is very vital issue in *Bdellovibrio* lifecycle as it needs to modify prey cell wall and synthesis of its own cell wall. Bd0047 and Bd0129 are important gene products that play an essential role in cell wall synthesis through forming DAP an essential product that involved in cell wall cross-linking in other bacteria.

With regard to the Bd0047 results, the transcriptional analysis of *bd0047* showed that this gene is expressed when *Bdellovibrio* start to grow inside the prey cell during the predatory lifecycle. It is also upregulated in various HI strains when compared to the attack phase of predatory *Bdellovibrio* (Figure 6- 7 and 6- 8). This shows that *bd0047* is expressed during growth phases so it may be a housekeeping gene that encodes a protein predicted to be part of the DAP/Lysine pathway which mean that this gene is involved in cell wall synthesis of *Bdellovibrio* rather than being involved in predatory processes.

The remarkable finding that the *bd0048* extra gene found only in *Bdellovibrio* (Figure 6-6- A Pale purple arrow with empty blue box and Figure 6- 10) has a shared operon with *bd0047* suggests that it might be act as novel DAP regulator or signals high levels of DAP synthesis via being cotranscribed.

Fluorescent tagging of Bd0047 showed that the protein has a central localisation within the cell in attack phase and during attachment while when *Bdellovibrio* begin to grow inside the prey, Bd0047 is diffuse throughout the cell and then returns back to the central localization (Figure 6-15, 6-16 and 6-17 and 6-18). It is important to mention that shape of attack phase has slightly "S shape" and this might be resulted from the mCherry tag interfering with *Bdellovibrio* cell wall synthesis. The central localization of DapB in attack phase and during attachment may be preparing precursors for crosslink synthesis for the cell wall, while when grown inside the prey diffuse to start in the cell wall synthesis as *Bdellovibrio*

elongate. Bd0047 is not important for invasion because of there were no huge differences in location of the Bd0047-mCherry in attack phase and during attachment to the prey cells. In HI cells, Bd0047 expressed fluorescence in all cell shapes at a central localization which suggested that they have general role making new self-wall synthesis of *Bdellovibrio* (Figure 6-19 and 6-20).

With respect to Bd0129 results, the transcriptional analysis of *bd0129* showed that the *bd0129* expressed at all time points of the predatory lifecycle and its upregulated in various HI strains compared to attack phase (Figure 6-24 and 6-25), that not suggest a predatory role but predicted to encode a housekeeping gene that required for DAP/Lysine pathway that make up the cell wall of *Bdellovibrio* and modify prey cell wall. The transcription of *bd0129* not lower when using the Δ *bd0134*KO RNA, this might be due to that *bd0129* still follow material from *bd0528* and this what keep the signal of *bd0129* on and it is transcribed. DapE of *Bdellovibrio* and *H. influenza* share same active site and most substrate recognition and binding which might suggest that DapE of both bacteria have same function and interaction (figure 6-22 and 6-23).

Fluorescent tagging of Bd129 revealed that the protein shows mostly bipolar localisation within the cell in attack phase and during attachment while when *Bdellovibrio* begin to grow inside the prey, the location of Bd0129 diffuse throughout the cell (Figure 6-31, 6-33 and 6-33). This suggest that the location of Bd0129 in attack phase near poles could be used for maintaining cell wall (No septal peptidoglycan activity but polar peptidoglycan synthesis) while when grown inside prey diffuse to start in cell wall synthesis even the Bd0129 mCherry tag led to some alteration of normal cell length and shapes of attack phase cells and elongated the predatory time course.

Distribution of the location of Bd0129- mCherry during attachment to prey cells showed not a huge difference than in attack phase which was mostly bipolar. If Bd0129 is important for invasion a big change would be expected in location of Bd0129- mCherry during the attachment. Ruby and Rittenberg (1984) showed that there was DAP addition to prey upon invasion so maybe it's not through de novo synthesis method and it could be by another method of DAP removal and reuse in prey. The location of Bd0129 in attack phase of predatory lifecycle was not matched to the resulted that obtained by Du and Lutkenhaus (2017) as they found that DapE of tagged with GFP without linker for *E. coli* after growth overnight was distributed near the poles, While distribution of the location of *Bdellovibrio* Bd0129- mCherry were bipolar, monopolar, diffuse throughout the cell and more than two foci.

In HI cells, Bd0129 expressed fluorescence and the location of fluorescence in most bean and long cells shapes of HI *Bdellovibrio* Bd0129- mCherry are near poles which suggested that they have a role in peptidoglycan synthesis and cell division (Figure 6-34 and 6-35). The noteworthy findings in HI *B. bacteriovorus* *bd0129-mcherry* strain is the point mutation in *bd1493* encoding BamA. The point mutation in BamA is very interesting due to BamA being a part of BAM (β -barrel assembly machinery) complex protein that responsible for assembly and export of outer membrane proteins. In Gram- negative bacteria, BAM complex consist of BamA which is a central and essential component that considered as an outer membrane protein itself that accompanied by accessory proteins BamB, BamC, BamD, and BamE (Knowles et al. 2009, Walther et al. 2009, Rigel and Silhavy 2012). BamA is composed of five polypeptide translocation associated (POTRA) domains that periplasmic domain of BamA that extend to C-terminal β -barrel of BamA that is immersed in the outer membrane (Figure 6-37) (Gu et al. 2016).

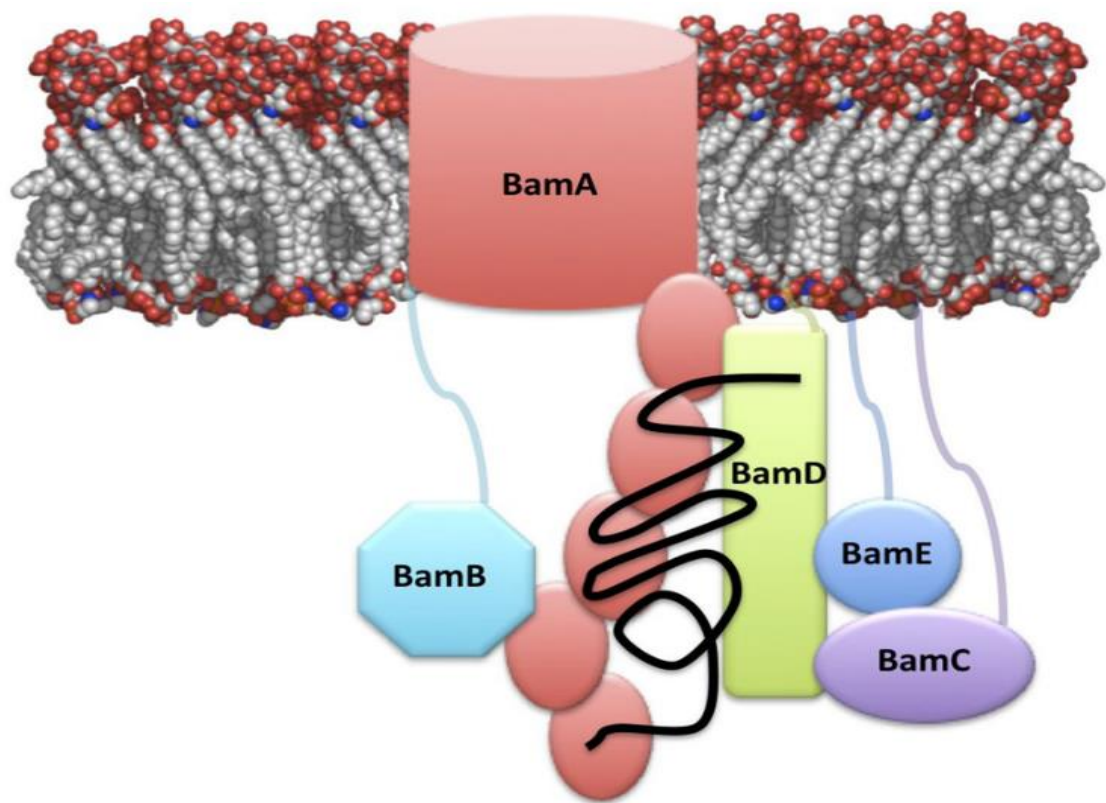


Figure 6. 37: The BAM complex of the Gram negative bacteria. Image taken from (Sandoval et al. 2011).

The fluorescent tag mCherry prevented the DapE from working properly possibly affecting production of DAP crosslinks that may shorter or longer than the normal or may be DAP crosslinks attached to outer membrane differently than the normal. This may have affected cell wall stability. The point mutation in BamA occurred in the fourth POTRA domain in N347T so may change the protein shape a little bit and possibly compensates for any wall stability effects, affecting Bam function, that were caused by the *Bd0129-mCherry* tag.

The deletion of *dapE* in other bacteria is lethal, and bacteria cannot grow without presence of DAP in media which mean that this enzyme is essential for DAP/Lysine pathway that making peptidoglycan cross-linking (Pavelka and Jacobs 1996, Karita et al. 1997, Nocek et al. 2010). Deletion of *bd0129* from *Bdellovibrio* also is impossible as for other non-predatory bacteria (Figure 6-35 A and B). This could

be because *Bdellovibrio* as a predatory bacteria do not get a sufficient amount of DAP supply early in growth from the invasion of prey, and need to use their own DAP or *Bdellovibrio* mutants cannot be supplied at invasion with extra DAP to get over DAP needed inside prey bdelloplast during growth as its not taken up through the prey envelope. Bd0129 is a housekeeping protein like in other bacteria. This chapter answers the question "Where does added DAP in prey cell in the 1984 paper of Ruby and Rittenberg come from" so the answer to this question is that DAP does not come from *Bdellovibrio* de novo synthesis pathway. So it must be being produced from prey materials.

6.5 Conclusions

This chapter has investigated some *Bdellovibrio* DAP synthesis genes which play essential roles in making materials for peptidoglycan cross-linking. From all the results of this chapter we can conclude the following

1. *bd0047* is expressed in both HD and HI lifestyles which mean that this gene has an important role in DAP/lysine pathway of *Bdellovibrio*.
2. The central localisation of fluorescent *Bd0047* during the predatory cycle and HI lifestyle indicated that this protein give the order to start synthesis of DAP.
3. *bd0048* that is only found only in *Bdellovibrio* may have a DAP regulator role as it is in a shared operon with *bd0047*.
4. *bd0129* is expressed in both HD and HI lifestyles and *Bd0129mCherry* fluorescence is also expressed in both HD and HI lifestyle near the poles indicated that this gene might have role in peptidoglycan crosslink synthesis and division of *Bdellovibrio*
5. *bd0129* is plays a vital role in predatory lifecycle of *Bdellovibro*, and its deletion is lethal either in presence or absence of external source of DAP.

6.6 Future work

1. Investigate the role of *bd0048* and if it essential for invasion in *Bdellovibrio* through examining the RT-PCR profile for this gene, C-terminal tag and deletion of this gene.
2. Test the effect of the point mutations in BamA (one of the Bam complex proteins that responsible for outer membrane protein export/assembly and all Bam complex proteins depend on BamA) that was detected in HI *B. bacteriovorus bd0129-mcherry* strain. Does such a point mutated BamA work well in wild type HD100?

3. Test if *bd0129-mcherry* tagging has any effect on the cell wall strength or stability or affects the length of predatory lifecycle through making not good quality of crosslink in cell wall. This can be done by testing wall stability by osmotic shocks.
4. Investigate the role of the other DAP/Lysine pathway genes in *Bdellovibrio* genome by construct a C-terminal fluorescence tag of these gene and if examine the deletion of these genes are possible in comparison with non-predatory bacteria.

7 Chapter Seven: Testing a link between a diguanylate cyclase *dgcD* (*bd3766*) and discovering restoration of predation- competence in *B. bacteriovorus* Δ *dgcB* (*bd0742*) mutant versus other *dgc* mutants (Δ *bd1434* and Δ *bd0367*) strains

As was pointed out in the Figure (3-17) of Chapter 3, there were three different point mutations found in the GGDEF gene *bd3766* in Δ *bd0742* suppressor strains. These are (tTc/tGc) encoded F394C, (cAa/cTa) encoded Q429L and (ctgctc/ctc) encoded LL439L. These point mutations were possibly the most likely suppressor candidates of Δ *bd0742* which is also a GGDEF gene. However, Hobley and co-workers found that deletion of *bd3766* gave no phenotype in their original study. In this chapter, I introduced the mutant and wild type GGDEF proteins and investigate what phenotypes resulted from their deletion with what I found about these point mutations.

7.1 Introduction to other GGDEF proteins in *Bdellovibrio*

Bd3766 (*DgcD*) is one of the four GGDEF protein in *B. bacteriovorus*. *Bd0742* is not the sole GGDEF protein in *B. bacteriovorus*, it encodes other GGDEF domain proteins that can be activated to function as diguanylyl cyclases (DGC) synthesising cyclic- di-GMP molecule. The DGC activity of *Bd3766* and ability to bind cyclic-di-GMP were confirmed previously (Rotem et al. 2015). The gene *bd3766* is not expressed from an operon as the two adjacent genes to *bd3766*, are in different orientations; these are *bd3765* and *bd3767* which encode hypothetical proteins (Figure 7-1).

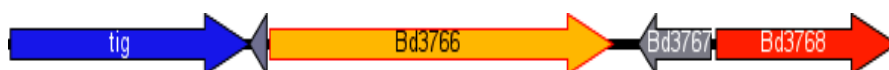


Figure 7. 1: The genomic context of *B. bacteriovorus* *bd3766* gene (<http://hactar.shef.ac.uk/xbase/>).

The transcription of *bd3766* was examined by RT-PCR, and it was expressed in continuously during the *Bdellovibrio* life cycle as expected (unpublished data) shown with permission from Dr. Carey Lambert in Figure (7-2).



Figure 7. 2: Reverse-transcriptase PCR (unpublished data with permission from Dr. Carey Lambert) showing transcriptional expression of *bd3766* throughout the predatory cycle. RNA was isolated at the indicated time points during one round of synchronous *Bdellovibrio* HD100 infection of *E. coli* host prey cells, and primers specific to the gene were used to amplify (RT-PCR, 27 cycles) 110 bp internal fragment from each transcript if present. AP = Attack phase (free-swimming *Bdellovibrio*); 15, 30, 45 = minutes since start of infection (attachment to and rounding of *E. coli* host cell into a bdelloplast structure); 1h, 2h, 3h, 4h = hours since start of infection (host cell resources being degraded and used for *Bdellovibrio* growth into a filament, followed by septation into multiple progeny and eventually lysis from host cell); HID2= host-independent strain RNA (No prey control); Ec = *E. coli* S17-1 RNA (no *Bdellovibrio* control); gen = *B. bacteriovorus* HD100 genomic DNA (positive control); NT = No template control; L = NEB 100 bp DNA ladder.

The encoded Bd3766 protein is 627 amino acids long and it contains two predicted transmembrane domains (TM1 and TM2 which run from amino acids 7-29, 429-448), four tetratricopeptide repeats (which runs from amino acids 147-180, 187-220, 227-260 and 307-340), a coiled coil region (which runs from amino acid 397-425) and a GGDEF domain (which runs from amino acids 451-624).

Tetratricopeptide repeats (TPR) is a structural motif that found in a wide range of proteins. It play role in protein-protein interactions and the assembly of multiprotein complexes (Das et al. 1998, D'Andrea and Regan 2003). Bd3766 is predicted to be a membrane bound protein due to the presence of these two transmembrane domains (The presence of a potential signal sequence was predicted as an alternative to TM) (Figure 7-3) (<http://smart.embl->

<http://smart.embl-heidelberg.de/>, <http://hactar.shef.ac.uk/xbase/>). Either prediction gives Bd3766 a location with TPR domains in the periplasm and GGDEF domain in the cytoplasm, but we will proceed with 2TM model for simplicity. The gene *bd3766*, unlike other GGDEF genes was found, not to be conserved in the related *B. bacteriovorus* tiberius genome (Hobley et al. 2012).

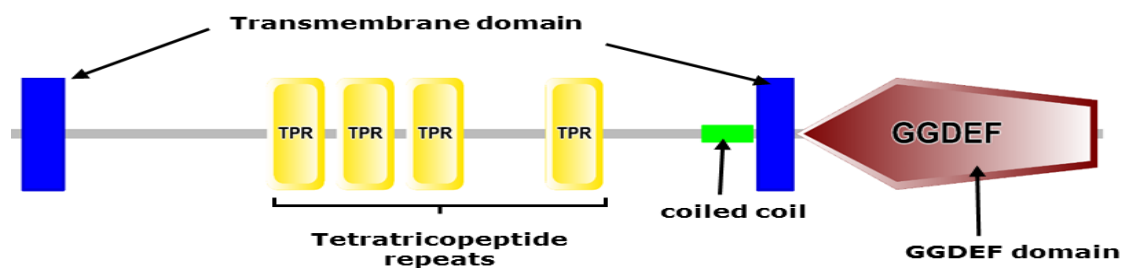


Figure 7. 3: The predicted domains of Bd3766 protein. The protein consisting of (from N-terminus to the C-terminus) transmembrane domain, a tetratricopeptide repeats region, a coiled-coil region, a transmembrane domain and finally the GGDEF domain (<http://smart.embl-heidelberg.de/>).

Hobley and co-workers found previously that deletion of *bd3766* produced a mutant strain that had no obvious phenotype in both host-dependent and host-independent growth modes; whereas, deletion of other GGDEF genes in *B. bacteriovorus* HD100 produced strains with observable phenotype that effected on its growth. Host-dependently grown *B. bacteriovorus* $\Delta bd03766$ strain was still able to produce plaques on the prey lawns which means that the deletion of this gene did not affect a plaque formation ability. A C-terminal fluorescent mCherry tag of this protein showed weakly but constant expression during the predatory lifecycle, while in host-independent cells, fluorescence was diffuse throughout cells with some bright fluorescent foci (Hobley et al. 2012). Because deletion of this gene resulted in a strain with no obvious phenotype Hobley and her co-workers did not do any more investigations on this gene.

Bd01434 (DgcC) is another *B. bacteriovorus* GGDEF domain proteins that function as diguanylyl cyclases synthesising cyclic-di-GMP molecule. The two adjacent genes to *bd1434* are also in different orientations; they are *bd1433* which encodes a hypothetical protein and *bd1435* which encodes putative NAD (FAD)-dependent dehydrogenases that plays a role in flavin adenine dinucleotide binding and oxidoreductase activity in the cell (Figure 7-4) (<http://hactar.shef.ac.uk/xbase/>).

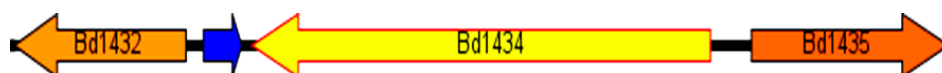


Figure 7. 4: The genomic context of *B. bacteriovorus* *bd3766* gene (<http://hactar.shef.ac.uk/xbase/>).

The expression of *bd1434* has been previously examined by RT-PCR, and it was continuously expressed during the *Bdellovibrio* life cycle as expected (unpublished data shown with permission from Dr. Carey Lambert in Figure (7-5)).



Figure 7. 5: Reverse-transcriptase PCR (unpublished data with permission from Dr. Carey Lambert) showing transcriptional expression of *bd1434* throughout the predatory cycle. RNA was isolated at the indicated time points during one round of synchronous *Bdellovibrio* HD100 infection of *E. coli* host prey cells, and primers specific to the gene were used to amplify (RT-PCR, 27 cycles) 108 bp internal fragment from each transcript if present. AP = Attack phase (free-swimming *Bdellovibrio*); 15, 30, 45 = minutes since start of infection (attachment to and rounding of *E. coli* host cell into a bdelloplast structure); 1h, 2h, 3h, 4h = hours since start of infection (host cell resources being degraded and used for *Bdellovibrio* growth into a filament, followed by septation into multiple progeny and eventually lysis from host cell); HID2= host-independent strain RNA (No prey control); Ec = *E. coli* S17-1 RNA (no *Bdellovibrio* control); gen = *B. bacteriovorus* HD100 genomic DNA (positive control); NT = No template control; L = NEB 100 bp DNA ladder.

Bd1434 (DgcC) is a 434 amino acid protein that is annotated as a GGDEF domain protein. Bd1434 contains a sole GGDEF domain (runs from amino acid 224-399) which indicates that the main function of this protein is synthesis of c-di-GMP, however it has a large stretch of upstream amino acids with unknown function, but which may regulate the GGDEF domain (Figure 7-6).



Figure 7. 6: The predicted domain of Bd1434 protein. The protein contains only a labelled GGDEF domain (Image taken from SMART database, <http://smart.embl-heidelberg.de/>). However a large-, potentially, regulatory region of the protein lies upstream.

Hobley et al. (2012) pointed out that Bd1434 is conserved between different *Bdellovibrio* strains including *B. bacteriovorus* Tiberius by genome sequence comparisons. The location of the Bd1434 protein during predatory growth was detected by mCherry tagging and Bd1434-mCherry was found to be cytoplasmically distributed and it is expressed in attack phase and early stages of invasion then the fluorescence became fainter as *Bdellovibrio* elongated inside the bdelloplast and the fluorescence faded when *Bdellovibrio* reached to septation stage. Bd1434-mCherry is also expressed in HI cells as a strong fluorescent focus at one pole of the cell and was not cytoplasmically distributed as in attack phase and in 60% of dividing HI cells the location of fluorescent foci were seen associated with division points which suggested that Bd1434 may be required for regulating cell division during predatory growth.

In Hobley's work, the diguanylyl cyclase activity of Bd1434 was tested by inhibition of *E. coli* MG1655 swimming and wild type Bd1434 strongly inhibited *E. coli* swimming (Hobley et al. 2012). Deletion of *bd1434* resulted in a mutant strain

with cells that were smaller and wider than wild type, with normal flagella, that were able to invade and kill *E. coli* prey cells at the same rates as the wild type. However, the *Δbd1434* deletion mutant strain was unable to grow as a host-independent culture when applying 1×10^7 cells of *Δbd1434* deletion mutant attack phase to test axenic growth on SPY media. The same number of 1×10^7 cells when applied for the *B. bacteriovorus* HD100, were able to grow axenically on SPY media. This meant that *bd1434* gene is definitely essential for the conversion to/or survival by *B. bacteriovorus* in axenic growth.

The only time that *Δbd1434* deletion mutant strain was able to grow axenically on SPY media was when it produced a single colony when 4.56×10^{11} cells of attack phase of this mutant was used on the SPY plate unlike 1×10^7 cells of attack phase for wild type *B. bacteriovorus* HD100. This suggested that a secondary mutation had happened and allowed the *Δbd1434* deletion mutant to grow axenically. This suppressor strain contained 10% biflagellate cells but in general did not differ visibly from wild type HI of *Bdellovibrio*. The complementation of *Δbd1434* deletion mutant with the wild type version of this gene resulted in a strain that was predatory with same wild type length and width and they regained the ability to carry out to axenic growth as HI strains like wild type (from a 1×10^7 inoculum).

Bd0367 (DgcA) is one of the GGDEF domain proteins in *B. bacteriovorus* that function as diguanylyl cyclases synthesising c-di-GMP molecule. The DGC activity of this domain and ability to binding the c-di-GMP were confirmed (Hobley et al. 2012, Rotem et al. 2015). Bd0367 is the sole GGDEF proteins that controls *Bdellovibrio* gliding motility which is essential for it to exit and movement out of the remnant prey shell to search for new prey cells and start the predatory cycle again. The two adjacent genes to *bd0367* are *bd0365* and *bd0368*. The upstream gene is *bd0365* which is a Leucyl-tRNA synthetase that has role in leucyl-tRNA aminoacylation and protein synthesis and the downstream gene is *bd0368* which

is cation efflux system protein that has a cation transmembrane transporter activity (Figure 7-7) (<http://hactar.shef.ac.uk/xbase/>). *bd0367* is conserved among various strains of *Bdellovibrio* (Hobley, Fung et al. 2012).



Figure 7. 7: the genomic context of the *B. bacteriovorus* *bd0367* gene (<http://hactar.shef.ac.uk/xbase/>).

Bd0367 expression was examined by RT-PCR, and it was expressed in a continuous manner throughout *Bdellovibrio* predatory life cycle as (unpublished data) shown with permission from Dr. Carey Lambert Figure 7-8).



Figure 7. 8: Reverse-transcriptase PCR (with permission from Dr. Carey Lambert) showing transcriptional expression of *bd0367* throughout the predatory cycle.

RNA was isolated at the indicated time points during one round of synchronous *Bdellovibrio* HD100 infection of *E. coli* host prey cells, and primers specific to the gene were used to amplify (RT-PCR, 27 cycles) 90 bp internal fragment from each transcript if present. AP = Attack phase (free-swimming *Bdellovibrio*); 15, 30, 45 = minutes since start of infection (attachment to and rounding of *E. coli* host cell into a bdelloplast structure); 1h, 2h, 3h, 4h = hours since start of infection (host cell resources being degraded and used for *Bdellovibrio* growth into a filament, followed by septation into multiple progeny and eventually lysis from host cell); HID2= host-independent strain RNA (No prey control); Ec = *E. coli* S17-1 RNA (no *Bdellovibrio* control); gen = *B. bacteriovorus* HD100 genomic DNA (positive control); NT = No template control; L = NEB 100 bp DNA ladder.

Bd0367 is a 343 amino acid protein that is annotated as a GGDEF domain protein. Bd0367 contains two domains: a REC domain (which run from amino acid 24-135) that located at N- terminus of the protein and GGDEF domain (which run from amino acid 148-321) (Figure 7-9).

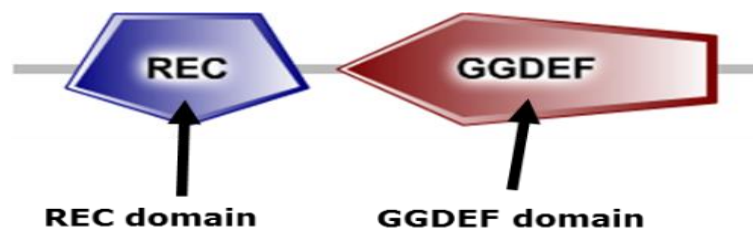


Figure 7. 9: The domains of Bd0367 protein. The protein consist of two domain, the REC domain and GGDEF domain (Image taken from SMART database, <http://smart.embl-heidelberg.de/>).

The REC domain of Bd0367 is a receiver domain act as a response regulators that is similar to WspR domain of other bacteria that regulates the biogenesis of flagella and pellicle formation in *Pseudomonas* (Hickman et al. 2005, Hickman and Harwood 2008). The activity of this domain may be controlled through phosphorylation by an as yet unknown histidine kinase (Hobley et al. 2012). Diguanylyl cyclase activity of Bd0367 was proven through inhibition of *E. coli* MG1655 flagellar swimming and also by measuring the level of the c-di-GMP that could be extracted from $\Delta bd0367$ strain that sharply dropped with deletion of this gene.

The deletion of *bd0367* was obtained as a host-independent strain and was not able to produce plaques on double YPSC *E. coli* prey lawn plate and they were also not motile in liquid broth. Gliding motility was abolished with deletion of *bd0367* giving a defect in the predatory escape from *E. coli* prey. This strain was able to enter prey cells and grow inside the prey forming new progeny at septation but unable to escape from prey remnant comparing with *Bdellovibrio* wild type HID13 that only need 10 mins only to escape after septation.

Hobley and co-workers found that fluorescent fusion Bd0367-mCherry was expressed in all stages of the predatory lifecycle at a low level and was also expressed in axenic growth and it was distributed diffusely throughout the cells.

The view that Bd0367 regulated the gliding motility was confirmed by work of Hobley and her co-workers when they complemented $\Delta bd0367$ mutant with the wild type version of *bd0367-mcherry* (complementation using *bd0367* without tag was not possible probably due to the effect of c-di-GMP on the *E. coli* cloning strains) which result in recuperating the ability of plaques formation on double YPSC *E. coli* prey lawn plate and restored the gliding motility and rapid predatory escape after septation that abolished by deletion of this gene but not flagellar motility that could be effected by the mCherry tag.

The level of c-di-GMP in the $\Delta bd0367$ deletion mutant strain compared to wild type *Bdellovibrio* HID13 cells (matched by wet cell weights) were measured and the results showed that the level of c-di-GMP in the $\Delta bd0367$ mutant is very low (0.4 ± 0.1 nM/mg wet weight of cells) in comparison with the control HID13 and HID26 (1.4 ± 0.1 nM/mg wet weight of cells). This indicated that deletion of this gene has negative effect on the c-di-GMP content that can be extracted from *Bdellovibrio* cells and this is another evidence that this gene has diguanylyl cyclase activity.

In this chapter, I test the predation ability of fluorescently tagged suppressor *bd3766** constructs made from all the $\Delta bd0742$ suppressor mutations in *bd3766* discovered in Chapter 3 where I put these into the original *B. bacteriovorus* $\Delta bd0742$ strain with construct controls (*WTbd3766-mCherry* and *WTbd0064-mCherry*) asking if these require a long time, like the original HI *B. bacteriovorus* $\Delta bd0742$ mutant strain, to kill *E. coli* pZMR100 or not. Also, because I hypothesised that the point mutations in the suppressor strains may now make the GGDEF domain produce high levels of c-di-GMP, these suppressor strains might start to kill the prey. Also, I investigated the ability of these suppressor constructs to inhibit the motility of the *E. coli* MG1655 to give a quick and direct answer suggesting the level of c-di-GMP in these suppressor *Bdellovibrio* stains.

Furthermore, I tested the predation ability of the $\Delta bd0742:bd3766Q429L$ strain (I choose this strain as a representative of other suppressor strains as they are in the same part of Bd3766 and have the same general effect) for 120 hrs on *E. coli* S17-1 and extending predation testing asking if $\Delta bd0742:bd3766Q429L$ strain was able to prey upon different types of prey other than *E. coli* S17-1 including *P. putida* and if this suppressor strain takes a long time to kill the prey like the original HI *B. bacteriovorus* $\Delta bd0742$ mutant strain or not. Also, I tested if prolonged incubation of overlay plates of $\Delta bd0742:bd3766Q429L$ strain with prey lawn can produce differences similar to the liquid predation and start to produce plaques or not.

7.2 Specific Research Aims

1. To test the ability of *Δbd0742::bd3766-F394C-mCherry*, *Δbd0742::bd3766-Q429L-mCherry* and *Δbd0742::bd3766-LL439L-mCherry* to prey on *E. coli* pZMR100 compared to *Δbd0742::WTbd3766-mCherry* and *Δbd0742:bd0064-mCherry* control strains.
2. To investigate if expressing the point mutated *bd3766* versions will affect the motility of the *E. coli* MG1655 indicating production of more or less c-di-GMP.
3. To test the ability of *Δbd0742:bd3766-Q429L* strains to prey on *E. coli* S17-1 compared to the wild type control HID26 testing over extended time periods.
4. To test the ability of *Δbd0742:bd3766-Q429L* strain to prey on *P. putida* compared to the wild type control HID26 testing over extended time periods.
5. To test the ability of *Δbd0742:bd3766-Q429L* strain to produce plaques on *E. coli* prey lawn plates versus predation and bdelloplast formation in liquid cultures.

7.3 Expected outcome

1. The point mutations causing changes (see below) in the coiled coil region (F394C) and in the transmembrane domain (Q429L and LL439L) of Bd3766 expressed from the genome of *Δbd0742:bd3766-Q429L-mCherry*, *Δbd0742::bd3766-Q429L-mCherry* and *Δbd0742::bd3766-LL439L-mCherry* strains are able to prey on *E. coli* pZMR100 faster than the control strains.

2. The point mutation in the coiled coil region (F394C) and in the transmembrane domain (Q429L and LL439L) of Bd3766 affect the motility of *E. coli* MG1655 by making more c-di-GMP.
3. $\Delta bd0742:bd3766-Q429L$ strain is able to prey on *E. coli* comparably to wild type control HID26.
4. $\Delta bd0742:bd3766-Q429L$ strain able to prey on *P. putida* comparably to wild type control HID26.
5. The point mutation in *bd3766* causes the regained ability of producing the plaques on a prey lawn for $\Delta bd0742:bd3766Q429L$ strain.

7.4 Results

7.4.1 Testing the ability of *Δbd0742* mutants with mCherry versions of naturally point mutated *bd3766** (*Δbd0742::bd3766-F394C-mCherry*, *Δbd0742::bd3766-Q429L-mCherry*, *Δbd0742::bd3766-LL439L-mCherry* strains) to lyse *E. coli* pZMR100 compared to wild type *Δbd0742::WTbd3766-mcherry* and *Δbd0742::bd0064-mCherry* strains controls.

In order to investigate if the point mutations that were found in *bd3766* gene in the genomes of the suppressor strains: *bd3766-F394C*, *bd3766-Q429L* and *bd3766-LL439L* have an effect on the predation ability of HI *B. bacteriovorus* *Δbd0742* mutant strain, fluorescent constructs of WT*Bd3776-mCherry*, *Bd3776-F394C-mCherry*, *Bd3776-Q429L-mCherry* and *Bd3776-LL439L-mCherry* were made using the Gibson assembly cloning assay (see Section 2.10.3).

pK18mobsacB plasmids containing WT*Bd3776-mCherry*, *Bd3776-F394C-mCherry* and *Bd3776-Q429L-mCherry* constructs were then transformed into *E. coli* S17-1 and conjugated from donor *E. coli* strains S17-1 into the original HI *B. bacteriovorus* *Δbd0742* mutant strain by single crossover with maintenance of plasmid by kanamycin selection. In addition to these strains, I also prepared another “mCherry” control fluorescent strain which is *Δbd0742::bd0064-mCherry*. *Bd0064* is a redundant PilZ family protein that can be expressed mCherry-tagged without phenotypic effects in *Bdellovibrio* (Willis et al. 2016). For assessing the ability of *Δbd0742::bd3766-F394C-mCherry*, *Δbd0742::bd3766-Q429L-mCherry* and *Δbd0742::bd3766-LL439L-mCherry* strains to lyse *E. coli* pZMR100 prey, predation assays were run using a standard starting *E. coli* pZMR100 inoculum of OD₆₀₀ 1.0 (10 ml containing 5.39 X10⁸ cells per ml of *E. coli*) with HI *B. bacteriovorus* *Δbd0742* mutant strains that contain constructs of point-mutant *bd3766**mCherry alongside control strains of HI *B. bacteriovorus* *Δbd0742::bd0064-mCherry* and *B. bacteriovorus* *Δbd0742::WTbd3766-mCherry* strains. The strains were matched by Lowry protein assay (500 mg protein). The

Lowry method (Markwell et al. 1978) was used to match cultures as no plaques counts are possible for the non-predatory original *B. bacteriovorus* $\Delta bd0742$ (see section 2.12.1). The predation assays were performed for 120 hr for all strains in triplicate.

It is apparent from Figure 7-10 A and B that the $\Delta bd0742::bd3766-F394C-mCherry$, $\Delta bd0742::bd3766-Q429L-mCherry$ and $\Delta bd0742::bd3766-LL439L-mCherry$ strains reduce the number of *E. coli* pZMR100 quickly, within 72 hrs (~ three log drop in CFUs of *E. coli* pZMR) (Figure 7-10) compared with control strains $\Delta bd0742::bd0064-mCherry$ and $\Delta bd0742::WTbd3766-mCherry$ which either start to decrease the number of *E. coli* pZMR100 prey only at 96 hr or 120 hrs respectively (~ two log drop in CFUs of *E. coli* pZMR 100 for $\Delta bd0742::bd0064-mCherry$ strain). This indicates that these point mutations in *bd3766*, arising naturally in the $\Delta bd0742$ original strain upon prolonged incubation with prey are now able to lyse the *E. coli* pZMR 100 again and faster than the control strains.

NB. Figure 7-10 (below) was separated to A and B because the point mutation (LL439L) in *bd3766* strain was isolated more recently than the other strains in this experiment so it is appears in Figure 7-10 as a B Figure.

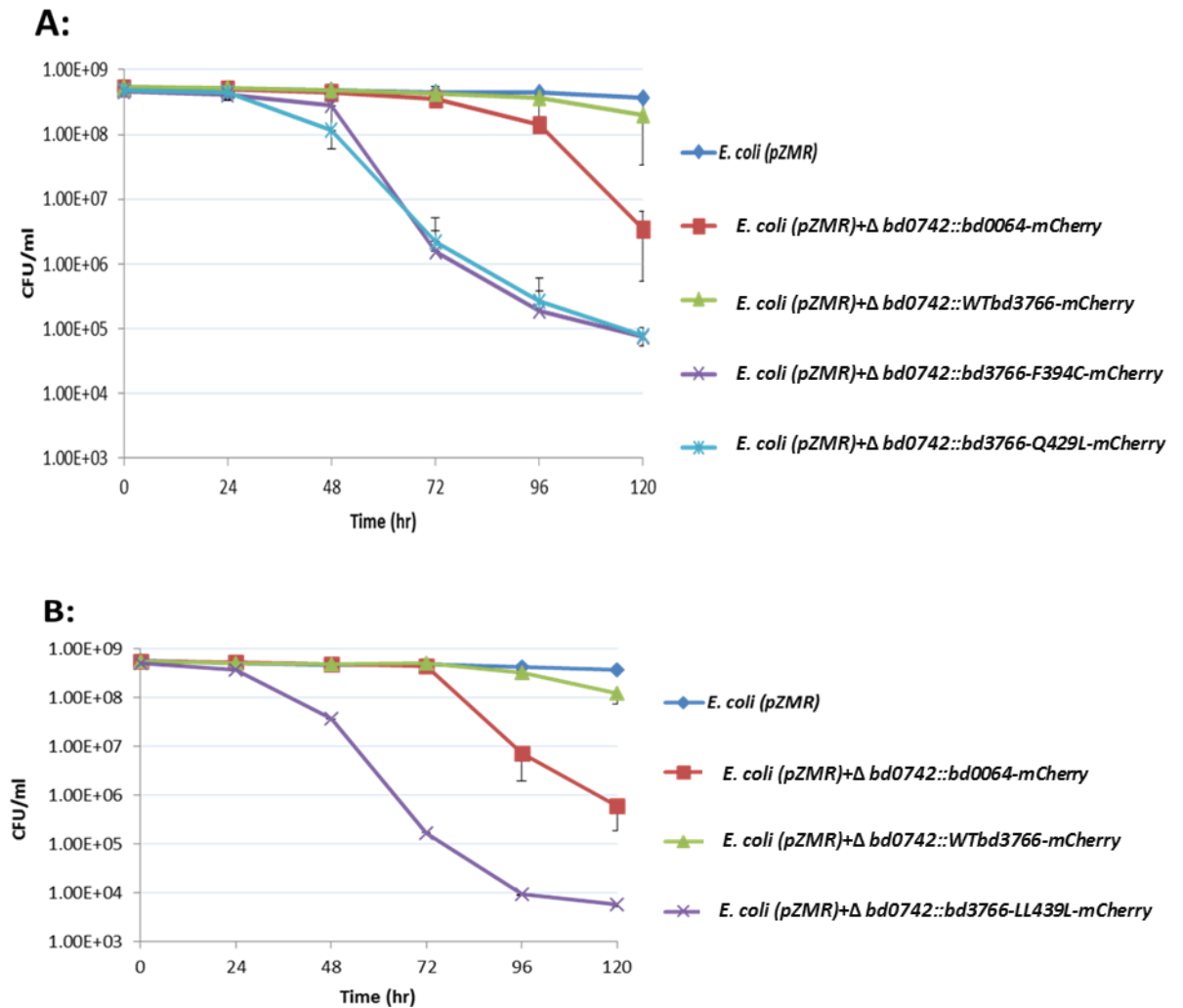


Figure 7. 10: The number of viable prey cell counts in predation assays. A: For Δ bd0742:: bd3766-F394C-mCherry and Δ bd0742::bd3766-Q429L-mCherry strains alongside with Δ bd0742::WTbd3766-mCherry and Δ bd0742::bd0064-mCherry strains on *E. coli* pZmr100 B: For Δ bd0742:: bd3766-LL439L-mCherry strain alongside with Δ bd0742::WTbd3766-mCherry and Δ bd0742::bd0064-mCherry strains on *E. coli* pZMR100 (all predation assays were performed with three biological repeats) and the error bars represent 1 SD from the mean. It is clear from this graph that all the strains containing the point mutations kill *E. coli* faster than the control strains.

7.4.2 Testing the effect of the point mutated Bd3766* GGDEF proteins on the motility of *E. coli* MG1655 as an assay for c-di-GMP production

To investigate if the point mutations found in *bd3766* gene play a role in regulating the motility of *E. coli* MG1655 through a c-di-GMP dependent mechanism and to find out what c-di-GMP dependent phenotypes can be mediated by *bd3766* point mutations, plasmids containing the wild-type (WT) of *bd3766* and genes encoding point mutated Bd3766* (F394C and Q429L and LL439L) were transformed into *E. coli* MG1655 in order to perform motility test to detect if there are any differences in motility between WTbD3766 (*E. coli* MG1655 pK18WT*bd3766-mCherry*), Bd3766-F395C (*E. coli* MG1655 pK18*bd3766-F394C-mCherry*), Bd3766-Q429L (*E. coli* MG1655 pK18*bd3766-Q429L-mCherry*) and Bd3766-LL429L (*E. coli* MG1655 pK18*bd3766-LL439L-mCherry*) strains. The cultures of each strain were grown under standard conditions, and motility tests carried out on semisolid (0.25% agar) medium plates.

The principle of this assay is that the *E. coli* PilZ protein (YcgR) binds c-di-GMP and in turn binds to FlhM of the *E. coli* flagellar motor and stops its rotation. So if extra or less c-di-GMP is made by *bd3766* variants changes in flagellar motility will be seen as the swarm size changes in soft agar.

It is obvious from Figure (7-11 A, B and C) that the zone diameter of swimming motility of Bd3766-F395C (2.51 cm), *Bd3766-Q429L* (2.46 cm) and Bd3766-LL439L (2.36 cm) were significantly reduced compared with the wild-type WTbD3766 swimming motility zone diameter (2.88 cm). The statistical analysis revealed that the swimming motility of each strain (Bd3766-F395C, Bd3766-Q429L and Bd3766-LL439L) was attenuated significantly by ~12% (*P* value 0.0064), ~15% (*P* value 0.0173) and ~16% (*P* value 0.0106) respectively when compared with the wild-type strain WTbD3766.

All these results were obtained from multiple measurements to avoid batch variants in agar. Each set of three experiments was carried out for all strains and control strain on one plate at the same time and repeated four times on the three separate occasions. These results demonstrated that the Bd3766* proteins negatively regulate bacterial swimming motility and I concluded that this is likely due to c-di-GMP-dependent post-translational regulation of the flagellum-based motility in *E. coli* MG1655 by the Bd3766 variant proteins making more c-di-GMP.

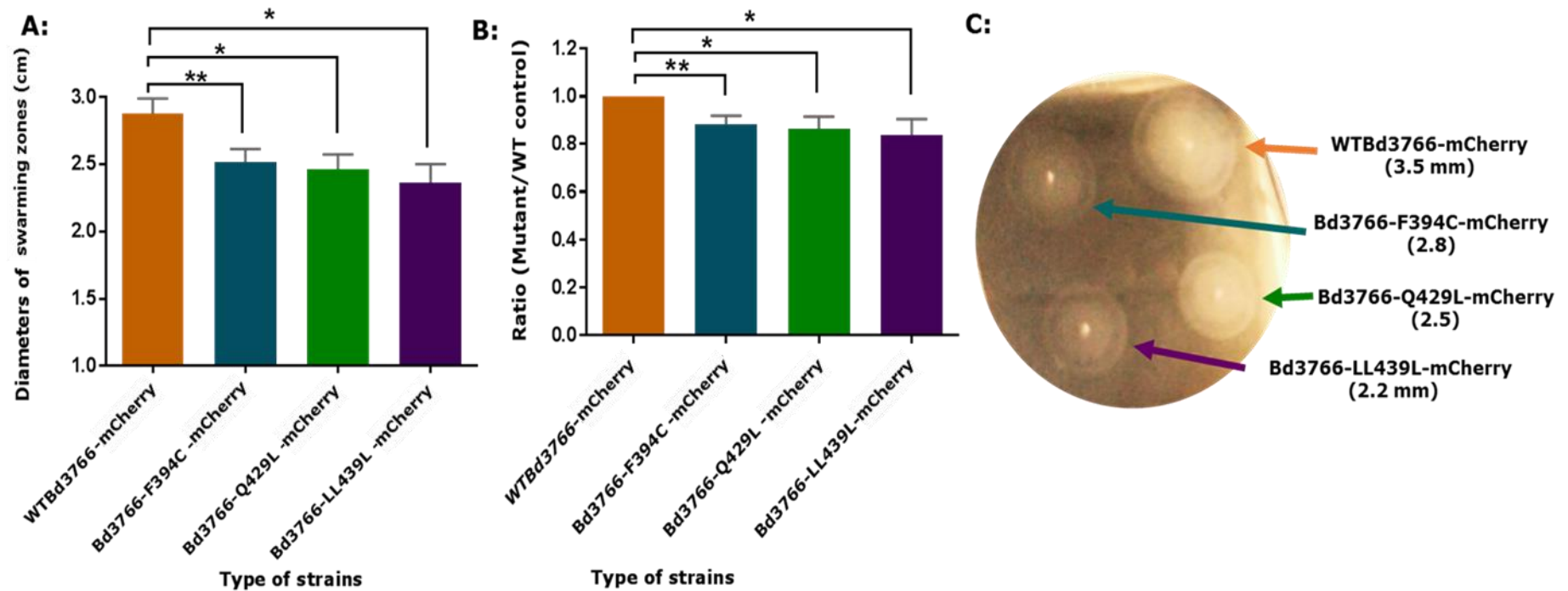


Figure 7. 11: Mean swarm size of motility assay of *E. coli* MG1655 strain expressing wild type Bd3766 and point mutated version of Bd3766.

A: Relative ratios of swimming zone diameters; **B:** Mean diameters of swarming zone. **C:** Photo of a typical plate of motility of *E. coli* MG1655 expressing wild type Bd3766-mCherry and Bd3766*-mCherry version. (Each set of measurements was carried out in one plate at same time and repeated in triplicate four times on the three different occasions to give the means). WTbD3766:*E. coli* MG1655 (pK8WTbD3766mCherry), Bd3766-F395C:*E. coli* MG1655 (pK18Bd3766-F394C-mCherry), Bd3766-Q429L: *E. coli* MG1655 (pK18Bd3766-Q429L-mCherry), Bd3766-LL439L:*E. coli* MG1655 (pK18Bd3766-LL439L-mCherry).

Because swarm plates rely on the growth of *E. coli* as well as motility, a growth check was carried out to investigate the effect of Bd3766*-mCherry versions on the growth of *E. coli* MG1655 and to see if there are any variations in growth of these strains comparing to the wild type version control. All these variant strains were grown together with the control strain that containing WTBd3766-mCherry and all cultures grown under standard condition with addition of kanamycin, as they contained the pK18mobsacB plasmid that is a kanamycin resistance vector. The OD₆₀₀ readings of each strain was measured every 30 minutes for 9 hrs (Figure 7-12).

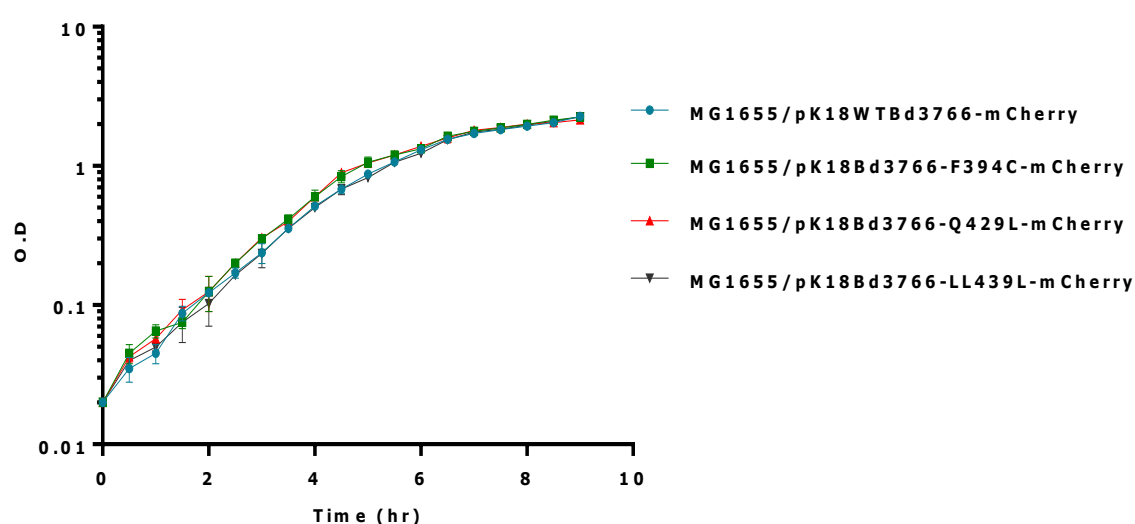


Figure 7. 12: The growth curve rate of *E. coli* MG1655 (pK18/WTBd3766mCherry) compared to the growth rate of *E. coli* MG1655 (pK18/Bd3766*-mCherry). OD₆₀₀ values were measured every 30 minute intervals for 9 hrs. Error bars show the standard deviation.

The growth curves clearly show that no growth defects can be seen from heterologous expression of pK18/Bd3766*-mCherry versions on the growth of the *E. coli* MG1655 comparing to the *E. coli* MG1655 (pK18WTBd3766mCherry). So the motility effect observed in Figure (7-11) was not due to growth changes but instead c-di-GMP changes affecting flagellar rotation.

7.4.3 Testing the ability of suppressor $\Delta bd0742:bd3766\text{-}Q429L$ strain to prey on *E. coli* S17-1 compared to the wild type host-independent *Bdellovibrio* control strain, HID26

In this section I wanted to test the predation ability of the $\Delta bd0742$ suppressor strain that contains the point mutation in *bd3766* encoding the amino acid change Q429L as a representative for other $\Delta bd0742$ suppressor strains. I did this as all point mutations were found within 100 bp (the region of *bd3766* results of genome sequencing displayed see section 3.6.8 in Chapter 3) and the fluorescent versions of $\Delta bd0742$ suppressor strains have a similar effects in section 7.4.1 above in this Chapter.

The suppressor strains that resulted from HI *B. bacteriovorus* $\Delta bd0742$ mutant strain that lysed *E. coli* and *A. baumannii* were filtered twice and cultured in a large volume by adding new prey. Genomic DNA was prepared and sent for genome sequencing and frozen stocks were also prepared from these prey-lysate cultures. *B. bacteriovorus* $\Delta bd0742$ suppressor strain that has a point mutation in the transmembrane region (Q429L) is named as a $\Delta bd0742:bd3766\text{-}Q429L$ strain. The predation assays were run using a starting *E. coli* S17-1 stationary phase inoculum of OD₆₀₀ 1.0 (10 ml containing 5×10^8 cells per ml of *E. coli* S17-1) with $\Delta bd0742:bd3766\text{-}Q429L$ and HID26 strains were matched by Lowry protein assay (500 mg protein). Lowry method (Markwell et al. 1978) was used to match cultures as no instantaneous plaques counts are possible for $\Delta bd0742:bd3766\text{-}Q429L$ (see Section 2.12.1 and CFU are not reliable). The predation assays were performed for 120 hr for both strains in triplicate. It is apparent from Figure (7-13 A and B) that $\Delta bd0742:bd3766\text{-}Q429L$ strain decreases the number of *E. coli* S17-1 within the 48 hr (~ 2.5 log drop in CFUs of *E. coli* S17-1) compared with the HID26 wild type strain (which is a positive predatory HI control) which rapidly decreased the number of *E. coli* S17-1 after only 24 hrs ($\sim$ three log drop in CFUs of *E. coli* S17-1) (Figure 7-13 A).

This meant that the point mutation in *bd3766* of $\Delta bd0742:bd3766-Q429L$ strain was active in predation from a very early time point in the assay compared to the original HI *B. bacteriovorus* $\Delta bd0742$ mutant strain which did not kill *E. coli* until suppressors arose at 96 hrs or more (Figure 3-7 B and C see Chapter 3, Section 3.6.2). The suppressor mutation affected the predation ability of $\Delta bd0742:bd3766Q429L$ strain in a positive way. The predators that resulted from the $\Delta bd0742$ suppressor preying on *E.coli* still had an HI like shape and were checked for longer time viability as attack phase cells by adding a fresh prey and observing whether they lyse the prey or not and they were lysed the fresh prey which was added. They were viable long term as predators.

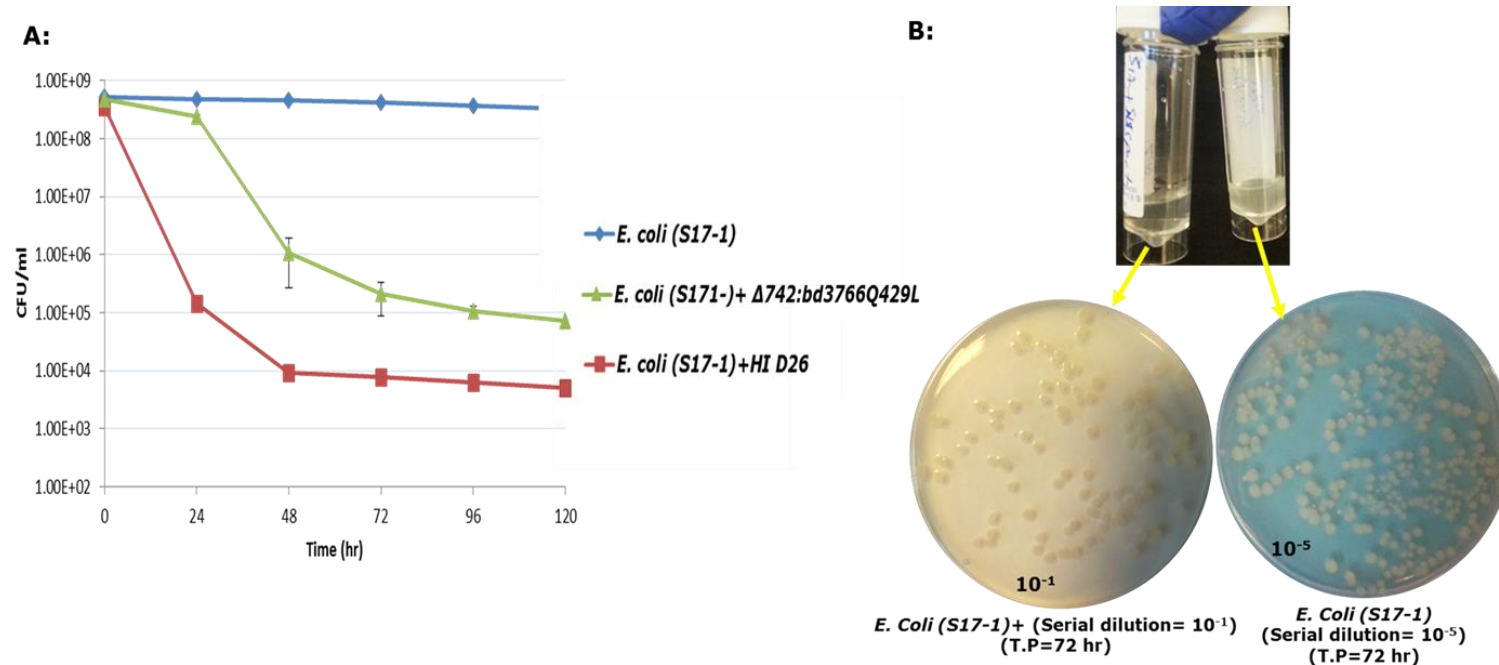


Figure 7. 13: Dynamics of predation of $\Delta bd0742:bd3766-Q429L$ strain were tested against wild-type (WT) host- independent (HI) *B. bacteriovorus* (HID26) strain (the predatory control) on *E. coli* S17-1 (from 3 biological repeats). (A): The Predation ability of $\Delta bd0742:bd3766-Q429L$ strain and HID26 strain on *E. coli* S17-1 prey were examined by drop in number of *E. coli* S17-1 over 120 hr. In liquid cultures, HID26 started to reduce the number of *E. coli* S17-1 during 24 hr while $\Delta bd0742:bd3766-Q429L$ strain was started to decrease the number of *E. coli* S17-1 during 48 hr (from three biological repeats in separate weeks) with the error bars represent 1 SD from the mean. **(B):** Tubes of *E. coli* S17-1+ buffer control and *E. coli* S17-1+ $\Delta bd0742:bd3766-Q429L$ strain infection and their serial dilution plates after 120 hrs.

7.4.4 $\Delta bd0742:bd376-Q429L$ strain was unable to prey on *P. putida* compared to wild type Host- independent *Bdellovibrio* control strain HID26

As discussed previously in chapter three, all *bd3766** suppressor strains used here were isolated after prolonged incubation on *E. coli* S17-1 or *A. baumannii* (but not other prey including *P. putida*) and suppressors arose that now preyed on *E. coli* or *A. baumannii*. Suppression was not seen for all types of prey (see Chapter 3, Section 3.6.3, Figure 3-9, 3-10 and 3-11). A new question which appeared from this work was: Is the suppressor strain arising from *E. coli* S17-1 and *A. baumannii* predation able to prey on other types of prey? I chose *P. putida* as a representative for all types of prey that I used previously in chapter three remembering that the original HI *B. bacteriovorus* $\Delta bd0742$ mutant strain was unable to prey on it and them.

The predation ability of $\Delta bd0742:bd3766Q429L$ strain to prey on *P. putida* was tested using a starting *P. putida* in stationary phase inoculum OD₆₀₀ 1.0 (10 ml containing 1.42×10^9 per ml for *P. putida*) with $\Delta 742:bd3766-Q429L$ and HID26 strains were matched by Lowry protein concentration assay (500 mg protein). Lowry method (Markwell et al. 1978) was used to match cultures as no instantaneous plaques counts are possible for $\Delta 742:bd3766-Q429L$ (see Section 2.12.1 and CFU are not reliable). The assays were carried out for 120 hr for both strains in triplicate. As indicated from Figure (7-14 A and B), $\Delta bd0742:bd3766-Q429L$ strain was unable to reduce the number of *P. putida* after 120 hrs compared with the HID26 wild type strain (which is a positive predatory control). HID26 rapidly decreased the number of *P. putida* during 72 hrs (~ three log drop in CFUs of *P. putida*) (Figure 7-14 A).

This showed that even though the point mutation (Q429L) was present in *bd3766* of the $\Delta bd0742:bd3766-Q429L$ strain, (allowing it to kill *E.coli* S17-1 of *A. baumannii* prey) it remained unable to kill *P. putida* so surprisingly it still shared

the same defect with the original HI *B. bacteriovorus* $\Delta bd0742$ mutant strain that was also unable to prey on *P. putida*. This suggest that it may not be just the simple loss of c-di-GMP levels that are important in predation when Bd0742 is absent.

Unfortunately as this was at the end of my project time did not allow me to test what was different about *P. putida* from *E.coli* and *A. baumannii*. (I tested total c-di-GMP levels in each prey strain once only-data not shown- but there was no significant difference, so more detailed studies are required).

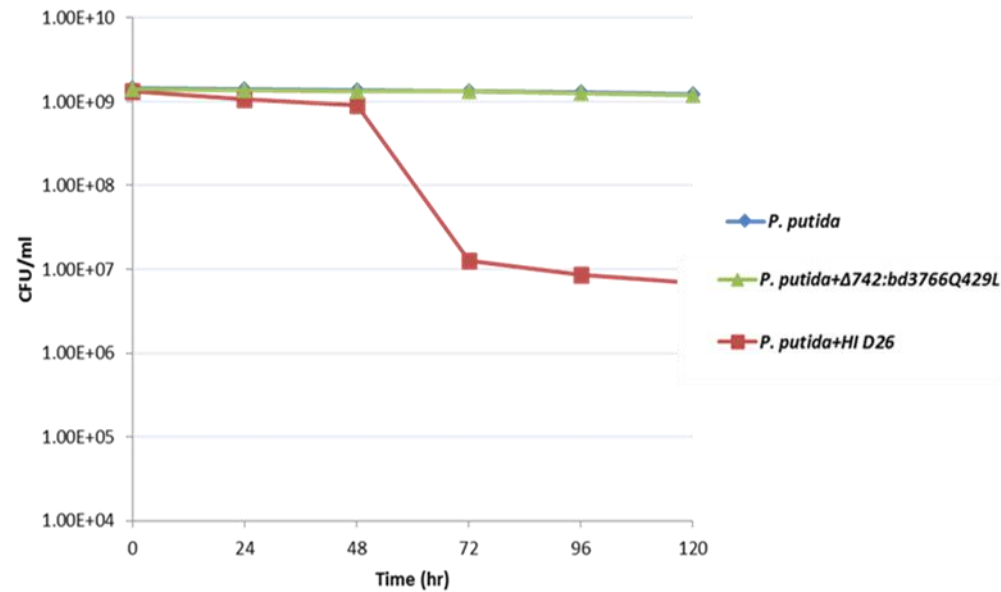
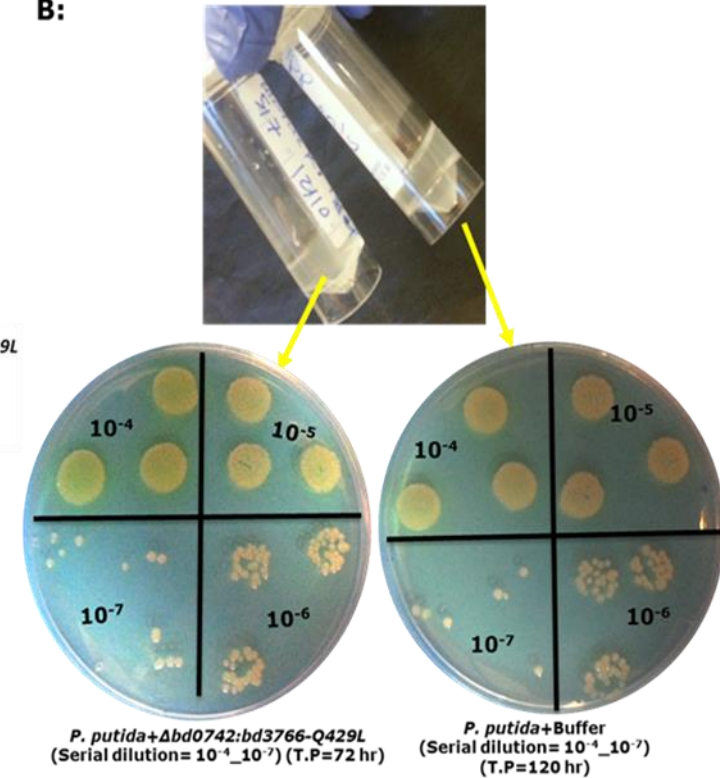
A:**B:**

Figure 7. 14: Dynamics of predation of Δ bd0742:bd3766-Q429L strain were tested against wild-type (WT) host- independent (HI) *B. bacteriovorus* (HID26) strain (the predatory control) on *P. putida* (from 3 biological repeats). (A): The Predation Δ bd0742:bd3766-Q429L strain and HID26 strain on *P. putida* prey were examined by drop in number of *P. putida* over 120 hr. In liquid cultures, HID26 started to reduce the number of during 72 hr while Δ bd0742:bd3766-Q429L strain was unable to kill *P. putida* (from three biological repeats in separate weeks) with the error bars represent 1 SD from the mean. **(B):** Tubes of *P. putida*+ buffer control and *P. putida*+ Δ bd0742:bd3766-Q429L strain infection and their serial dilution plates after 120 hrs.

7.4.5 Despite restoring *E. coli* predation in liquid, the $\Delta bd0742:bd3766Q429L$ strain did not restore the ability to produce plaques on an *E. coli* prey lawn

After finding that predatory $\Delta bd0742$ suppressor strains result from prolonged incubation of *B. bacteriovorus* $\Delta bd0742$ on liquid *E. coli* S17-1 prey, I tested the predation ability of these suppressor strains to lyse some genera of prey and found that in liquid predation assay the predation ability was restored for some strains (see section 7.4.3 and 7.4.4 in this Chapter). However, I noted that there was no plaque production on plates which was surprising, even where liquid predation occurred for some types of prey.

Therefore, one of the questions that had been raised and I needed to answer is about the ability of $\Delta bd0742:bd3766-Q429L$ suppressor strain to produce plaques on prey lawn. Here, I test the ability of $\Delta bd0742:bd3766-Q429L$ strain to produce plaques when this strain is plated on lawns of *E. coli* S17-1 and incubated at 29 °C to see if this strain able to produced plaques over extended times as seen in liquid predation by $\Delta bd0742:bd3766-Q429L$ which show when the cultures were left for more than 24 hr (Figure 7-14). Despite the extended incubation period (21 days), $\Delta bd0742:bd3766-Q429L$ strain was still unable to produce the plaques on *E.coli* prey lawns (Figure 7- 15). It has conclusively been shown that the suppression that happened in the genome of the original HI *B. bacteriovorus* $\Delta bd0742$ mutant strain that allowed $\Delta bd0742$ suppressor strains to emerge and able to restore partially the predation ability of these strains and lysing some prey types in liquid, however plaque formation ability was not restored.

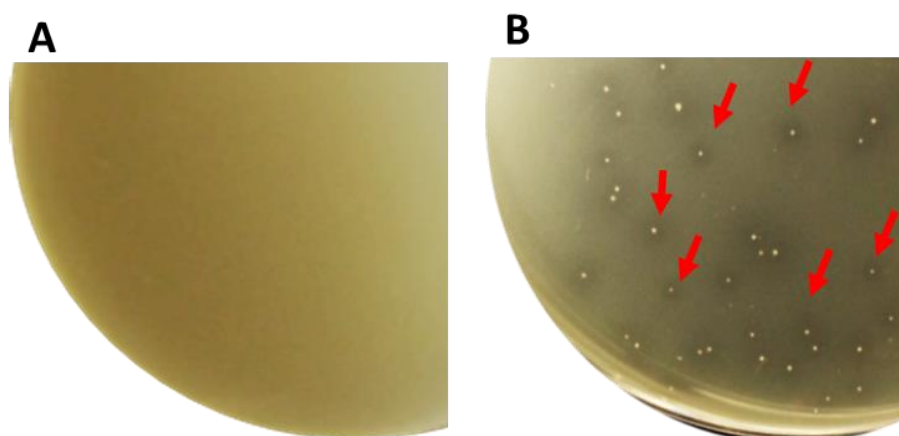


Figure 7. 15: The plaques plates assay of $\Delta bd0742:bd3766-Q429L$ strain on double layer YPSC overlay plates containing prey (A: $\Delta bd0742:bd3766-Q429L$ strain and B: HI D26). The suppression that happened in HI *B. bacteriovorus* $\Delta bd0742$ mutant strain that resulted in liquid predation of *E. coli* by suppressor strains was unable to restore the plaque formation on *E. coli* S17-1 lawns after incubation for 3 weeks compared with control strain HI D26.

7.5 Conclusions

1. I have found that $\Delta bd0742$ expressing the point mutated Bd3766*proteins such as $\Delta bd0742::bd3766-F394C-mCherry$, $\Delta bd0742::bd3766-Q429L-mCherry$ and $\Delta bd0742::bd3766-LL439L-mCherry$ were able to lyse *E. coli* pZMR100 quickly (within 72 hrs). The control strains which I used were the original HI *B. bacteriovorus* $\Delta bd0742$ mutant strain expressing as a single cross over Bd0064 or Bd3766 wild type as mCherry fusions ($\Delta bd0742::WTbd3766-mCherry$ and $\Delta bd0742::bd0064-mCherry$ strains). These two control strains exposed to *E. coli* prey which about at 96 hrs or more start lysing that prey, this must again be due to the onset of suppression in the genome of the HI *B. bacteriovorus* $\Delta bd0742$ mutant strain as it has the same 96 hrs kinetics as that original suppression-for the supplied extra gene whether Bd0064 -a non-GGDEF gene or Bd3677 a GGDEF wild type gene).

2. The motility of *E. coli* MG1655 was reduced by the suppressor point mutations in Bd03766 – but not as much by the wild type Bd3766, when plasmid expressing constructs were transformed in the *E. coli* MG1655. This means that these Bd3766* variant proteins made more c-di-GMP than Bd3766 as there is an inverse relationship between the motility of *E. coli* and the level of c-di-GMP.
3. I have found that the *Δbd0742:bd3766-Q429L suppressor* strain is now a selective predator for some type of Gram-negative prey as it is able to prey on *E. coli* but not able to prey on *P. putida*. Additional to that, I found that the point mutation made *Δbd0742:bd3766-Q429L* lyse *E. coli* within 48 hrs compared with the original HI *B. bacteriovorus Δbd0742* mutant strain that was unable to lyse *E. coli* until the compensatory point mutation in *bd3766* happened but both strains share the same inability to lyse *P. putida*.
4. I found that *Δbd0742:bd3766-Q429L* strain is unable to produce plaques on prey lawns even after longer incubation time which meant that surprisingly the point mutation in *bd03766* has a positive effect on the predation ability of the *Δbd0742 suppressor* strains in liquid cultures but not on solid surface like the prey lawn.

7.6 Footnote Experiments

7.6.1 Testing Bd3766* suppressor variants of $\Delta bd0742$ for any activity in $\Delta bd0367$ and $\Delta bd1434$ GGDEF deletion mutants

As I found in chapter three, point mutations that had occurred in the *bd3766* gene that enabled the HI *B. bacterivorius* $\Delta bd0742$ mutant strain to regain the predation ability in liquid cultures on some types of prey.

Important note: Fluorescently tagged suppressor *bd3766** constructs were made from all the $\Delta bd0742$ suppressor mutations in *bd3766* discovered in Chapter three.

Bd3766 is one of the GGDEF domain proteins in *Bdellovibrio* and all the point mutations that we have $\Delta bd0742$ suppressor strains occurred in the DNA coding for coiled coil region and transmembrane domain of Bd3766 which might indicate that these point mutations cause these Bd3766 proteins to produce more c-di-GMP which induces the predation ability of HI *B. bacteriovorus* $\Delta bd0742$ mutant strain (See overall discussion for model).

Because I thought that the point mutation in Bd3766 will make the *Bdellovibrio* produce more c-di-GMP so adding an extra copy of the point mutation Bd3766* variants might have the same general effect that happened in the case of the *bd0742* deletion strain for the other GGDEF mutants ($\Delta bd1434$ and $\Delta bd0367$ strains). Therefore testing the effect of adding an extra copy of *bd3766-Q429L-mCherry*, (a representative Bd3766* strain), in the original $\Delta bd1434$ mutant and $\Delta bd0367$ mutant strains is important to see if it helps to restore the ability of the original $\Delta bd1434$ mutant strain to grow as a host- independent colonies on SPY agar, and enables $\Delta bd0367$ mutant strain to escape from residual of the prey cell within the normal time range of the wild type and restore predatory ability.

7.6.2 Specific Research Aims

1. To investigate the effect of adding an extra copy of *bd3766-Q429L-mCherry* to other GGDEF deletion strains namely the original $\Delta bd1434$ mutant strain and test if this point mutation able to restore the ability of $\Delta bd1434$ mutant and grow as a Host- Independent culture.
2. To investigate the effect of adding an extra copy of *bd3766-Q429L-mCherry* to other GGDEF deletion strains namely the original $\Delta bd0367$ mutant strain and test if this point mutation enable the $\Delta bd0367$ mutant to finish the predatory cycle and escape after septation from *E. coli* within the normal range of the wild type HID26 the control strain.

7.6.3 Expected outcome

1. Addition of *bd3766-Q429L-mCherry* to the $\Delta bd1434$ mutant strain will restore the ability of $\Delta bd1434$ mutant to grow as a Host- Independent cells.
2. Addition of *bd3766-Q429L-mCherry* to $\Delta bd0367$ mutant strain will restore the ability of $\Delta bd0367$ mutant strain to finish the predatory cycle and escape after septation from *E. coli* prey within the normal range of the wild-type HID26 control strain.

7.6.4 Results:

7.6.4.1 The effect of an additional copy of *bd3766-Q429L-mCherry* supplied to $\Delta bd1434$ mutant strain

Bd0742 is not the only the active GGDEF protein in *B. bacteriovorus*, there is also Bd1434 (DgcC) whose deletion results in loss of ability of *B. bacteriovorus* to grow as a host-dependent culture. In order to clarify if the point mutation in Bd3766-Q429L (transmembrane region) has an effect on the $\Delta bd1434$ mutant strain and if this point mutation play rescue role with $\Delta bd1434$ mutant strain, for that reason, wild type WTbd3776-mCherry and Bd3776Q429L-mCherry constructs were generated and conjugated into *B. bacteriovorus* $\Delta bd1434$ original mutant strain by single crossover with maintenance by of kanamycin selection for the pK18mobsacB single cross over plasmid. The resultant plaques from these conjugations were grown under standard conditions for 24 hr. After that *B. bacteriovorus* $\Delta bd1434::WTbd3766-mCherry$ and *B. bacteriovorus* $\Delta bd1434::bd3766-Q429L-mCherry$ strains were tested for ability to grow as host-independently (HI) with using *B. bacteriovorus* HD100 and $\Delta bd1434$ mutant strain (this strain was unable to grow host- independently and grow only as a HD culture) as a controls.

As shown in Figure (7-16) HI *B. bacteriovorus* HD100 was able to form host-independent (HI) colonies (2.5×10^7 cells applied) after the 10 days incubation and $\Delta bd1434$ mutant strain was unable to form the HI colonies (4.3×10^{10} cells applied) even the plates left in incubator for 20 days, while *B. bacteriovorus* $\Delta bd1434::WTbd3766-mCherry$ and *B. bacteriovorus* $\Delta bd1434::bd3766-Q429L-mCherry$ strains HI colonies took about 16 days to form colonies (2.5×10^7 and 2.1×10^7 cells applied respectively). Their colonies were smaller than the HI *B. bacteriovorus* HD100 colonies and these colonies resulted from the extra copy of WTbd3766-mCherry or bd3766-Q429L-mCherry added to genome of *Bdellovibrio*. This indicated that the point mutation in *bd3766* (Q429L) has an effect on the $\Delta bd1434$ mutant and restore ability to form HI colonies but this results simply

from the extra copy of *bd3766* (either wild type or point mutant versions). So for the $\Delta bd1434$ mutant, in contrast to the $\Delta bd0742$ mutant strain, adding the gene with the point mutations in *Bd3766* has no different effect to wild type *Bd3766* on the ability of restoring HI $\Delta bd1434$ mutant strain (Figure 7-16). The likely reason for this is in the discussion.

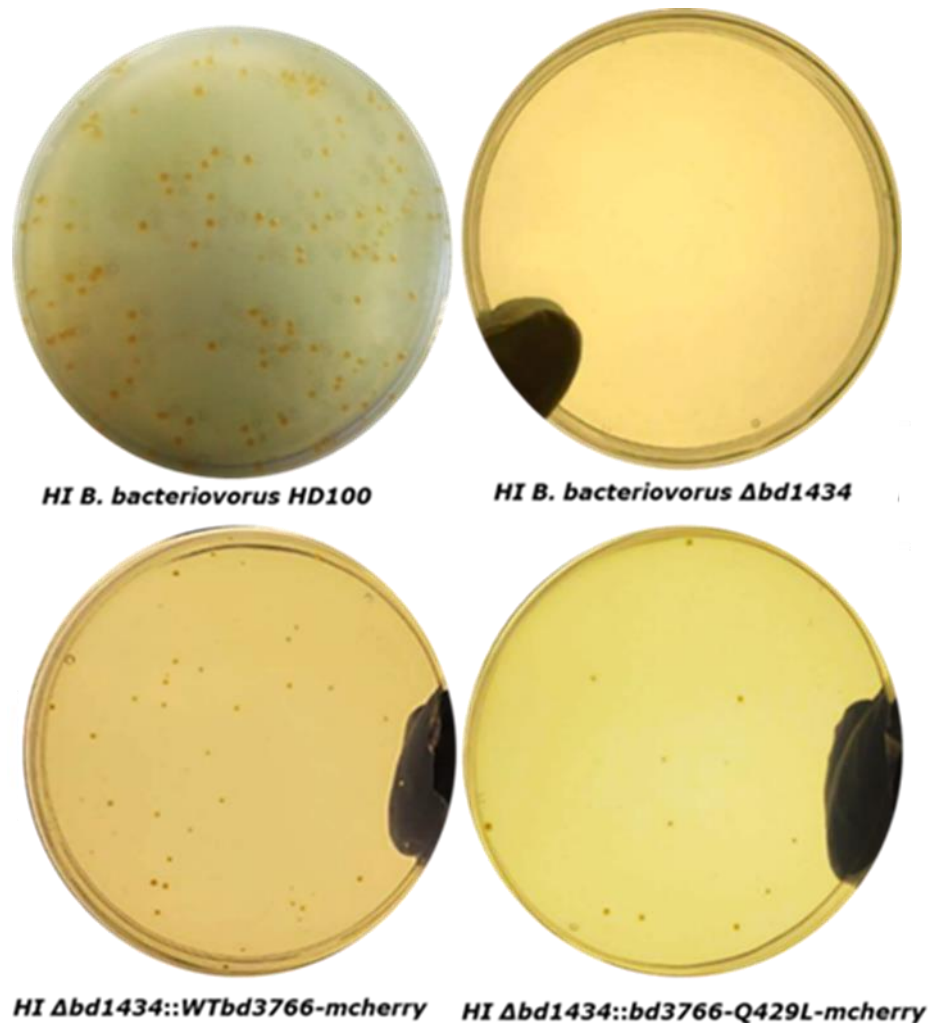


Figure 7. 16: The axenic growth of HI *B. bacteriovorus* HD100, *B. bacteriovorus* $\Delta bd1434$, *B. bacteriovorus* $\Delta bd1434::WTbd3766$ -mCherry and *B. bacteriovorus* $\Delta bd1434::bd3766$ -Q429L-mCherry strains on SPY agar plates. HI colonies of *B. bacteriovorus* HD100 take approximately ten days to form. In contrast, *B. bacteriovorus* $\Delta bd1434$ was unable to form colonies on SPY agar plates after 20 days. The addition of an extra copy of either *WTbd3766*-mCherry or *bd3766*-Q429L-mCherry to *B. bacteriovorus* $\Delta bd1434$ resulted in growth of HI colonies on SPY agar over a period of approximately sixteen days.

As expected both HI *B. bacteriovorus* $\Delta bd1434::WTBd3766-mCherry$ and HI *B. bacteriovorus* $\Delta bd1434::bd3766-Q429L-mCherry$ strains showed faint fluorescence in all cell morphotypes with foci of bright fluorescence within the HI cells. The morphology of both of the strains showed no obvious differences between them (Figure 7-17). Because of the diverse morphology of the HI cells it is impossible to predict if the bright poles were potential predatory poles.

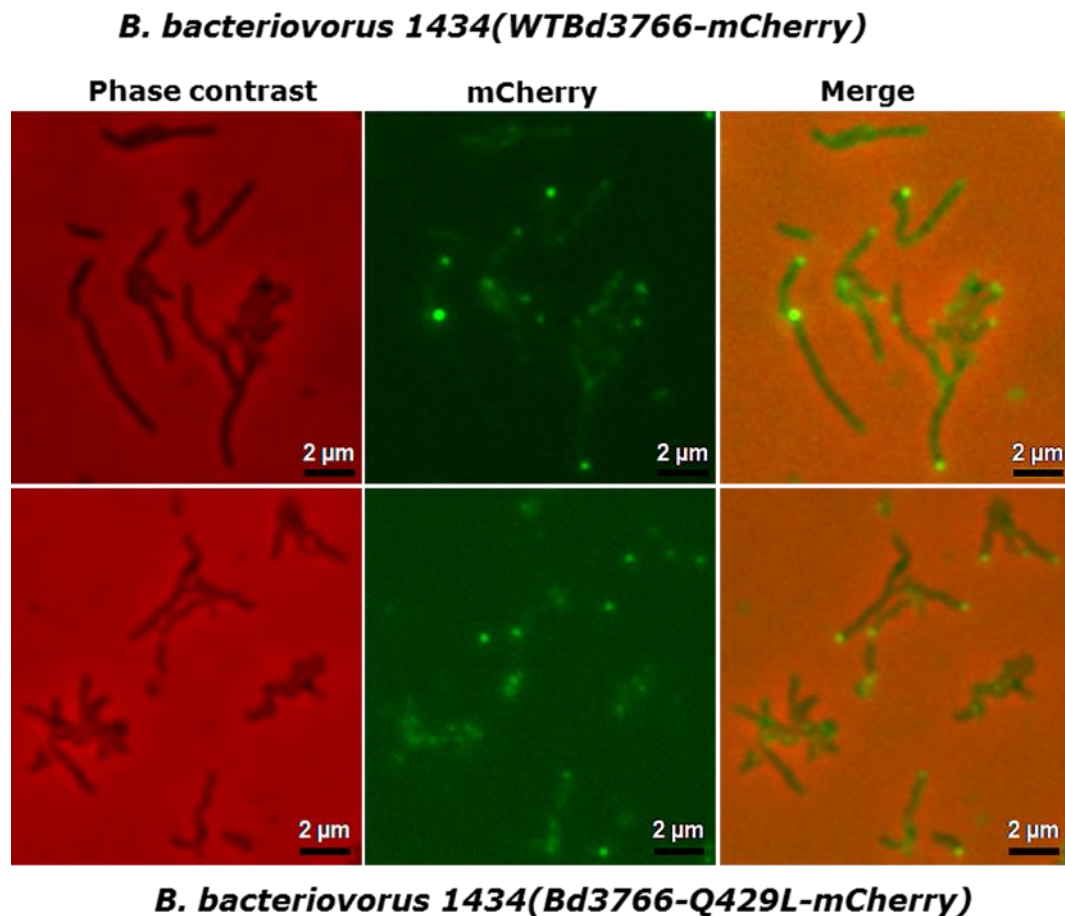


Figure 7. 17: Images of HI *B. bacteriovorus* 1434::*WTBd3766-mCherry* and HI *B. bacteriovorus* 1434::*bd3766-Q429L-mCherry* strains. Both of the strains expressed faint fluorescence in host- independent growth phase in all cell morphotypes with foci of bright fluorescence within the HI cells. The images were all taken with 1.7 second fluorescent exposure and maximum gain.

7.6.4.2 Testing the effect of addition extra copy of the *bd3766* and *bd3766-Q429L-mcherry* in $\Delta bd0367$ mutant strain

To investigate if the point mutation in *Bd3766-Q429L* (transmembrane region) has an effect on the other GGDEF mutant and if this point mutation played compensatory role with another GGDEF domains mutant strain in *Bdellovibrio*, in this case the $\Delta bd0367$ mutant strain, wild type WT*Bd3776-mCherry* and *Bd3776Q429L-mCherry* constructs were generated and conjugated into HI *B. bacteriovorus* $\Delta bd0367$ original mutant strain ($\Delta bd0367$ mutant isolated as a HI and cannot grow as a HD) by single crossover with maintenance by of kanamycin selection. After the HI colonies of both HI *B. bacteriovorus* $\Delta bd0367::WTbd3766-mCherry$ and HI *B. bacteriovorus* $\Delta bd0367::bd3766-Q429L-mCherry$ strain were grown on SPY plates, they were picked in to PY broth with kanamycin to keep plasmid selection. The experiments were carried out along with HI *B. bacteriovorus* *bd0064-mCherry* strain that was used as possible wild-type control which should escape bdelloplasts, but it did not turn out to be ideal. Fluorescent images were taken at specific time points: 1hr, 4 hrs and 9 hrs to see if the HI *B. bacteriovorus* $\Delta bd0367::WTbd3766-mCherry$ and HI *B. bacteriovorus* $\Delta bd0367::bd3766-Q429L-mCherry$ were able to finish the predatory cycle. Maximilian Harris a master student in Sockett laboratory found previously that $\Delta bd0367$ mutant strain was unable to exit from the bdelloplast, even after 19 hours (Hobley et al. 2012). Although, it is very hard to quantify for HI cultures during test predations, predation assays were still performed.

The images were taken 1hr after the start of the infection revealed that the control strain HI *B. bacteriovorus* *bd0064-mCherry* had a lot of autobdelloplasts which makes accurate measurement difficult, and fewer *Bdellovibrio* attaching to prey cells (Figure 7-18 A). The majority of HI *B. bacteriovorus* $\Delta bd0367::WTbd3766-mCherry$ and HI *B. bacteriovorus* $\Delta bd0367::bd3766-Q429L-mCherry$ were attached to prey cells and more formed bdelloplasts (Figure 4-18 B and C). This was unexpected but time did not allow further controls to be tested. As $\Delta bd0367$

strain was Kanamycin sensitive, therefore a parallel test could not be performed with these Kanamycin resistant strains as Kanamycin selection can delay predatory processes. Bd0367 has previously been phenotype as not completing predation (Hobley et al. 2012).

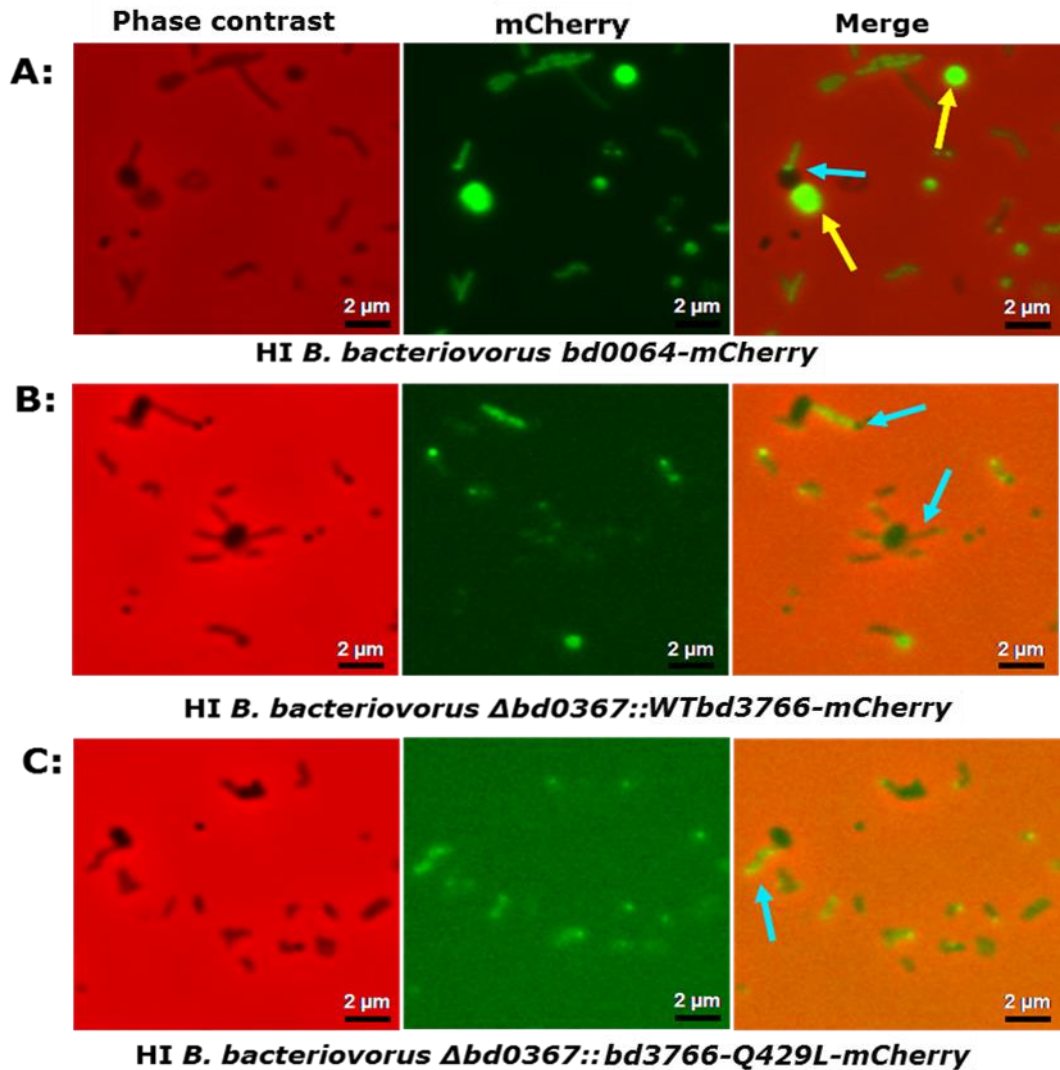


Figure 7. 18: Images from the qualitative examination the HI predation assays taken at 1 hr on *E. coli* pZmr100 prey of: A: HI *B. Bacteriovorus* *bd0064-mCherry*, B: HI *B. bacteriovorus* $\Delta bd0367::WTbd3766-mCherry$ C: HI *B. bacteriovorus* $\Delta bd0367::bd3766-Q429L-mCherry$. HI *B. Bacteriovorus* *bd0064* has a lot of autobdelloplast (yellow arrows) and low number had attached to prey cells (blue arrows). In contrast to that, HI *B. bacteriovorus* $\Delta bd0367::WTbd3766-mCherry$ and HI *B. bacteriovorus* $\Delta bd0367::bd3766-Q429L-mCherry$ had a lot of attached to prey cells (blue arrows).

The images taken at 4 hrs, from the beginning of the experiment showed that there were a lot of true bdelloplasts (grey arrows) seen in the culture of HI *B. bacteriovorus* *bd0064*-mCherry with a lot of the autobdelloplast (yellow arrows) (Figure 4-19 A) whereas, both HI *B. bacteriovorus* $\Delta bd0367::WTbd3766$ -mCherry and HI *B. bacteriovorus* $\Delta bd0367::bd3766$ -Q429L-mCherry form a lot of bdelloplast (grey arrows) (Figure 4-19 B and C).

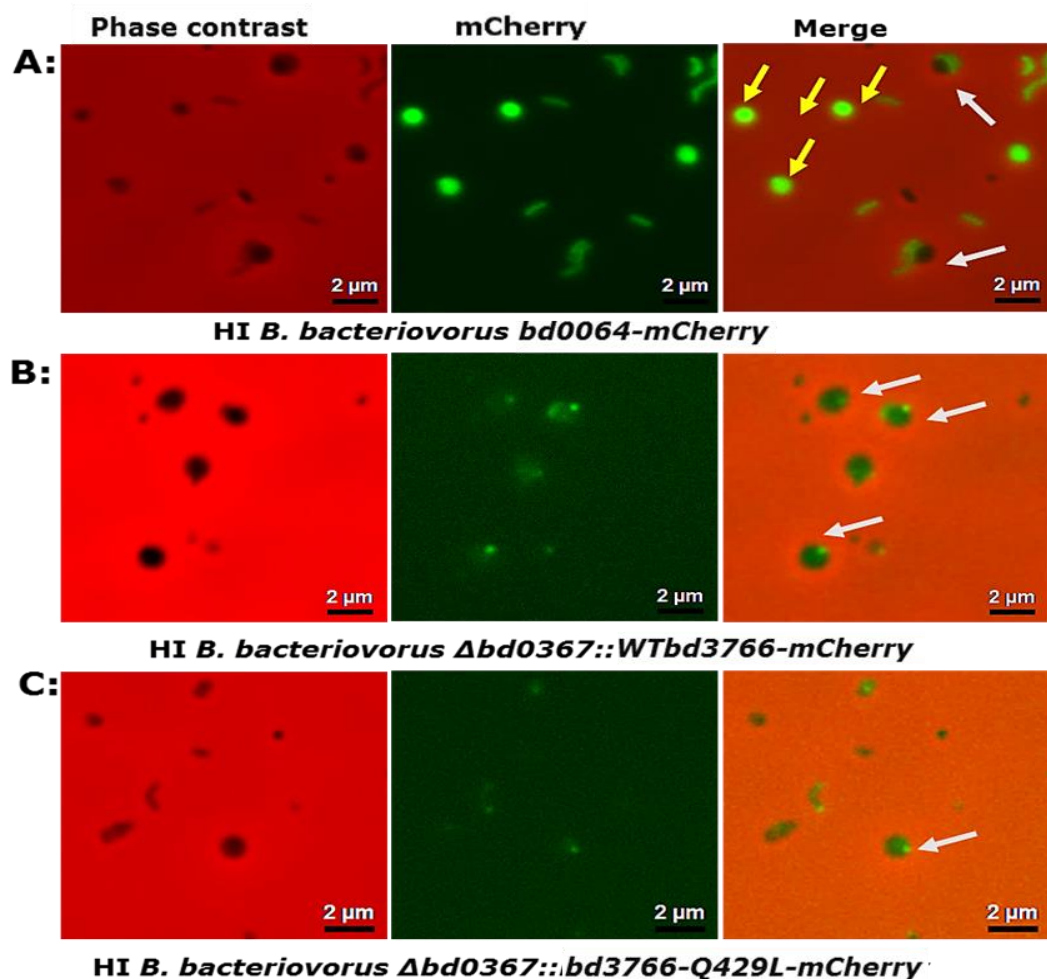


Figure 7. 19: Images from the qualitative examination the HI predation assays taken at 4 hrs on *E. coli* pZmr100 prey of A: HI *B. Bacteriovorus* *bd0064*-mCherry, B: HI *B. bacteriovorus* $\Delta bd0367::WTbd3766$ -mCherry C: HI *B. bacteriovorus* $\Delta bd0367::bd3766$ -Q429L-mCherry. HI *B. Bacteriovorus* *bd0064* has a lot of autobdelloplast (yellow arrows) and a lot of bdelloplast formed (grey arrows). In contrast to that, HI *B. bacteriovorus* $\Delta bd0367::WTbd3766$ -mCherry and HI *B. bacteriovorus* $\Delta bd0367::bd3766$ -Q429L-mCherry had a lot of bdelloplast formed (grey arrows).

At 9 hrs after the beginning of the experiment, images presented that there were a lot of bdelloplast visible (grey arrows) in the culture of HI *B. bacteriovorus* *bd0064-mCherry* in addition to a lot of the autobdelloplasts (yellow arrows) (Figure 7-20 A), both HI *B. bacteriovorus* $\Delta bd0367::WTbd3766-mCherry$ and HI *B. bacteriovorus* $\Delta bd0367::bd3766-Q429L-mCherry$ cultures had cleared the prey cells with new progeny of *Bdellovibrio* released (white arrows) (Figure 7-20 B and C). It was very difficult to do this experiment accurately, as the cultures of the HI *B. bacteriovorus* *bd0064-mCherry* strain contain a lot of the autobdelloplasts. Therefore the ratio of the attack phase cells to prey cells at the start of the experiment will not be equal that found in both HI *B. bacteriovorus* $\Delta bd0367::WTbd3766-mCherry$ and HI *B. bacteriovorus* $\Delta bd0367::bd3766-Q429L-mcherry$, and these may increase by early autobdelloplasting. Despite this caveat, the early finishing of the predatory cycle and clearance of prey that seen in both HI *B. bacteriovorus* $\Delta bd0367::WTbd3766-mCherry$ and HI *B. bacteriovorus* $\Delta bd0367::bd3766-Q429L-mCherry$ may result solely from the extra copy of *WTbd3766-mCherry* and *bd3766-Q429L-mCherry* and does not rely on mutational change in *Bd3766*.

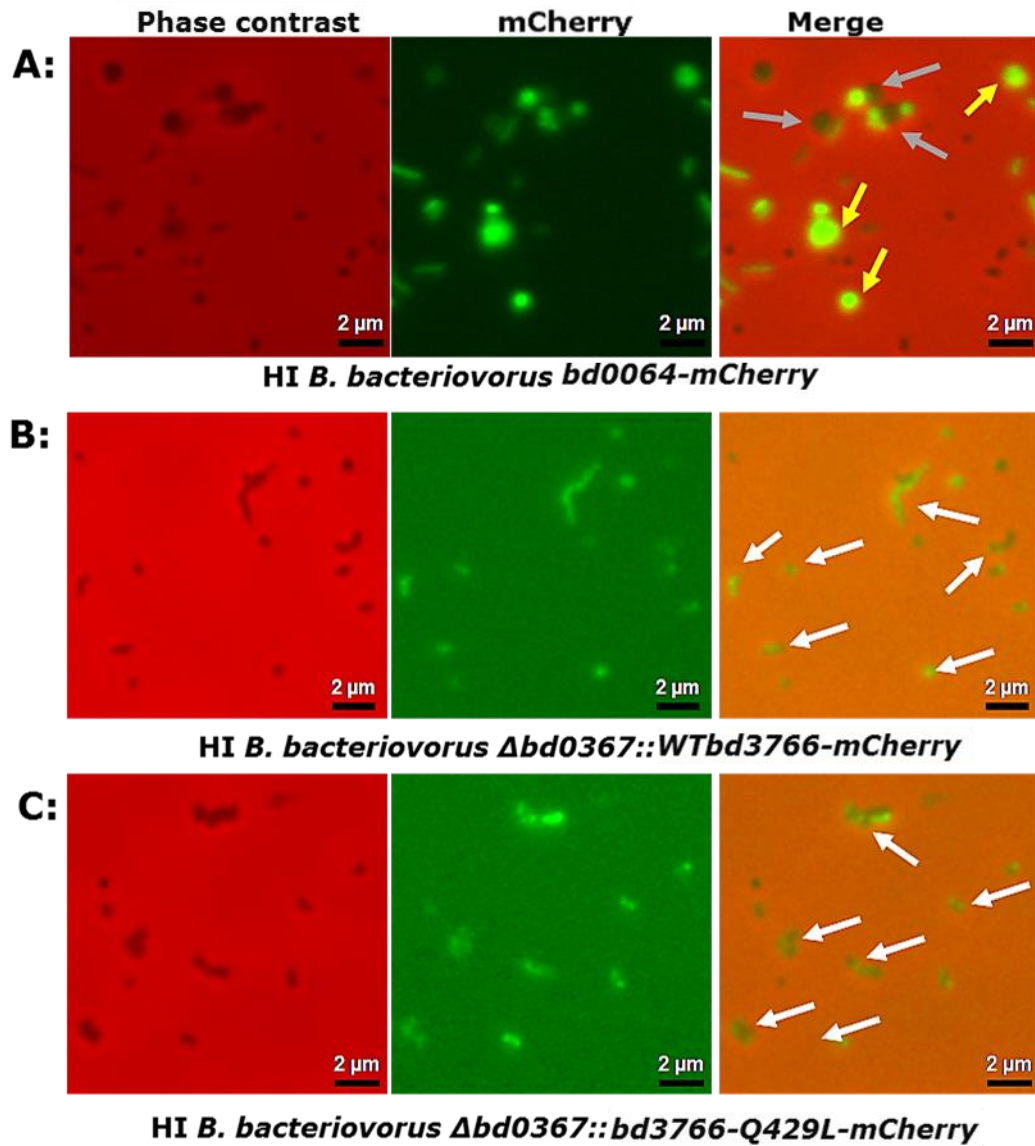


Figure 7. 20: Images from the qualitative examination the HI predation assays taken at 9 hrs on *E. coli* pZmr100 prey of A: HI *B. Bacteriovorus* *bd0064-mCherry*, B: HI *B. bacteriovorus* $\Delta bd0367::WTbd3766-mCherry$ C: HI *B. bacteriovorus* $\Delta bd0367::bd3766-Q429L-mCherry$. HI *B. Bacteriovorus* *Bd0064* has a lot of autobdelloplast (yellow arrows) and with a lot of Bdelloplast still last (grey arrows). In compare to, HI *B. bacteriovorus* $\Delta bd0367::WTbd3766-mCherry$ and HI *B. bacteriovorus* $\Delta bd0367::bd3766-Q429L-mCherry$ had a lot of free exit new progeny with absences of the bdelloplast (white arrows).

7.6.5 Conclusions

1. The ability of the original $\Delta bd1434$ mutant strain to grow as a HI resulted from adding extra copy of WT *bd3766-mCherry* or *bd376-Q429L-mCherry* to the $\Delta bd1434$ mutant strain which mean that extra copies of either WT *bd3766* or *bd376Q429L* were able to suppress the phenotype that was produced by deletion of *bd1434*. Therefore, the *point mutations in *Bd3766* are specific for suppression of $\Delta bd0742$.
2. The ability of $\Delta bd0367$ WT*bd3766-mCherry* and $\Delta bd0367$ *bd376Q429L-mCherry* strain to finish the predatory cycle and exit from prey cells also seemed to result from adding extra copy of WT*bd3766* or *bd376Q429L-mCherry* to $\Delta bd0367$ mutant strain which indicates again that extra copies of either WT *bd3766* or *bd376Q429L-mCherry* was able to suppress the phenotype that was resulted from deletion of $\Delta bd0367$. For that reason, the *point mutations in *Bd3766* are specific for suppression of $\Delta bd0742$.

The reasons why these *Bd3766** point mutations may be needed to achieve suppression in $\Delta bd0742$ is explained in the overall discussion.

7.7 Discussion

7.7.1 Does the point mutation in *bd3766* gene make Δ *bd0742* suppressor strain to be a selective predator?

Bd3766 is unique to the *B. bacteriovorus* HD100 genome and not found in *B. bacteriovorus* Tiberius genome while the other four GGDEF domain proteins in *B. bacteriovorus* are found also in *B. bacteriovorus* Tiberius genome. The gene product of *bd3766*, unlike other GGDEF genes, is an unusual GGDEF protein differing from other GGDEF proteins in *Bdellovibrio* by having additional to the GGDEF domain, four TPR and two transmembrane domains that might play a role in expressing "extra" diffusible c-di-GMP (see Section 7.4.2), in some unknown physiological conditions, possibly requiring another factor to bind to the TPR domains (see overall discussion). However as found here in studying the deletion of the *bd0742* gene, point mutations were needed in Bd3766 for it to work in the liquid predation suppression. The deletion of *bd3766* did not result in an obvious phenotype in HD and HI lifestyles so this suggests that *bd3766* is one of the genes that was obtained in more recent times by lateral transfer into the HD100 genome (Hobley et al. 2012).

This could mean that Bd3766* proteins work as a contingency for Bd0742 in the Δ *bd0742* suppressor strain and HI *B. bacteriovorus* Δ *bd0742* mutant strain as the loss of the *bd0742* gene encoded protein is a lower in the concentration of c-di-GMP and Bd3766* production of c-di-GMP increase the levels and thus the c-di-GMP signalled predatory ability of *Bdellovibrio* is partially tested so it is able to prey on *E. coli*. Solano et al. (2009) found that deletion of all 12 GGDEF genes from *S. enteritidis* resulted in a strain unable to synthesise c-di-GMP and complementation of *stm4551* (one of the 12 GGDEF genes in *S. enteritidis*) was able to recover the most of the phenotypes produced by deletion of GGDEF genes except cellulose production.

For an unknown reason these compensatory point mutations in Bd3766 happened only in HI *B. bacteriovorus* $\Delta bd0742$ mutant strain when grown for prolonged time on *E. coli* or *Acinetobacter*, but did happen with *P. putida* as prey. It could be something different present in the cell envelope of *P. putida* versus *E. coli*/*Acinetobacter* or content of amino acid in wall of these prey or possibly some enzymes secreted from by *P. putida* that might be prevent the natural selection for the Bd3766 point mutations.

7.7.2 Point mutations in Bd3766* negatively regulated the swimming motility of *E. coli* MG1655

One of the survival mechanism in many Gram-negative bacteria is swimming motility, and this motility is often suppressed by the high level of c-di-GMP (Hengge 2009, Romling et al. 2013). The level of c-di-GMP affects *E. coli* motility through binding to *E. coli* PilZ protein (YcgR) that in turn binds to flagellar motor and braks its rotation and this is seen in swarm size change on soft agar (Fang and Gomelsky 2010, Paul et al. 2010).

The point mutation in Bd3766* reduces the swimming motility of *E. coli* MG1655 (Figure 7-11) presumably by more c-di-GMP being made and binding to the YcgR PilZ protein that binds at the *E. coli* flagella motors and results in slowing down flagellar rotation. The only cause of this slowdown of swimming motility of *E. coli* MG1655 were the point mutations in the Bd3766 coiled coil region (F394C) and transmembrane domain (Q429L and LL439L) so if these point mutation produce low c-di-GMP, the result is normal flagellar rotation, while if these point mutations produce more c-di-GMP, the result is slow flagellar rotation that slows down swimming motility. The results suggest that Bd3766 point mutations have a role in motility attenuation by Bd3766-F395C, Bd3766-Q429L and Bd3766-LL439L compared to WT Bd3766 in *E. coli* MG1655 and these Bd3766* point mutations produce more c-di-GMP which affects *E. coli* MG1655 motility. Why Bd3766 does

not produce as much c-di-GMP as the point mutant forms when expressed alone in *E.coli* is mentioned in the general discussion.

7.7.3 Unexpectedly point mutations in *bd3766* do not restore the ability of $\Delta bd0742$ suppressor strain to produce plaques on plates

Hobley and co-worker previously pointed out that the deletion of *bd0742* led to a loss of liquid predation and plaque formation ability (Hobley et al. 2012). The point mutations that occurred in *bd3766**s restored the predation ability of the $\Delta bd0742$ suppressor strain in liquid cultures, but it made this strain a selective predator for some Gram-negative bacteria. The point mutation that occur in *bd3766* however did not restore the plaque formation ability for the $\Delta bd0742$ suppressor strain even after long incubation periods. The possible reason for this finding could be that the osmolarity on the solid surface is higher than in liquid culture and the *bd3766* point mutations were selected to let *Bdellovibrio* to survive in liquid culture. Another possible explanation for this finding is that this strain might have lost their ability to glide on the solid surface due to deletion of the *bd0742* so they are unable to glide out of the prey cell and exit and start the cycle again and infect a new prey cells in lawn plate.

7.7.4 *Bd3766 point mutants work as a contingency for *Bd0742* but extra copies of WT *Bd3766* or *Bd3766** work for two other GGDEF mutants in different physiological growth states.**

Deletion of *bd1434* and *bd0367* resulted in strains unable to grow as a Host-independent for *bd1434* and unable to finish predatory cycle (after predatory growth) and escape prey like the wild-type HI *Bdellovibrio*. As found in section (3.6.2 and 3.6.3), point mutations in *bd3766* were able to suppress partially the phenotype that results from deletion of *bd0742* and the predation ability was restored for some types of prey. Addition of an extra copy of the wild-type *bd3766* or *bd3766**point mutation to the original $\Delta bd1434$ mutant and $\Delta bd0367$ mutant strain were able to suppress the phenotypes that result from deletion of *bd1434*

and *bd0367* genes. This clearly demonstrated that these two phenotypes resulting from deletion of *bd1434* and *bd0367* can be compensated by adding extra copies of WT *bd3766* or *bd3766**. This means that the point mutations in *bd3766** are specific for Bd0742 only but not for other GGDEF proteins. The possible explanation for this situation that Bd3766 has the natural ability to produce c-di-GMP as an emergency response to keep *Bdellovibrio* surviving via TPR regions binding a signal (unknown) changing transmembrane and coiled coil regions of Bd3766 shape to activate the GGDEF activity (see general discussion) Here for Bd0742 point mutations in *bd3766* occur and bypass this normal signal based process thus producing more c-di-GMP without TPR being activated. The Bd3766 signal-based activation in different growth conditions was tested for the three GGDEF mutant suppression experiments so the Bd3766 activation was not the same in all cases (see overall discussion).

7.8 Future work

1. Investigating the gliding ability of *Δbd0742* suppressor strain to understand if gliding defect is the reason why this strain is unable to produce plaques on the prey lawn.
2. Investigate what the signal is that activates Bd3766 to make c-di-GMP- is it a protein or other type of signal?
3. Investigate the effect of the addition of an external (chemical) source of c-di-GMP for predation assay and see if this addition will make predation happen earlier than previous predation assays.
4. Investigate outer membrane content differences of the types of the prey used in this study to realise if differences in them can be a reason for make *Δ bd0742* suppressor selective predators for some type of prey (This is difficult to do but 5. may help).
5. Prepare vesicles from *E. coli* membrane and add them to *P. putida* culture and see if predation of *P. putida* can happen.
6. Gliding motility videos are require to see if the HI *B. bacteriovorus* *Δbd0367::WTbd3766-mCherry* and HI *B. bacteriovorus* *Δbd0367::Bd3766-Q429L-mCherry* strain are able to escape within the normal time range of the *HID13* control.

8 Chapter Eight: Investigation the role of CpoB in peptidoglycan modification during *Bdellovibrio* invasion and sealing of a prey entry pore

8.1 Introduction

Earlier in this study, the possibility of cytoplasmic synthesis of a cell wall component- DAP, and its involvement in prey invasion was investigated. It was found that this is not the direct role of Bd134 (ASKa) nor the DAP synthesis pathway proteins DapE and DapB. Towards the end of the study, Sam Greenwood, a Masters student in the Sockett laboratory found evidence for an unusual location of a periplasmic, peptidoglycan-synthesis-regulatory protein in *Bdellovibrio*. This regulates penicillin-binding proteins in other bacteria and it uses DAP-containing peptidoglycan (PG) units to build new wall during invasion of the prey and formation of the bdelloplasts.

8.2 Penicillin-binding proteins (PBPs)

Penicillin-binding proteins (PBPs) are (usually) membrane-associated macromolecules which play vital roles in the cell wall synthesis process, and they are found in all peptidoglycan-containing organisms (Macheboeuf et al. 2006). PBPs belong to the family of acyl-serine transferase enzymes and are all inhibited by the β -lactam antibiotics (such as penicillins), which bind to their active sites (Sauvage et al. 2008, Sauvage et al. 2008). PBPs can be divided into two type: High molecular mass (HMM) PBPs and Low molecular mass (LMM) PBPs according to their molecular weights. As most studies of this family of protein have focussed on *E. coli*, the initial focus will be on introducing *E. coli* PBPs.

High molecular mass (HMM) PBPs are peptidoglycan synthases responsible for peptidoglycan polymerization and addition into the previous cell wall , and they include PBP1A, PBP1B, PBP2, PBP3 while low molecular mass (LMM) PBPs are involved in peptidoglycan hydrolysis, and PBP4, PBP5, PBP6, PBP7 (Goffin and Ghuysen 1998, Ghosh et al. 2008). HMM PBPs are divided into two classes

depending on the ability of the single polypeptide to catalyse different reactions: Class A (Bifunctional) are able to catalyse the N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) and cross-linking of adjacent stem peptide, so they have glycosyltransferase (CTase) and transpeptidases (TPase) activities. The class B (Monofunctional) have only the transpeptidase activity (Goffin & Ghuysen, 2002). *In vivo*, LMM PBPs act either in DD-endopeptidation (responsible for D-Ala-mDAP cross-links) like PBP7 in *E. coli* or DD-carboxypeptidation (responsible for removing the terminal D-Ala of the peptidoglycan) like PBP5 in *E. coli*. Additional to that, there is some LMM-PBPs which have DD-endopeptidase and DD-carboxypeptidase activity like PBP4 (Ghosh et al. 2008).

8.2.1 Penicillin-binding protein 1

Penicillin-binding proteins 1 are one of the high molecular mass (HMM) PBPs and they are divided into two type; PBP1A and PBP1B. Both of them are bifunctional enzymes that have transpeptidase (TPase) and glycosyltransferase (CTase) activities (Goffin and Ghuysen 2002). PBP 1A consist of one transmembrane domain (TM), glycosyltransferase (GTase) domain and non-catalytic ODD (outer membrane docking domain) domain and transpeptidases domain, while PBP1B consists of the same domains in PBP 1A but has UB2H (UvrB domain 2 homolog) domain instead of ODD before the TGase and TPase domains (Figure 8-1) (Typas et al. 2011).

Bertsche et al. (2005) and Born et al. (2006) found that both PBP1A and PBP1B of *E. coli in vitro* were able to synthesise peptidoglycan by polymerizing lipid II by transpeptidases and glycosyltransferase assay from radiolabelled lipid II. The peptidoglycan (PG) that is synthesised by PBP1A has glycan strands approximately 20 disaccharide units in length with approximately 18–26% of peptides present in cross-links (Born et al. 2006). In contrast, PBP1B synthesise peptidoglycan

containing glycan strands that have more than 25 disaccharide units in length with approximately 50% of the peptides present in cross-links (Bertsche et al. 2005).

PBP1A and PBP1B are mainly involved in cell elongation and cell division respectively in non- predatory bacteria like *E. coli* and both of them are anchored in the inner membrane. PBP1A interacts with LpoA and PBP1B interacts with PBP3, FtsN, MipA and LpoB (Typas et al. 2011). These interactions have been shown to be required for cell division associated role of PBP1B.

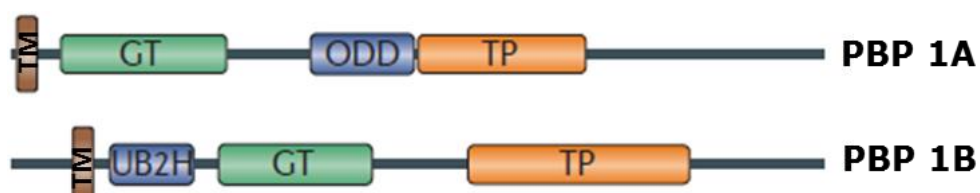


Figure 8. 1 Schematic representation of conserved TGase and TPase domains in PBP1A and PBP1B of *E. coli* with newly evolved ODD an UB2H domains in PBP1A and PBP1B respectively. Transmembrane domains are a green colour, Glycosyltransferase (GT) domain are a green colour box. Transpeptidase (TP) domains are the peach colour box .ODD and UB2H dominos are purple colour (Typas et al. 2012).

The deletion of either one of the genes encoding PBP1A or PBP1B protein in *E. coli* resulted in mild phenotype in growing cells, but deletion of genes encoding both of PBP1A and PBP1B together were lethal for *E. coli* (Denome et al. 1999, Banzhaf et al. 2012). A similar phenomenon in *Streptococcus pneumoniae* was noticed when either PBP1A or PBP1A was deleted, the cells able to tolerate that deletion while deletion of both genes (encoding both of PBP1A and PBP1B) produce a strain with abnormal septum positioning (Hoskins et al., 1999; Paik et al., 1999).

8.3 Outer membrane proteins (Lpo) are vital for PBP1A and PBP1B function in *E. coli*

In *E. coli*, the roles of PBP1A and PBP1B in the cell required the presence of cognate outer membrane lipoproteins. There are two outer membrane proteins: LpoA and LpoB which are vital for PBP1A and PBP1B activities, respectively (Paradis-Bleau et al. 2010, Typas et al. 2010). In *E. coli*, the PBP1B and LpoB complex is needed to recover the peptidoglycan after lysozyme-induced spheroplast formation (Ranjit and Young 2013).

The LpoB directly interacts with PBP1B through a specific small non-catalytic domain that is located before the GTase and TPase domains whereas, the LpoA interaction with PBP1A happens at a specific small non-catalytic domain that is situated between the GTase and TPase domains, called ODD (Figure 8-1). PBP1A and LpoA complex localise to the side wall, and PBP1B and LpoB complex also localises at the division site during septation, and the localisation of LpoB requires the activity of PBP3 (Typas et al. 2010). In low pH media with presence of the LpoA and LpoB the cognate partner of both PBP1A and PBP1B exhibit carboxypeptidase (CPase) activity (ability to hydrolyse the terminal D-alanine residue of the pentapeptide stem through DD-carboxypeptidase activity) (Egan et al. 2015).

In an *E. coli* strain that lacks PBP1B or LpoB, the overexpression of isolated ODD resulted in cell death by cell lysis and this indicated that the expressed ODD domain enter in competition with PBP1A in order to bind LpoA (Typas et al. 2010). The activity of both PBP1A and PBP1B are restricted by their interactions with the outer membrane activators LpoA and LpoB respectively. The interaction of LpoA with PBP1A stimulates the TPase activity of PBP1A and increases the rate of the cross-linking by 20% in the peptidoglycan product (Typas et al. 2010) with increase in D-Ala incorporation six fold that is accompanied by 1.5 fold increase in GTase activity (Lupoli et al. 2014). LpoA has two-domains, a N-terminal

tetratricopeptide repeat (TPR) domain and a C-terminal domain and in vivo the presence of the C-terminal domain is enough to bind and activate PBP1A (Typas et al. 2010, Jean et al. 2014).

In contrast, the interaction of LopB with PBP1B led to stimulating both TPase activity and GTase activity (Eight-fold increase) of PBP1B with a reduction in the glycan chain length. In addition, there is an increase in the rate of cross-linking by 20% in peptidoglycan product with an increase in D-Ala incorporation rate by 1.5 fold (Egan et al. 2014, Lupoli et al. 2014). *E. coli* cell growth require of PBP1A-LpoA or PBP1B-LpoB complex and the deletion of one of the *lpo* genes in the absence of the PBP1A or PBP1B led to *E. coli* lysis that is accompanied with bulges near the mid-cell division site (Typas et al. 2010).

8.4 Tol-Pal system in *E. coli* its and relationship with peptidoglycan synthesis

Tol-Pal system is a family of proteins that consist of five proteins that localise in the inner and outer membranes and span through the periplasmic space in Gram-negative bacteria. This system maintain the integrity of the outer membrane and proper septation during cell division. The genes that encoded this family are from the same operon. These proteins are TolQ, TolR, TolA, TolB and Pal (Figure 8-2) (Webster 1991).

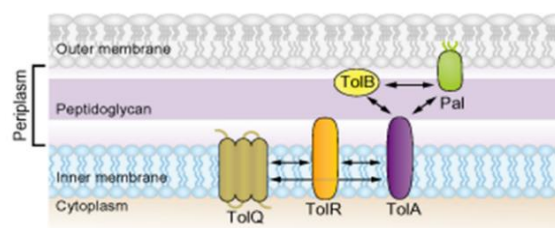


Figure 8. 2: Diagrammatic representation of *E. coli* Tol–Pal complex organization system that modified from (Santos et al. 2014).

TolQ, TolR and TolA proteins are inner membrane protein that forms a complex. TolQ and TolR have the ability to energise TolA, through stimulating conformational change in its periplasmic domain there by using the protein motive force to promote the outer membrane constriction (Cascales et al. 2000, Bonsor et al. 2007). TolB is a periplasmic protein that interacts with both C-terminal domains of TolA and with Pal, which is an outer membrane lipoprotein (Bonsor et al. 2009). Dependent on interaction with the divisome complex, each Tol- Pal system protein during cell division is located at the midpoint of bacterial cell. Deletion any one of the five Tol- Pal system encoding genes results in a tol-pal phenotype which has a sever effect on the outer membrane stability and delays its constriction during cell division. This causes failure of separation of daughter cell from mother cell that result in long chain of cells (Cascales et al. 2002, Gerding et al. 2007). Furthermore, Gray et al. (2015) suggested that the activation of PBP1B by LpoB includes the Tol system proteins. To date, there is limited research which clearly demonstrates the mechanism which the Tol system uses to promot outer membrane construction and the coordination between the OM invagination, facilitated by Tol system, and septal PG synthesis processes.

8.5 Protein CpoB (Coordinator of peptidoglycan synthesis and outer membrane constriction, associated with PBP1B) in *E coli*

Coordinator of peptidoglycan synthesis and outer membrane constriction, associated with PBP1B (CpoB) is a soluble protein that is encoded by the *ybgF* gene that is located and conserved at the end of the *tol-pal* gene operon (Krachler et al. 2010, Gray et al. 2015)). CpoB is conserved in the *tol* operon in all Proteobacteria and other Gram-negative bacteria but not in *Chlymadiae* which are an intracellular parasite (Sturgis 2001). CpoB protein was assessed for cellular location in *E. coli* by using C-terminal fusion mCherry, and CpoB_{EC}-mCherry is found in pre-divisional cells at envelope constriction and during cell division progress at mid of the cell. The location of CpoB indicates that it plays a role in cell division (Gray et al. 2015). CpoB_{EC} interacted with TolA_{EC} by pulldown His tag

assay and CpoB_{Ec} binds to region between the UB2H and TPase domains of PBP1B_{Ec} (Figure 5-1) (Krachler et al. 2010, Gray et al. 2015). This results in partial inhibition of the TPase activity of PBP1B that was stimulated by LpoB *in vitro*, which indicates a specific complex is formed between CpoB, TolA, PBP1B, and LpoB. CpoB is still able to localize to the septum in the absence of Tol A or PBP1B, but failed to localize when PBP3 was inhibited by aztreonam during the later stage of cell division (Gray et al. 2015).

Past research showed that deletion of the gene encoding CpoB did not produced an *E. coli* strain with perturbation phenotype in outer membrane, as happens when deleted any of *tol-pal* genes. This suggested that CpoB is not essential for the function of Tol-Pal genes and $\Delta cpoB$ strain showed increased sensitivity toward the β -lactams antibiotics including cefsulodin that blocks PBP1B activity (Yousif et al. 1985, Vianney et al. 1996).

In recent research using improved microscopy and fluorescent labels, Gray and co-workers pointed out that CpoB_{Ec} is important for envelope integrity and a strain with $\Delta cpoB$ showed increase in the blebbing of the *E.coli* outer membrane. In addition, a strain with $\Delta cpoB \Delta lpoA$ exhibited a severe defect in the outer membrane with elevation in outer membrane blebbing with leaking periplasm and cell lysis; while a strain lacking both CpoB and PBP1A grew normally (Gray et al. 2015).. In addition, they found that the N-terminal TPR domain of LpoA has an additional function that compensates for the absence of CpoB (Gray et al. 2015)

Gray and co-workers also found that the activity of PBP1B in *E. coli* is regulated by CpoB through signals received from the Tol system. This acts on coordination between the production of the new cell wall and the inward movement of the outer membrane. Failure of coordination is seen between the cell wall production and the movement of the outer membrane with membrane crumbling, and cell death by disruption of CpoB_{Ec} activity (Gray et al. 2015).

8.6 Does *Bdellovibrio* have the same machinery as *E. coli* to control cell division?

B. bacteriovorus does not divide by binary fission (Figure 1.5) but it septates as a filament while usually inside a prey cell bdelloplast. Bioinformatic analysis and BLAST analysis of *Bdellovibrio* genes showed that *Bdellovibrio* have genes encoding penicillin binding protein (PBP) 1A and 1B and also, have most genes that encode the Tol-Pal system and have CpoB but do not encode the Outer membrane proteins Lpo A and B that control the activity of PBP1 (Figure 8-3). This means that there are other genes that might be control PBP1 activities.

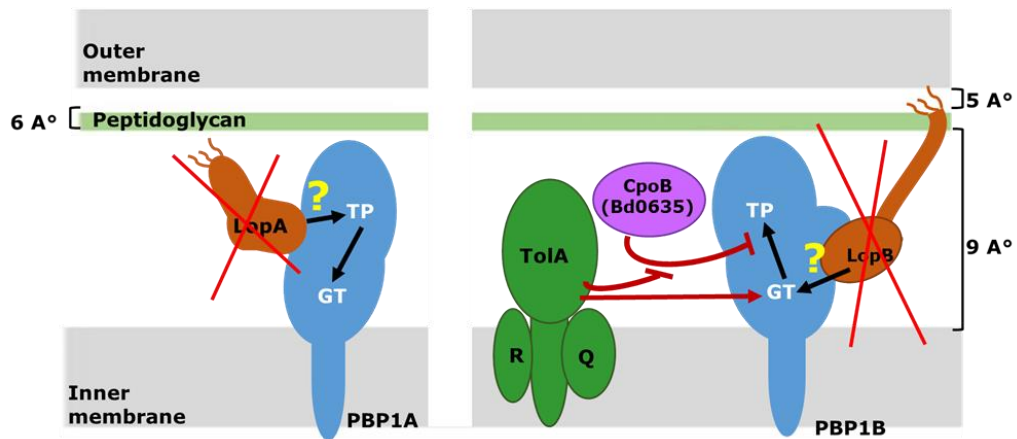


Figure 8. 3: The PBP1 regulation proteins in *Bdellovibrio* differ from *E. coli*. *Bdellovibrio* has both PBP1A and PBP1B and most genes of Tol-Pal system but it lacks the LpoA and LpoB and might have other gene-products that stimulate the TP and GT activities of the PBP1 and that may act in concert with CpoB. This Figure modified from (Ruiz 2016).

8.7 CpoB_{Bd} (Bd635)

CpoB_{Bd} is a 223 amino acid protein that was annotated initially as a conserved hypothetical protein. It consists of DUF3153 (which runs from amino acid 4-140) domain, coiled coil domain (which runs from amino acid 27-147) and Tetratricopeptide repeat domain (TPR-6) (which runs from amino acid 176-208) (<http://smart.embl-heidelberg.de/>). (Figure 8-4).

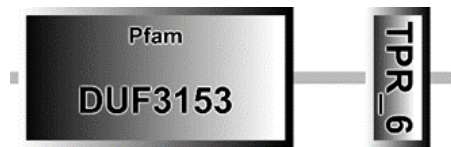


Figure 8. 4: Domains of CpoB_{Bd} protein. The protein is consisting of DUF3153, coiled coil and finally the TPR-6 domain (<http://smart.embl-heidelberg.de/>).

BLAST analysis shows that CpoB_{Bd} is conserved in epibiotic *B. exovorus* but also invasive strains *B. bacteriovorus* Tiberius and other *Bdellovibrio* species Like *Bdellovibrio* sp. ArHS, *Bdellovibrio bacteriovorus* W and *Bdellovibrionales bacterium RIFCSPHIGO2_02*. Bd0635 has a conserved YbgF domain and it belongs to TPR-6 superfamily (Figure 8-5) and *B. bacteriovorus* HD100 genome was found to encode single gene with similarity to the *E. coli* *cpoB* (<https://blast.ncbi.nlm.nih.gov/>).

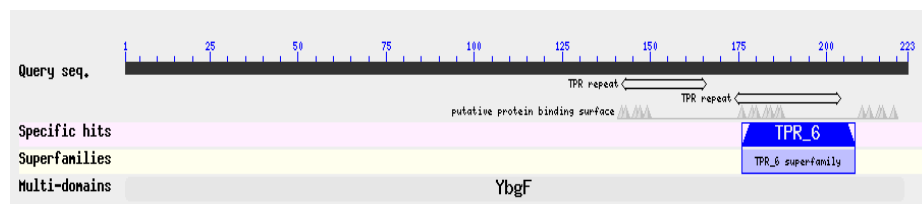


Figure 8. 5: Blast analysis showed the Conserved domains of CpoB_{Bd} (<https://blast.ncbi.nlm.nih.gov/>).

The genomic context showed that *cpoB_{Bd}* lies between two genes that are in same orientation. The upstream gene is *bd0634* that annotated as a hypothetical protein. The downstream gene is *bd0636* (*gcp*) which is O-sialoglycoprotein endopeptidase that vital for the formation of a threonylcarbamoyl group on adenosine in tRNAs and the other two genes upstream of *bd0634* are *bd0632* and *bd0633* which are *tolB* and *pal* genes respectively and the rest of Tol-Pal system genes of *Bdellovibrio* are scattered through the genome. (Figure 8-6) (<http://hactar.shef.ac.uk/xbase/>).



Figure 8. 6: The genomic context of *B. bacteriovorus* *cpoB_{Bd}* gene (<http://hactar.shef.ac.uk/xbase/>).

This is in contrast to *E. coli*, where all the *tol-pal* gene system found in same operon cluster and at the end of that cluster the *E. coli* *cpoB* gene is located (Figure 8-7).

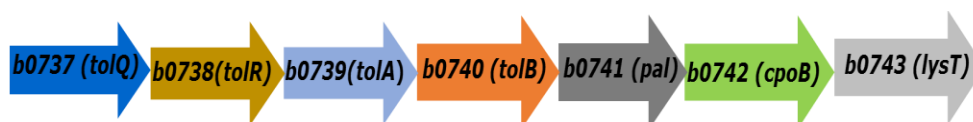


Figure 8. 7: The genomic context of *E. coli* of *cpoB* with *tol-pal* cluster (<http://hactar.shef.ac.uk/xbase/>).

The subcellular localisation of CpoB_{Bd} is predicted by the CELLO website (<http://cello.life.nctu.edu.tw/>) and the results (Figure 8-8) revealed that, as expected, CpoB_{Bd} has a periplasmic location with the predictive score of 2.557.

CELLO Prediction:

Periplasmic	2.557 *
Cytoplasmic	0.855
Outer.Membrane	0.759
Extracellular	0.715
Inner.Membrane	0.114

Figure 8. 8: The prediction of CpoB_{Bd} location by CELLO. The localisation of CpoB_{Bd} is periplasmic (<http://cello.life.nctu.edu.tw/>)

The presence or absence of the secretion signal peptides in CpoB_{Bd} amino acid sequence was tested using SignalP 4.1 server (<http://www.cbs.dtu.dk/services/SignalP-4.1/>). The results can be seen in Figure

(8-9) and shows that CpoB_{Bd} includes a signal sequence that starts from 1-19 amino acids. This indicates that this protein is secreted from the periplasm and out of the *Bdellovibrio* and this fit with the CELLO prediction.

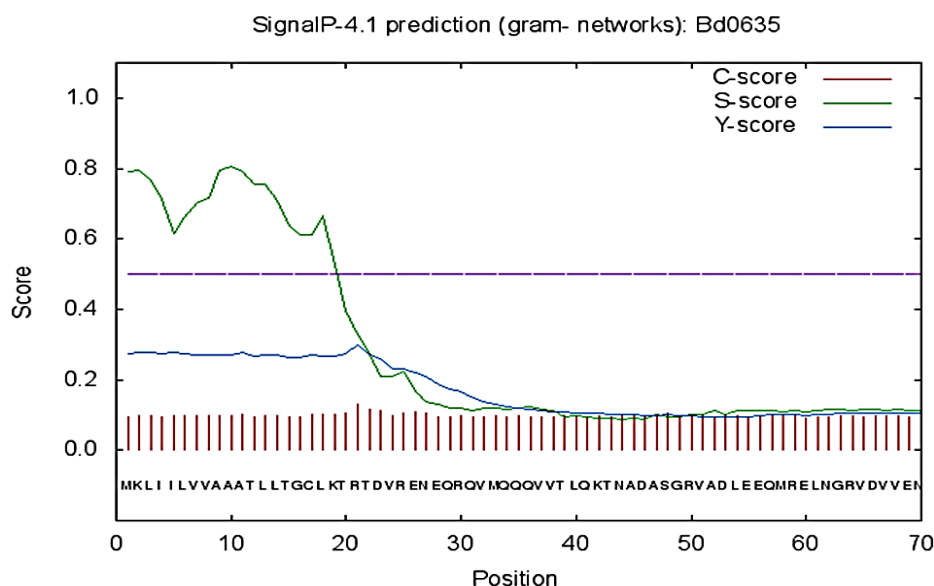


Figure 8. 9: SignalP 4.1 server result of CpoB. The threshold values for signal peptides is a purple dashed line, and the green line rests above this point until amino acid 19 which mean that Bd0635 encodes a signal sequence of 19 amino acids (<http://www.cbs.dtu.dk/services/SignalP-4.1/>).

The shared operons for upstream or downstream genes of CpoB_{Bd} were examined using (<http://operons.ibt.unam.mx/OperonPredictor/>) and it was predicted that *cpoB_{Bd}* is encoded within an operon with *bd0634* upstream and also with *bd06363* (*gcp*) downstream were investigated. Also, it will be tested if there is any shared operon between *bd0633* (*pal*) and *bd634* will be investigated additionally in this chapter.

8.8 Specific Research Aims

1. To examine the transcription of *B. bacteriovorus cpoB_{Bd}* gene throughout the predatory lifecycle.
2. To investigate if there are shared operons between *bd0633* (one of the Tol-Pal system genes that play role in cell wall through outer membrane constriction) and *bd0634* and *cpoB_{Bd}* of *B. bacteriovorus*.
3. To assess the cellular localisation of CpoB_{Bd} during the predatory lifecycle.
4. To investigate if CpoB_{Bd} has any role in sealing the hole that formed during entry of the *Bdellovibrio* to prey cell.
5. To assess the cellular localisation of CpoB_{Bd} during the host- independent lifestyle.
6. To isolate an in-frame deletion strain of *cpoB_{Bd}* and assay the phenotypes of the mutant strain in both HD and HI lifestyle, comparing to *E. coli cpoB* phenotypes.

8.9 Expected outcome

1. Transcription of *cpoB_{Bd}* expresses discontinuously during the predatory lifecycle of *B. bacteriovorus* predatory cycle linking to a cell division role.
2. If CpoB_{Bd} is exported into the prey periplasm and modifies the prey cell wall, the fluorescent tag of CpoB_{Bd} will be visible in attack phase and bdelloplast (It will also be tested it in host-Independent cells as they have a proposed autobdelloplast phase).
3. CpoB_{Bd} in *Bdellovibrio* plays a similar role to the *E. coli* CpoB in cell wall division.
4. Deletion of *cpoB_{Bd}* is possible and if a deletion strain were obtained, is the deletion strain will still be able to of perform the full predatory process.

5. Deletion of *cpoB_{Bd}* will be viable but may effect on host-independent cell wall synthesis growth of the deletion strain comparing with Wild-type host-independent of *Bdellovibrio*.

8.10 Results

8.10.1 Transcription of *cpoB_{Bd}* in host- dependent (HD) life cycle and host-independent growth mode

The expression of *cpoB_{Bd}* during the predatory life cycle of *Bdellovibrio* was examined using RNA samples isolated at indicated time points of the predatory life cycle of *B. bacteriovorus* strain during one round of synchronous *Bdellovibrio* infection of *E. coli* host cells (Figure 8-8). The expression profile of *cpoB_{Bd}* appear weak in attack phases and starts to rise in 15-30 mins then drops at 45 mins. The expression increases again at 1-2 hrs and drop in 3-4 hrs again of the predatory lifecycle of *Bdellovibrio*. This expression pattern suggests that CpoB is needed throughout the predatory cycle and not just at invasion or replication.



Figure 8. 10: Reverse-transcriptase PCR is showing transcriptional expression of *cpoB_{Bd}* throughout the predatory cycle (diagram above of stages). RNA was isolated at the indicated time points during one round of synchronous *Bdellovibrio* HD100 infection of *E. coli* host prey cells, and primers specific to the gene were used to amplify (RT-PCR, 27 cycles) 89 bp internal fragment from each transcript if present. AP = Attack phase (free-swimming *Bdellovibrio*); 15, 30, 45 = minutes since start of infection (attachment to and rounding of *E. coli* host cell into a bdelloplast structure); 1h, 2h, 3h, 4h = hours since start of infection (host cell resources being degraded and used for *Bdellovibrio* growth into a filament, followed by septation into multiple progeny and eventually lysis from host cell); Ec = *E. coli* S17-1 RNA (no *Bdellovibrio* control); gen = *B. bacteriovorus* HD100 genomic DNA (positive control); NT = No template control; L = NEB 100 bp DNA ladder.

To test if *cpoB_{Bd}* was expressed and has a role in HI growth mode of the *Bdellovibrio*, RT-PCR was achieved using total RNA matched at 10 ng/μL isolated from the attack phase and different wild-type HI strains (HID2, HID13, HID22 and HID26) (Figure 8-11). As it is clear from (Figure 8-11) that *cpoB_{Bd}* expression level is higher in all HI strains in comparison to attack phase (non- growing) and

this matched the results of Microarray data of Lambert et al. (2010) that found out the expression of this gene during HI growth 6.26 fold higher than in attack phase.

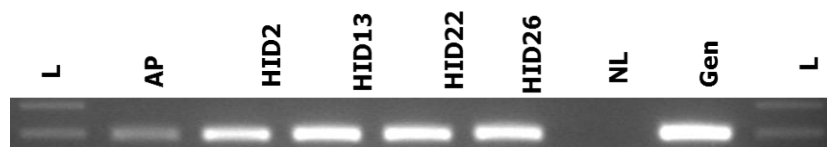


Figure 8. 11: Reverse-transcriptase PCR showing relative transcription expression of the *B. bacteriovorus* HD100 *cpoB_{Bd}* during attack phase and host-independent growth. RNA was isolated from *B. bacteriovorus* HD100 attack phase (AP) cells and four host-independent strains (HID2, HID13, HID22, HID26) at OD600 0.6 and the RNA concentration was matched to 10 ng μ l⁻¹. Primers specific to gene were used to amplify (RT-PCR, 30 cycles) a 100 bp internal fragment from each transcript if present. Gen = *B. bacteriovorus* HD100 genomic DNA (positive control); NT = no template control; L = NEB 100 bp DNA ladder.

8.10.2 The *cpoB_{Bd}* is coexpressed in an operon with *bd0633* (*paL* gene)

RT-PCR was performed in order to test whether *cpoB_{Bd}* and *bd0634* and whether *bd0634* and *bd0633* (*paL*) are co-transcribed in an operon using matched amounts of RNA (10 ng/ μ L) isolated from *B. bacteriovorus* HD100 strain at one time point during the predatory lifecycle (1hr). A 700 bp and 536 bp products indicate that *bd0633* and *bd0634* are all in the same operon and *bd0634* and *bd0635* also transcribed from the same operon which might have associated function (Figure 8-12 and 8-13).

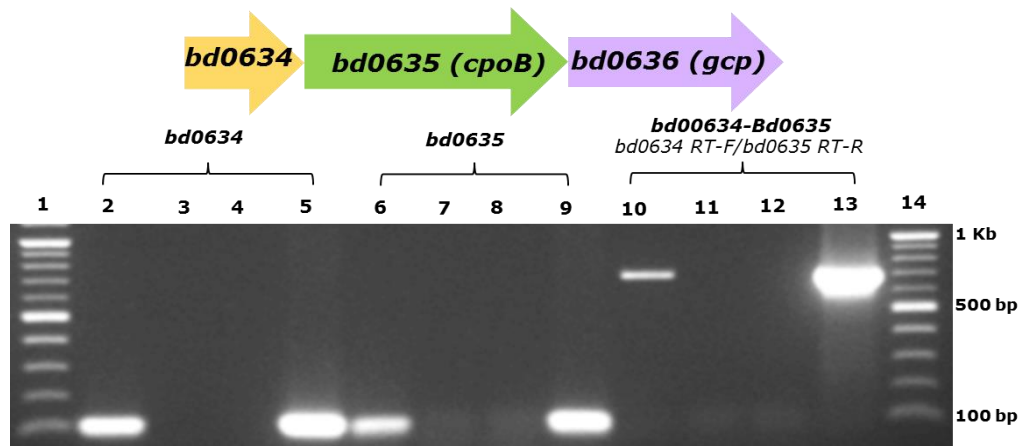


Figure 8. 12: RT-PCR shows that the *B. bacteriovorus* genes *bd0634* and *cpoB_{Bd}* are co-transcribed. Lanes 1 and 14: NEB 100 bp ladder; Lanes 2, 6 and 10: predator and prey total RNA at 1hr; Lanes 3, 7 and 11: *E. coli* S17-1 RNA; Lanes 4, 8 and 12: No template; Lanes 5, 9 and 13: *B. bacteriovorus* HD100 genomic DNA. A 700 bp band found in lane 10 indicates that there are co-transcription between *bd0634* and *bd0635* genes.

The results from all co-transcription RT-PCR analysis of *bd0633*, *bd0634* and *cpoB_{Bd}* suggest that the products of these genes have associated functions and may participate with CpoB_{Bd} in peptidoglycan modification.

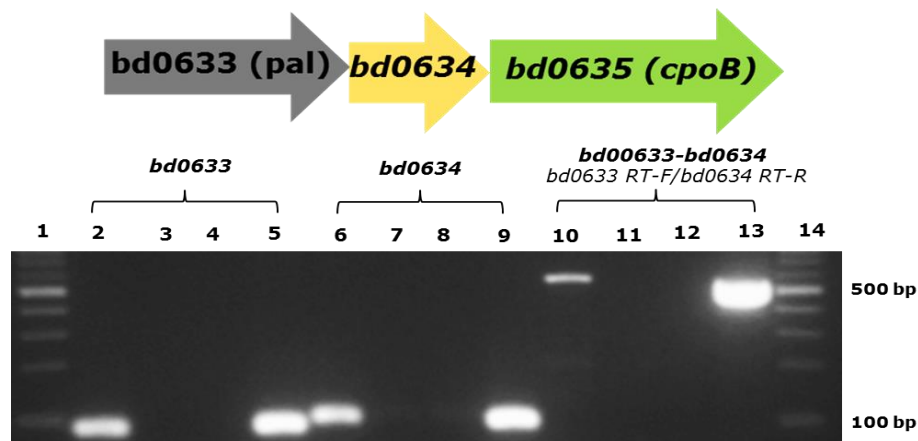


Figure 8. 13: RT-PCR shows that the *B. bacteriovorus* genes *bd0634* and *bd033* are co-transcribed. Lanes 1 and 14: NEB 100 bp ladder; Lanes 2, 6 and 10: predator and prey total RNA at 1hr; Lanes 3, 7 and 11: *E. coli* S17-1 RNA; Lanes 4, 8 and 12: No template; Lanes 5, 9 and 13: *B. bacteriovorus* HD100 genomic DNA. A 536 bp band found in lane 10 indicates that there are co-transcription between *bd0633* and *bd0634* genes.

8.10.3 The *cpoB_{Bd}* and *bd0636* genes are not co-transcribed

In order to assess if *cpoB_{Bd}* and *bd0636* are co-transcribed in an operon, RT-PCR was carried out using matched amount of RNA (10 ng/μL) isolated from *B. bacteriovorus* HD100 strain during predatory lifecycle (1hr). The result showed that there is no observable operon between *cpoB_{Bd}* and *bd0636* genes (Figure 8-14).

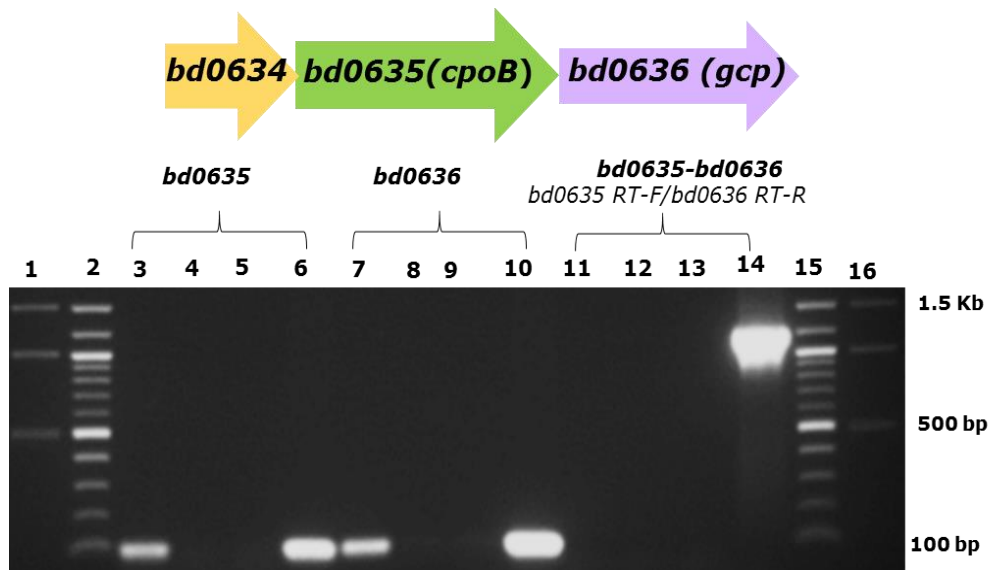


Figure 8. 14: RT-PCR shows that the *B. bacteriovorus* genes *cpoB_{Bd}* and *bd0636* are not co-transcribed. Lanes 1 and 16: NEB 1000 bp ladder; Lanes 2 and 15: NEB 100 bp ladder; Lanes 3, 7 and 11: predator and prey total RNA at 1hr; Lanes 4, 8 and 12: *E. coli* S17-1 RNA; Lanes 5, 9 and 13: No template; Lanes 6, 10 and 14: *B. bacteriovorus* HD100 genomic DNA. No band found in lane 11 indicates that there are no co-transcription between *bd0635* and *bd0636* genes.

8.10.4 Localisation of CpoB_{Bd} -mCherry assessed by fluorescently tagged mCherry in *B. bacteriovorus*

To observe localisation of CpoB_{Bd}-mCherry during the predatory lifecycle, a C-terminal fluorescently tagged *B. bacteriovorus* *cpoB_{Bd}* strain was generated by MSci student Sam Greenwood. CpoB_{Bd} -mCherry was constructed by fusion of a 669-bp ORF fragment of *bd0635* lacking the stop codon, cloned in frame with *mCherry* in the plasmid pAKF56 using *E. coli* DH5α as a host. pAKF56::CpoB_{Bd} -

mCherry plasmid tagged protein was fluorescently heterologously expressed in *E. coli* (Figure 8-14) and localised seemingly cytoplasmically with a spotted pattern in the cell. This may be due to excess protein that result from high copy number of the intermediate clone pAKF56::CpoB_{Bd} -mCherry plasmid. While expressed in *E. coli* from conjugateable pK18::CpoB_{Bd} -mCherry protein fluorescence was cytoplasmically located (Figure 8-16).

CpoB_{Bd} -mCherry construct was transformed into *pK18mobsacB* plasmid and conjugated from donor *E. coli* strain S17-1 into *B. bacteriovorus* by single crossover, and maintained using kanamycin selection.

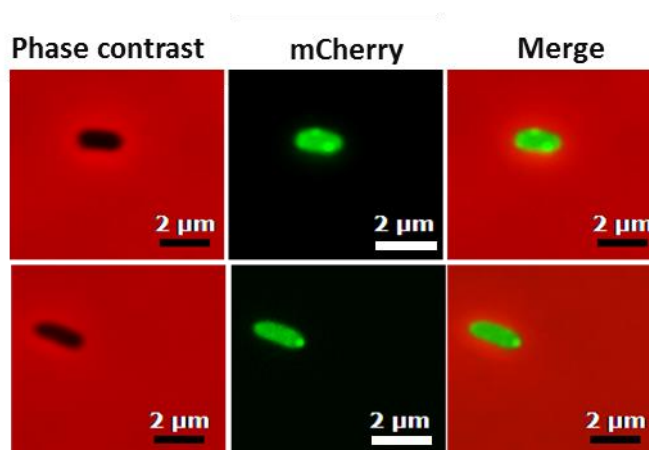


Figure 8. 15: Heterologous expressing of pAKF56::CpoB_{Bd} -mCherry in *E. coli* DH5α in stationary phase that grown in YT broth for 16 hours with appropriate antibiotic. The fluorescence is visible spread cytoplasmically with a spotted pattern in the cell.

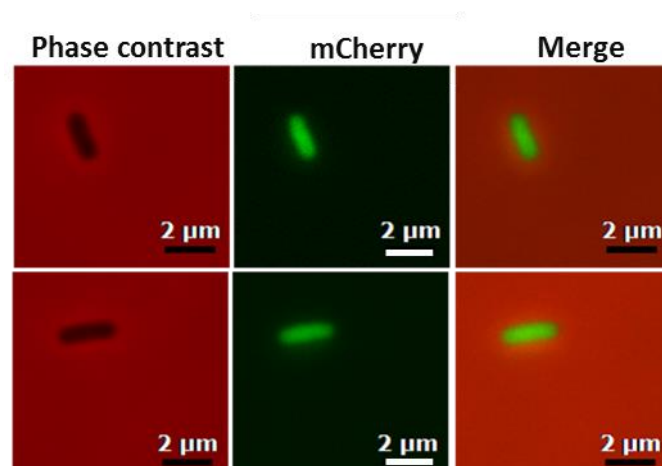


Figure 8. 16: Heterologous expressing of pK18::CpoB_{Bd} -mCherry in *E. coli* DH5α in stationary phase that grown in YT broth for 16 hours with appropriate antibiotic. The fluorescence is visible spread within the cytoplasm.

During Sam Greenwood project, he noticed that CpoB_{Bd} is located at the site of *Bdellovibrio* entry to prey cells as patches. As a result of use being unable to look for the location of PBP1B itself during the predatory lifecycle (it is lethal for *Bdellovibrio* this gene-product is tagged with mCherry), therefore CpoB_{Bd} was used as a proxy to find where PBP1B may be acting during the *Bdellovibrio* lifecycle.

To determine the location of CpoB_{Bd} throughout the predatory lifecycle, a synchronous infection was set up and fluorescent images taken at specific intervals throughout the lifecycle. It was observed that the attack phase cells show visible fluorescence with different sites at different times within the cell. Interestingly, the majority of the early attached *Bdellovibrio* to *E. coli* (at 15 min) showed the localisation of CpoB_{Bd} -mCherry to be diffuse through the cell (~51%), with the remainder mainly monopolar (~33%) at the back of the *Bdellovibrio* at the pole away from the prey and fewer bipolar (~15%) and rest (~1.3%) showing a single spot near but not at pole (Figure 8-17).

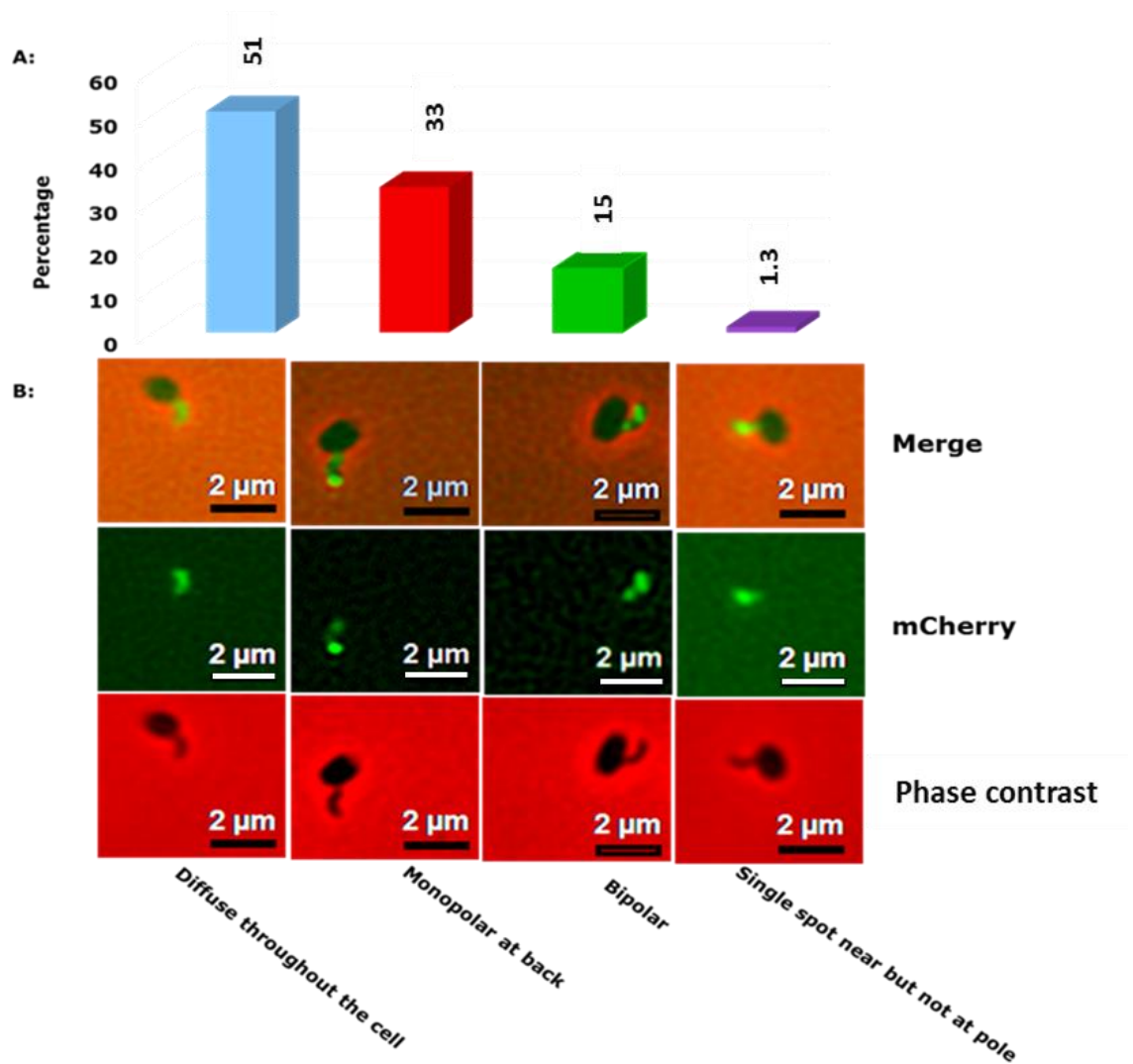


Figure 8. 17: Examples of Location of CpoB_{Bd} -mCherry during the attachment to the prey. A: The overall distribution of CpoB_{Bd} -mCherry fluorescence in *B. bacteriovorus* during attachment (The total cells no =150) from three biological repeats. B: Image of *B. bacteriovorus* HD100 *cpoB_{Bd}*- *mCherry* cells during attachment (scale bar = 2 μm). 1.5 Second exposure with maximum gain.

Surprisingly, the location of CpoB_{Bd} -mCherry in (non- dividing) attack phase *Bdellovibrio* cells at 30 mins of contact with/invasion into prey was mostly monopolar spot at the back of the *Bdellovibrio* (~ 64.8 %) and the rest were diffuse throughout the cell (~ 17.6 %), bipolar (~ 12.5 %) with few showing more than two foci (~ 5.8 %) (Figure 8-18). The location of CpoB_{Bd} -mCherry in *B. bacteriovorus* at 15 mins and 30 mins suggested that this this protein may have

a PBP1B-regulatory role in filling and rebuilding the hole in the prey peptidoglycan wall (Kuru et al. 2017) that the *Bdellovibrio* made to enter to the prey cells.

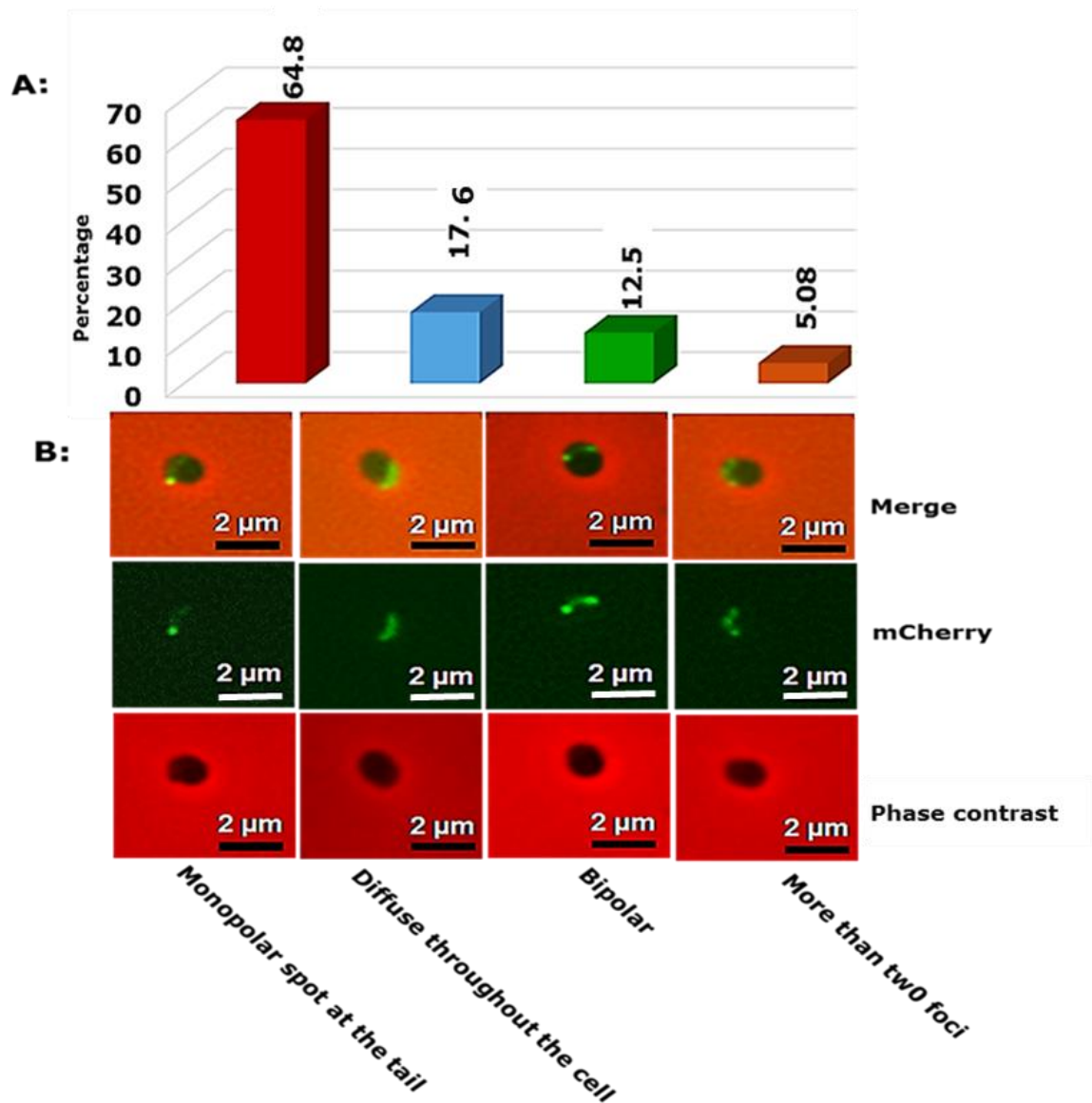


Figure 8. 18: Examples of Location of CpoB_{Bd} -mCherry at 30 mins. A: The overall distribution of CpoB_{Bd} -mCherry fluorescence in *B. bacteriovorus* at 30 mins time point (The total cells no =242) from three biological repeats. B: Image of *B. bacteriovorus* HD100 cpoB_{Bd} -mCherry cells at 30 mins (scale bar = 2 μm).1.5 Second exposure with maximum gain. Note that the monopolar spot at “tail” cell example is just entering the prey with the “tail” pole still outside when the CpoB is localised there. The cells which have fully entered are changing CpoB-mCherry localization.

With regard to the later time points of the predatory lifecycle, after 30 minutes, the fluorescence CpoB_{Bd} was present throughout the cytoplasm with patches of fluorescence in the prey cell wall coincident with the site of *Bdellovibrio* entry (Figure 8-19). The results of the fluorescent time course showed that CpoB_{Bd} - mCherry is continuously expressed at all the time points and this is matching the RT-PCR profile that was seen expressing throughout the predatory lifecycle (Figure 8-10). Furthermore, the results from the fluorescent time course suggested that CpoB_{Bd} - mCherry is required to regulate PBP1B filling in and rebuilding the entry hole into the prey cell (fluorescent patches spot with high expression) rather than for cell elongation and division (as the expression of the fluorescent expression is lower along the *Bdellovibrio* growing in the bdelloplast) (Figure 8-19). Testing any role in *Bdellovibrio* filament septation requires further tagged markers for septation such as FtsA-TFP which there was insufficient time to make at the end of the study. This question still remains to be studied.

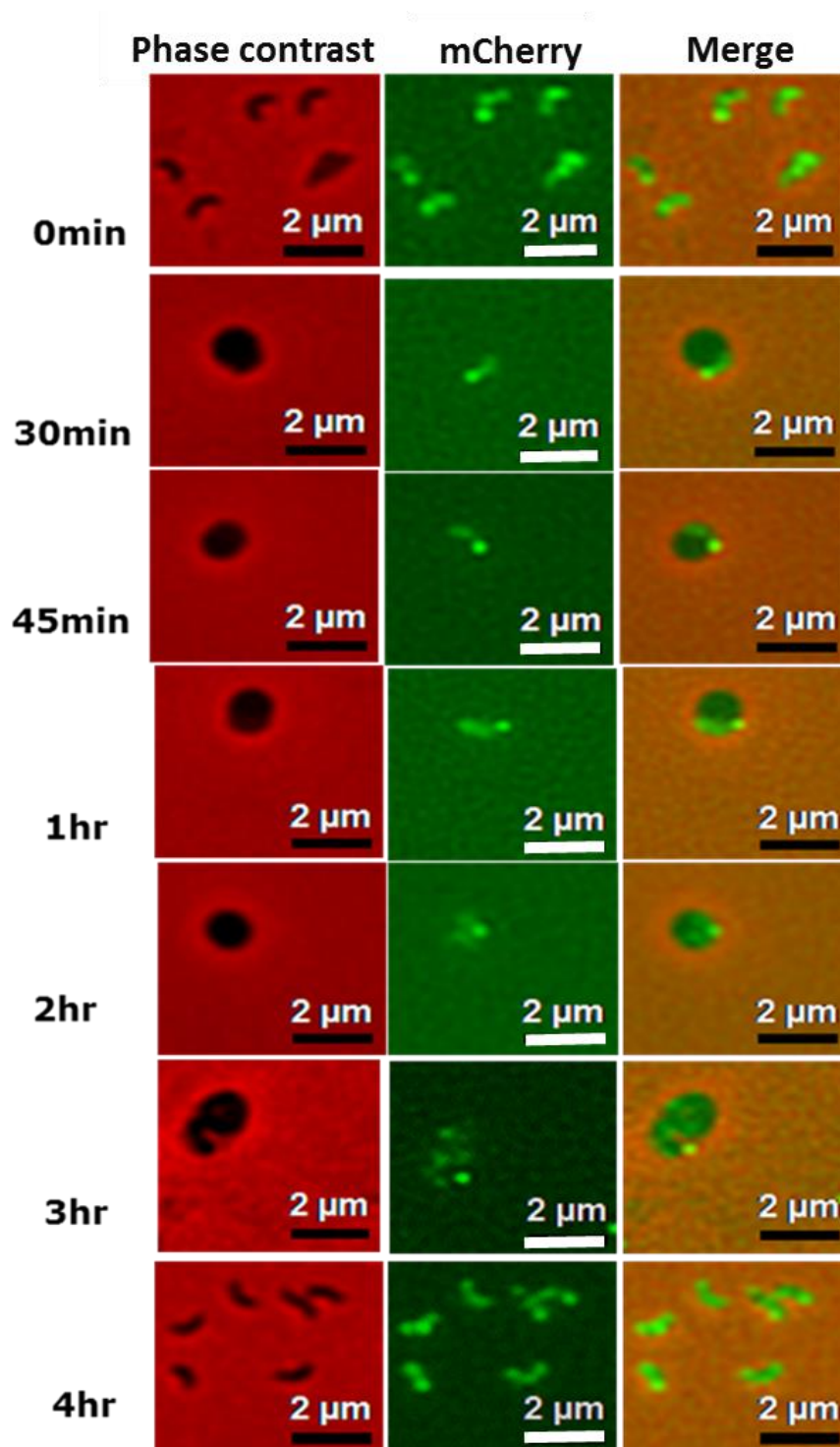


Figure 8. 19: The fluorescence pattern of *B. bacteriovorus* HD100 *cpoB_{Bd}*-mCherry during the whole predatory lifecycle on *E. coli* strain pZMR100. The bright sphere of CpoB-mCherry at the periphery of the bdelloplast is seen at 45mins-3hrs. 1.5 second exposure with maximum gain.

8.10.5 The dual colour *Bdellovibrio* strain gives direct evidence that CpoB_{Bd} -mCherry plays a role in sealing the *Bdellovibrio* entry hole

The fluorescent time course of *B. bacteriovorus* *cpoB_{Bd} -mCherry* strain showed that *B. bacteriovorus* left fluorescent spot patches at entry hole in prey cell that might act as a plug for sealing the *Bdellovibrio* entry hole. The location of the CpoB_{Bd} -mCherry during the predatory lifecycle possibly associated with plug formation at the site of entry of *Bdellovibrio* into prey cell (Figure 8-15) needed further confirmation of which compartment of CpoB_{Bd} was in during the predatory lifecycle.

Therefore, to test the idea that *cpoB_{Bd}* plays a role in sealing the entry point, an *E. coli* S17-1 *pK18::bd0064-teal* strain that encodes a PilZ domain c-di-GMP protein was conjugated in *B. bacteriovorus* *cpoB_{Bd} -mCherry* strain by single crossover to produce dual colour *Bdellovibrio*. The resultant exconjugants were picked up and grown under the standard condition for 24 hrs and fluorescent time course repeated for three times (Figure 8-16). It is clear from the Figure (8-20) TFP and mCherry fluorescence localised to the same place in attack phase, and 15 mins. The obvious differences started to be seen at 30min-1hrs; the fluorescent patches seen in the mCherry channel (false labelled green colour) but absent in the TFP channel (blue teal colour) in the prey-bdelloplast cell envelope. This gives a clear view that CpoB_{Bd} is secreted from *Bdellovibrio* into the prey cell envelope, it is therefore likely to regulate PBP1B to act to close the entry hole. As CpoB_{Bd} alone is not a peptidoglycan active enzyme it is much more likely that it works via participation of another gene product such as PBP1B.

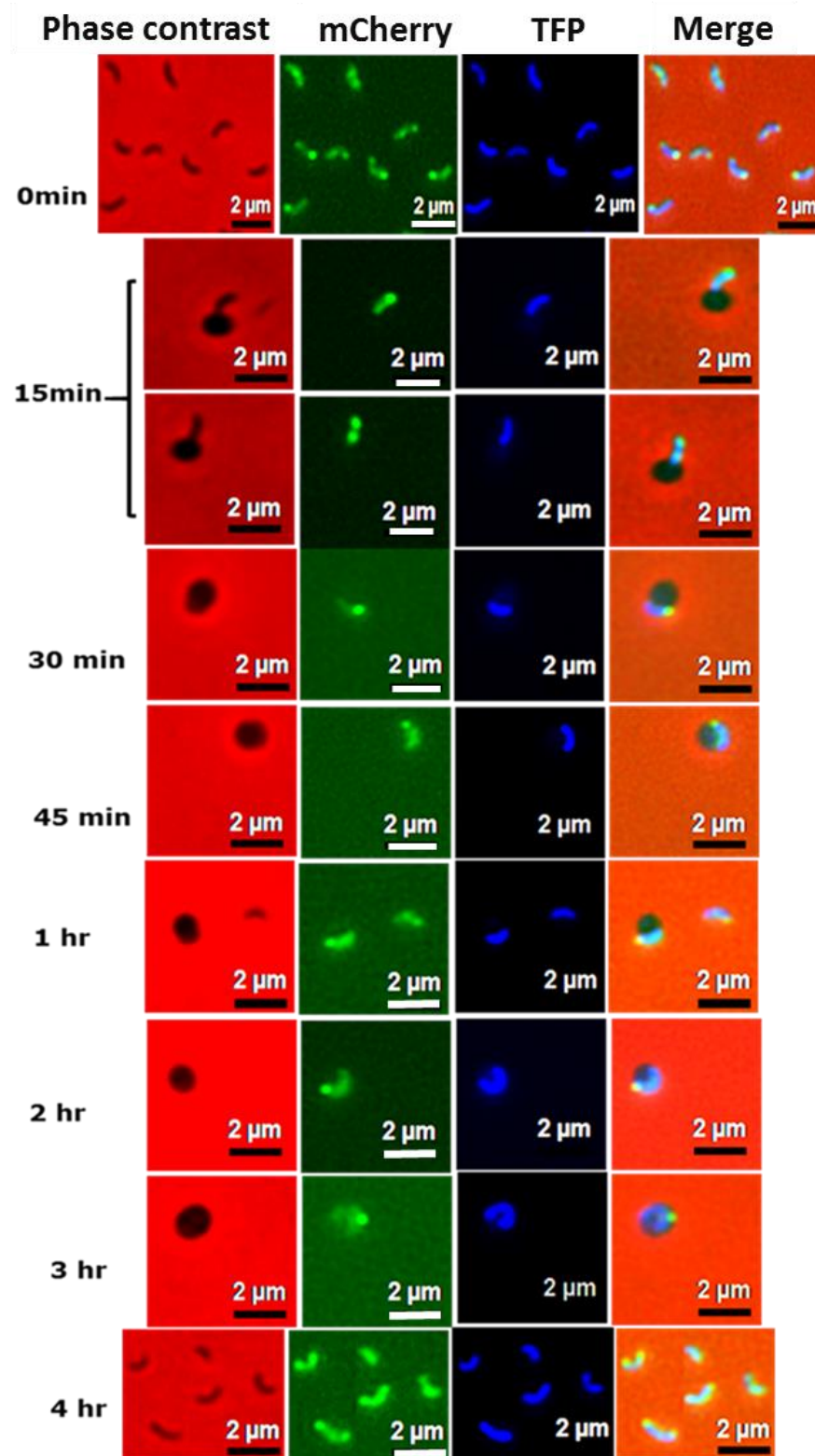


Figure 8. 20: The dual colours of *B. bacteriovorus* *cpoB_{Bd}* -mCherry - *bd0064-teal* tags fluorescence pattern during the whole predatory lifecycle *E. coli* pZMR100. One and half second exposure for mCherry and 0.6 second exposure for TFP with maximum gain.

8.10.6 CpoB_{Bd} of *Bdellovibrio* may play a role in filling and rebuilding the entry hole of the *Bdellovibrio*

As the previous results from the mCherry and the dual colour predator-prey fluorescent time courses showed that the CpoB_{Bd}-mCherry fluorescence spot start to appear at 30 mins, when the *Bdellovibrio* complete its entry to the prey cell. Therefore, to give clues from where these fluorescent spots form during the very early predatory life cycle of the *Bdellovibrio*. Thus the location of CpoB_{Bd}-mCherry before the *Bdellovibrio* finish the invasion completely and enter to the prey cell were followed in both mCherry and the dual colour fluorescent time courses. As can be seen from (Figure 8-21) CpoB_{Bd}-mCherry is located at the non-invading end of the *Bdellovibrio*, where the single sheathed flagellum is located.

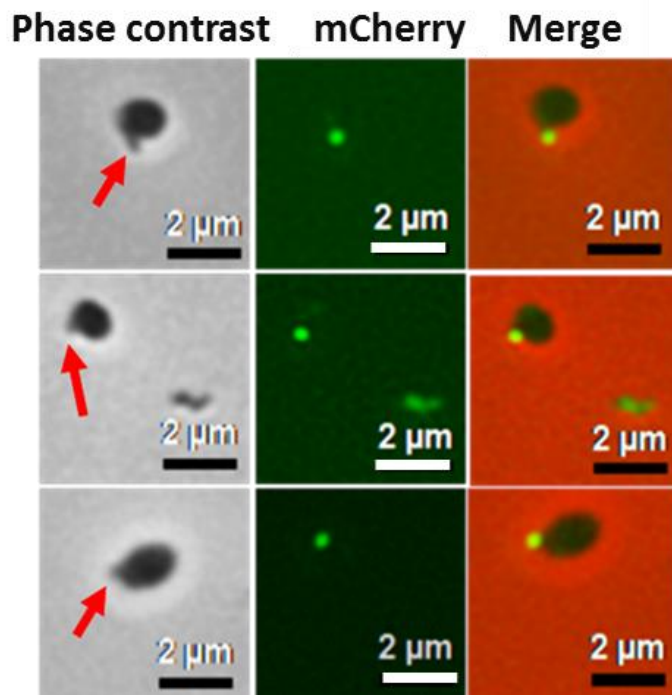


Figure 8. 21: Location of CpoB_{Bd}-mCherry at 30 mins in not completely invaded *Bdellovibrio* (red arrow in brightfield) in to the prey cells in the fluorescence *B. bacteriovorus* HD100 CpoB_{Bd}-mCherry. Image taken at 1.5 Second exposure with maximum gain (cell No. = 18 from three biological repeat).

The location of CpoB_{Bd} in dual colour (mCherry and TFP) fluorescence is seen in mCherry channel at the non-invading end of the *Bdellovibrio* but not seen in the TFP channel (Figure 8-22). It can therefore be assumed from the location of CpoB_{Bd}-mCherry that this protein has a fill in and rebuild role, essential for the predatory lifecycle of the *Bdellovibrio*.

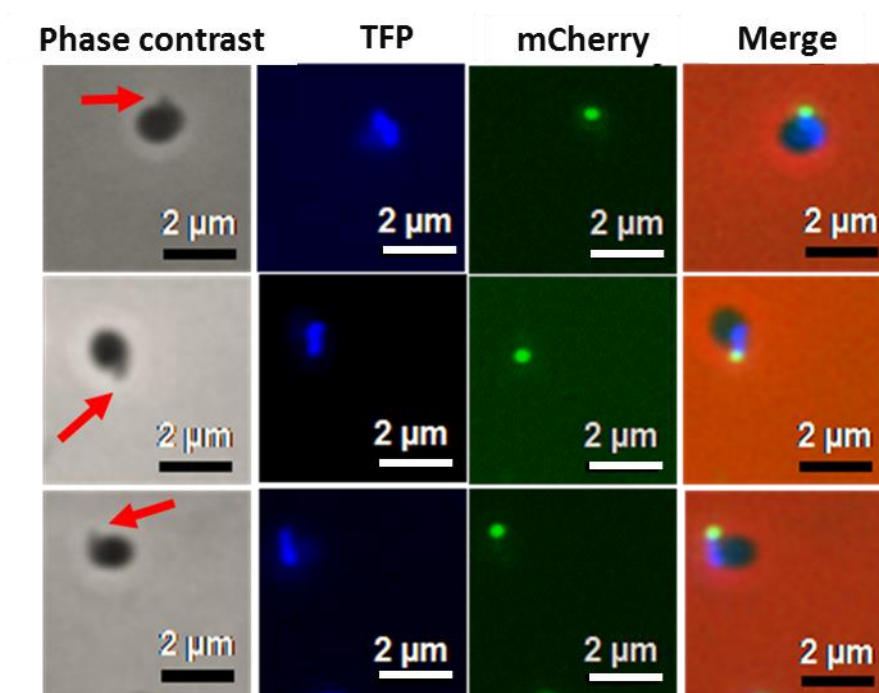


Figure 8. 22: Location of CpoB_{Bd} -mCherry at 30 mins in not completely invaded *Bdellovibrio* (red arrow in phase contrast) entering prey cells in the dual colours of *B. bacteriovorus* cpoB_{Bd} -mCherry - bd0064-teal tags fluorescence. Image taken at 1.5 Second exposure with maximum gain (cell no.= 12 from three biological repeat).

8.10.7 The fate of the robust fluorescent CpoB-mCherry spot that formed in prey wall after the end of the predatory lifecycle

One of the questions that emerges from the results of the time courses of the CpoB is: Where are these spots ending up? Also, what are the fate of these spots after the *Bdellovibrio* progeny exit from the residual shell of the remnant prey cell? Thus, in this section the focus will be on the sequence of the positions of the spots, the predatory time course is run and images at 5 hrs were taken to reveal the fate

of these spots at the end of predation. As shown in Figure (8-23) that there are many physically robust fluorescent- CpoB-mCherry labelled spherical structures that remain within the residual broken remnants of the prey cells after the end of the predatory lifecycle. This meant that when the *Bdellovibrio* progeny exit the spot is released with the prey shell residue and the importantly, the CpoB-mCherry labelled spheres are robust as if they have a membrane and cell wall around them, retaining the CpoB-mCherry.

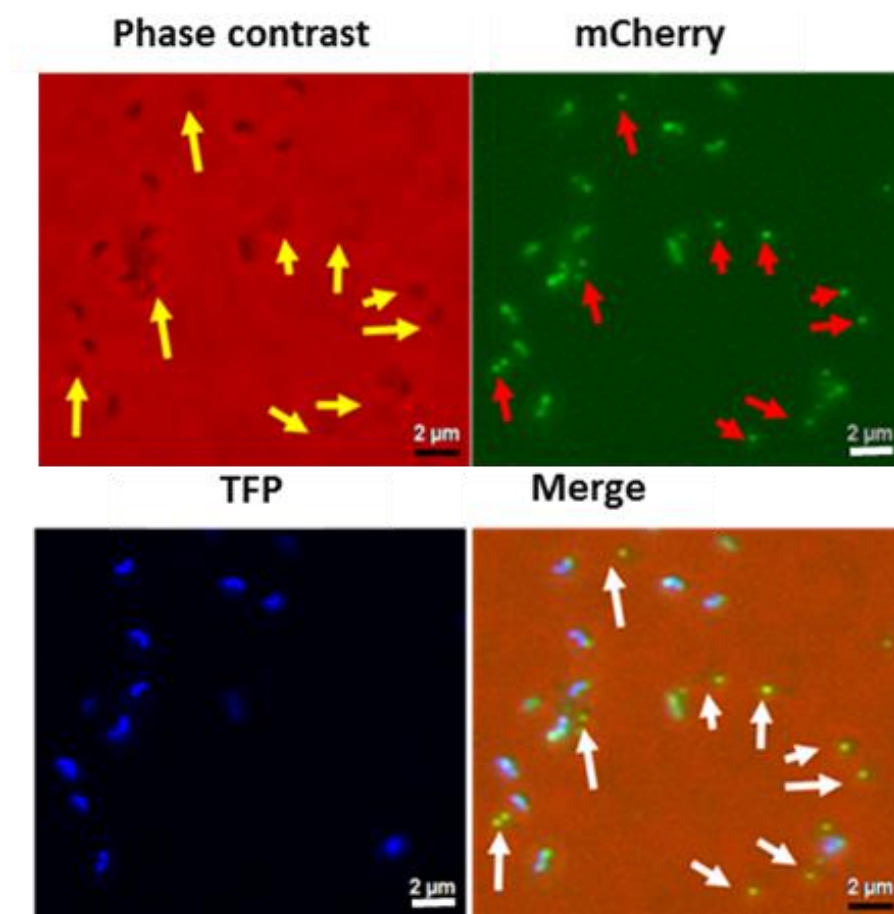


Figure 8. 23: The field of view of released *Bdellovibrio* cells and prey bdelloplast debris at 5hrs after predator exit at the end of the predatory life cycle for the dual colour of *B. bacteriovorus* *cpoB_{Bd}* -mCherry - *bd0064-teal* tagged cells. Fluorescence revealed that there were many fluorescence spots within broken prey envelope residues that could be found in mCherry channel (red arrows) but that were not found the TFP channel which predominantly showed whole attack phase predator *Bdellovibrio* (dark in phase/brightfield and blue in TFP channel as labelled cytoplasmically by Bd0064mTeal).

8.10.8 CpoB_{Bd} -mCherry protein is expressed in host-independent cells

For completeness, to investigate if *bd0635*-mcherry was expressed in HI growing cell and to find out if the HI cell has the same patches seen in predatory time course, *B. bacteriovorus cpoB_{Bd} -mCherry* strain was grown host-independently (HI). The growing HI *B. bacteriovorus cpoB_{Bd} -mCherry* strain expressed the fluorescence within all various cell types and shapes (Figure 8-24), with a lot of patches of fluorescence within the HI cells. There were numerous long HI cells than is usual for wild-type HI *Bdellovibrio*. This indicates that mCherry tag affects the function of Bd0635 which may be required for cell division, and in these HI cells this demand for CpoB_{Bd} function in cell division is increased compared to HD growth. This is also partly supported by the results of Microarray data which showed that CpoB_{Bd} expressed during HI growth is 6.26 fold higher than in matched RNA concentrations from attack phase (Lambert et al. 2010). Strikingly, there were CpoB_{Bd} -mCherry spots seen in round “autobdelloplast” like structures in the HI cultures. The autobdelloplast may form at the ends of long filamentous HI cells supported by septum-and envelope-limited lysis with internalised *Bdellovibrio* carrying out a predatory cycle to release motile flagellate attack phase cells (Sackett unpublished data). These attack phase cells could travel away from the stationary long HI cells and find a new niche in which to prey. The presence of CpoB supports the suggestion of autopredation in these structures.

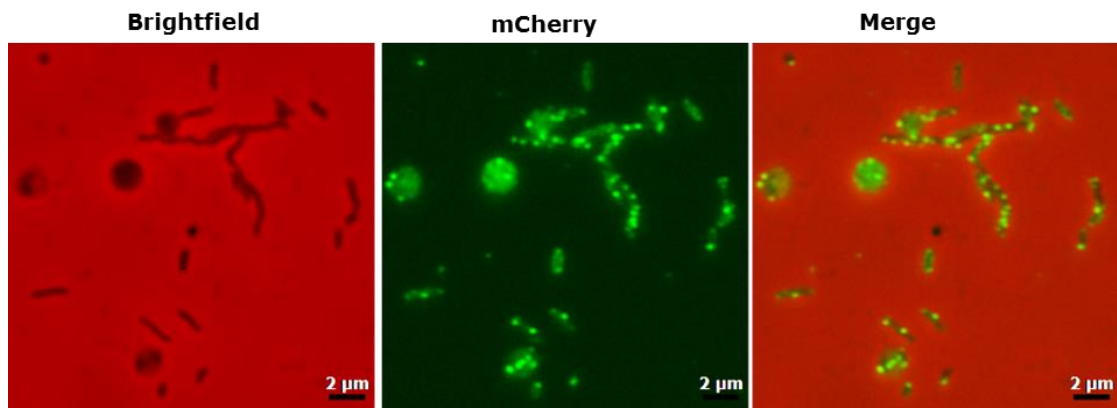


Figure 8. 24: HI *B. bacteriovorus cpoB_{Bd}* -mcherry strain. *cpoB_{Bd}* -mcherry expressed in Host- Independent growth phase in all cell type with a lot of patches of fluorescence within the HI cells. The images were taken with 0.3 second fluorescent exposure and maximum gain.

The DAPI stain of the HI *B. bacteriovorus cpoB_{Bd}* -mcherry showed that the fluorescent patches are not in same places of the DNA location mainly in the rounded cells (Figure 8-25). Due to the asynchrony of growth in HI cultures, it was not possible to follow autopredation events in a microscopic time course as could be done for HD cells. HI filamentous cells do carry out synchronous and asynchronous division at several septa as well as the autobdelloplasting at the ends. The HI septa may require different levels of CpoB than septa in HD dividing cells which are internal to prey.

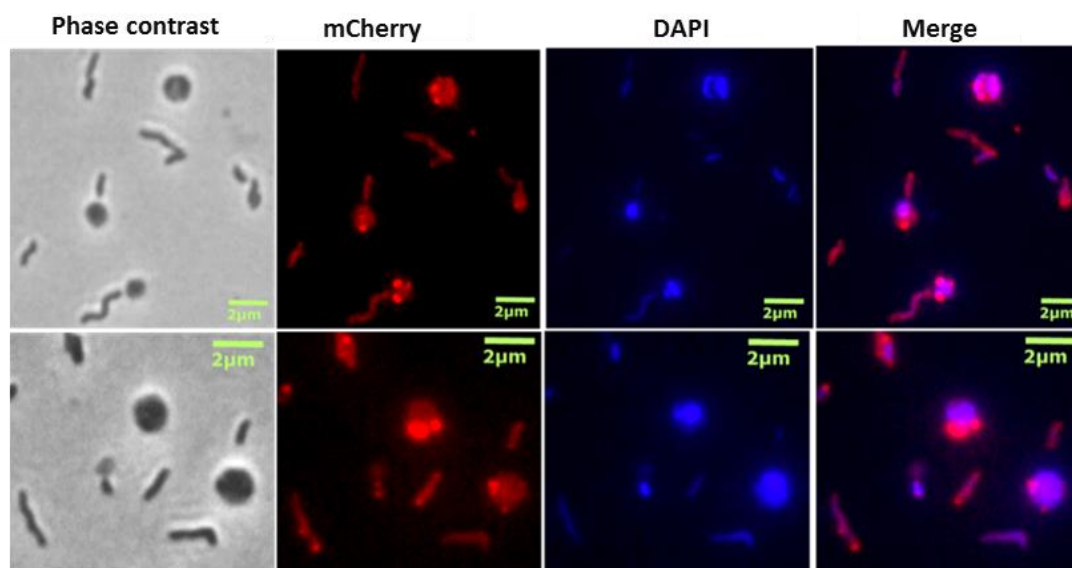


Figure 8. 25: HI *B. bacteriovorus* *cpoB_{Bd}* -*mcherry* cells were stained with DAPI strain. HI cells were stained with DAPI and imaged with 1.5 seconds fluorescent exposure for mCherry imaging and 0.05 seconds for DAPI images.

8.10.9 Deletion of *cpoB_{Bd}* is lethal for *Bdellovibrio*

In order to check the function of the *cpoB_{Bd}* gene to non-predatory bacteria such *E. coli* (where it is not essential) and if it has any role in predation of *B. bacteriovorus*, a silent knockout deletion strain of *cpoB_{Bd}* was made. A deletion plasmid for *cpoB_{Bd}* construct was made using Gibson assembly cloning (see Section 2.10.3) to combine the 1 kb region upstream and downstream of the gene of interest and the plasmid pK18*mobsacB*. The construct of Δ *cpoB_{Bd}* was confirmed by sequencing, and transformed into *E. coli* S17-1 then conjugated in *B. bacteriovorus* HD100, recombining by single crossover and maintained initially by kanamycin selection. Four different conjugation in HD and three different conjugation in Wild type HI (HI D13, HI D22 and HI D26) strains were made.

As *Bdellovibrio* has two growth modes (predatory HD or axenic HI) and *E. coli* has only one growth mode, construction of a Δ *cpoB_{Bd}* strain in both growth modes was tested. The resultant *Bdellovibrio* plaques from the HD conjugation were grown as host- dependent cultures, and then initial kanamycin selection was

removed and strains were screened for gene replacement and plasmid loss using 5% sucrose due to sucrose suicide selection. All the exconjugants were sensitive to kanamycin and 208 prey-lysate cultures were screened (from four different conjugations) using Dream Taq plaque- PCRs for initial testing for a HD-grown deletion strain of this gene. All of the exconjugants that were screened gave 1641 bp the wild type *cpoB_{Bd}* band, which meant that the HD growth exconjugants had reverted to wild-type strains and no mutant HD strain could be obtained (Figure 8-26 A).

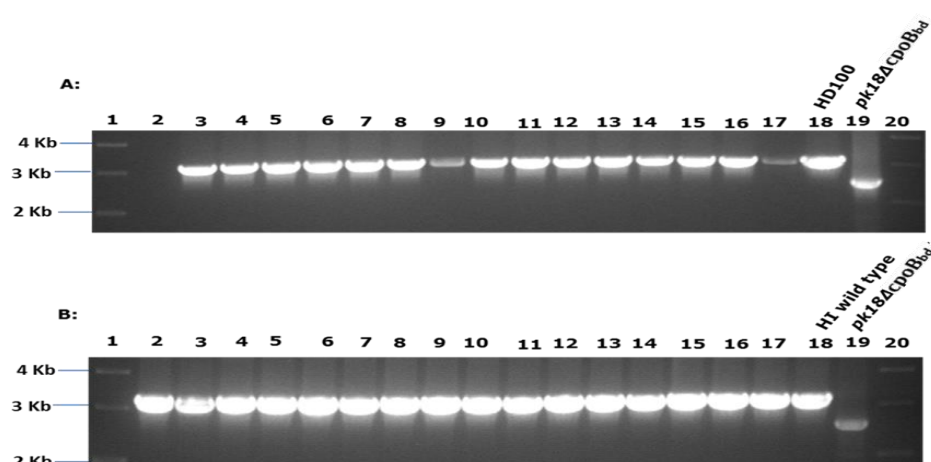


Figure 8. 26: Agarose gel showing amplified products from initial PCR screen of genomic DNA testing for potential HD or HI *cpoB_{Bd}* knockout strains. A: PCR of potential knockouts of HD *cpoB_{Bd}* using Taq polymerase PCR and primers *bd0635-F* and *bd0635-R*. Lane 1, 20 are 1 Kb ladder. Lane 3-17 are potential knockout show band of wild HD100 that seen at 1641 bp. Lane 18, 19 are HD100 control and *pk18Δ cpoB_{Bd}*. B: PCR of potential knockouts of HI *bd0635* using Taq polymerase PCR and primers *bd0635-F* and *bd0635-R*. Lane 1, 20 are 1 Kb ladder. Lane 2-17 are potential knockout show band of wild HI that seen at 1641 bp. Lane 18 and 19 are HD100 control and *pk18 Δ cpoB_{Bd}*.

As a result of HD screening of $\Delta cpoB_{Bd}$, the construction of *cpoB_{Bd}* was conjugated in the wild type *B. bacteriovorus* HI strains (HID13, HID22 and HID26) and grew cells as host- independent cultures and screened for gene replacement and plasmid loss using 5% sucrose. All the exconjugants were sensitive to kanamycin and 133 broths were screened (from three different conjugations) using Dream

Taq colony PCR for initial testing to try to obtain a HI deletion strain of this gene. All of the exconjugants that were screened gave the wild type band of 1641 bp, which indicated that the exconjugants had reverted to wild-type strains and no mutant strain can be obtained. This suggested that the deletion of *cpoB_{Bd}* was not possible under either HI or HD growth conditions (Figure 8-26 B).

As a much higher numbers of prey-lysates and broths were unsuccessfully screened to try to obtain the mutant strain of *cpoB_{Bd}* than is required to get other *Bdellovibrio* mutants in either HI or HD growth; it simply suggests that CpoB_{Bd} is essential in both growth modes of *B. bacteriovorus*.

This suggests that CpoB_{Bd} senses changes in *Bdellovibrio* envelope resulting from it entering prey as it squeezes through the pore in the prey PG (Evans et al. 2007), possibly using the Tol-Pal proteins. Then it regulates plug formation via PBP1B and closes this hole. CpoB_{Bd} may also play a regulatory role in cell division in HI (and possibly HD) lifestyles. Therefore *cpoB_{Bd}* may be vital for viability for several reasons, as a mutant would not allow the hole to seal, causing diffusion of resources away from inside the prey, and also affecting growth/replication of *Bdellovibrio*.

8.11 Discussion

In Gram-negative non- predatory bacteria, the role of CpoB was not clearly understood until Gray et al. (2015) suggested the role of CpoB is responsible for coordinating of the PBP1B activity and the Tol system to maintain effective PBP1B function and keeping cell envelope integrity during division. Also, it had previously proved impossible to get viable PBP1A mCherry tag gene and PBP1B mCherry tag, mean up there was no way to study peptidoglycan activity directly but CpoB was useful as it is known regulator of PBP1B in non- invasive bacteria.

Bioinformatics analysis showed that CpoB_{Bd} is conserved in all the *Bdellovibrio* family and also found in most bacteria and location of CpoB_{Bd} in *B. bacteriovorus*

is in periplasm and it has a signal peptide. Interestingly, the results of the fluorescent time course of the differences in *B. bacteriovorus* CpoB_{Bd}- mCherry versus the location of *B. bacteriovorus* known cytoplasmic bd0064-TFP showed a possible CpoB secretion role into prey which is unique to predatory bacteria.

The result of the RT-PCR transcriptional data profile showed that the expression of *cpoB_{Bd}* is continuously expressed during predatory lifecycle with increased in expression level in 15-30 mins when *Bdellovibrio* start to attach to the prey and form bdelloplast and level of expression also increased in 1hr-2hr when the bdelloplast start to elongate to form sausage shape before the division and progeny formation (Figure 8-10). This profile could fit with CpoB_{Bd} having a regulatory role in peptidoglycan synthesis and modification of the predator and the prey cell walls at different times (Early time 15- 30 mins plug formation post infection and 1- 2 hrs self-wall modification for *Bdellovibrio* growth-cell prior to septation).

The fluorescent microscopy confirmed that the CpoB_{Bd} was expressed during all stages of predatory life cycle that matched the RT-PCR transcription (Figure 8-10 and 8-19). There was an obvious fluorescent spot patch forming a possibly plug-like sphere at site of *Bdellovibrio* entry to the prey. The dual fluorescent colour of *Bdellovibrio* time course results (Figure 8-20) revealed that the fluorescent spot patches like plug at site of *Bdellovibrio* entry to the prey cell is formed by CpoB_{Bd} and likely its associated proteins (such as PBP1B and Tol Pal?) and its intensity being high at the prey envelope patches and lower in growing bdelloplast suggested that this protein is required for closed the entry hole. Furthermore, the location of CpoB_{Bd} -mCherry at 30 mins, when *Bdellovibrio* that still not completed its entry to prey cells, in both mCherry and the dual colour fluorescence time courses (Figure 8-21 and 8-22) showed that spot of the fluorescence in the "tail" of the *Bdellovibrio* which indicated that a robust vesicular structure containing the CpoB_{Bd} fluorescence might be released from the flagellar pole at the "tail" of

Bdellovibrio. All fluorescence data suggested that this CpoB protein is associated with a prey envelope pore-filling and rebuilding role to close the hole, that the *Bdellovibrio* made it to enter the prey cells and start the predatory lifecycle. The fate of this fluorescent spot after the end of the predatory cycle was indicates that these fluorescent spot-compartments were robust- most likely they have cell wall as well as being membranous vesicles that contain the CpoB (Figure 8-23).

Remarkably, the data from Figures (8-24 and 8-25) showed a lot of fluorescence patches within the HI cells, increasing in long cells indicated that the mCherry fusion affected *cpoB_{Bd}* function and HI cell division (vegetative and also in rounded self-bdelloplast) and its usage more in the HI than HD and this is fitted with the results of HI RT-PCR profile that showed level of expression of *cpoB_{Bd}* upregulated in HI growing cells, and this confirmed also Microarray data.

Interestingly, despite screening high numbers of predatory lysates, no deletion mutations can obtained with axenic growth in *cpoB_{Bd}* (Figure 8-26 A and B), these results suggested that *cpoB_{Bd}* is essential in both HD and HI lifestyle mode. In non- predatory bacteria like in *E. coli* deletion of CpoB is possible but results in outer membrane blebs with periplasmic leakage with increased sensitivity to Beta lactam antibiotics (Gray et al. 2015). In *Bdellovibrio*, CpoB activity may be require for a specialised membrane bleb to form, possibly with an internal cell wall, and that this is may be associated with the invasion and sealing inside prey of the predatory bacteria.

8.12 Conclusions

The results of this chapter have confirmed the very important role of CpoB_{Bd} protein for *Bdellovibrio* prey-entry, and it is essential for its predatory behaviour and also in Host- independent life. The result of this chapter clearly showed the following:

1. CpoB_{Bd} plays a role in *Bdellovibrio* predatory life cycle through likely regulating the forming of the plug that closes the hole that *Bdellovibrio* made when it enter into the prey cell so *Bdellovibrio* able to signal and be nourished inside prey without loss of significant materials leaking away, so it can finish the predatory life cycle.
2. The dual colour *Bdellovibrio* *cpoB_{Bd}-mcherry-bd0064-teal* showed clearly that CpoB_{Bd} is involved in making fluorescent plug that is seen in the predatory life cycle.
3. The 30 mins location of the CpoB_{Bd} -mCherry in non-completely fully entered *Bdellovibrio* revealed that fluorescent spot patches are found in non- invading end of *Bdellovibrio*. This suggests that this protein has a filling in and rebuilding role to close the entry hole that made it start its predatory life cycle. Interestingly this is the place where *Bdellovibrio* has its flagellum and there are reports of flagellar shedding in *Bdellovibrio* on prey entry (Lambert et al. 2006, Evans et al. 2007, Iida et al. 2009).
4. HI *B. bacteriovorus* CpoB_{Bd} -mCherry strain showed a lot of fluorescent patches spots and their place not colocated with DAPI stain which might suggest that this protein also has a role in cell division of filamentous (septum) cells and it is also seen in rounded cell structures (autobdelloplast) were it could be having a role in division or structure of entry vesicles if *Bdellovibrio* use normal predatory entry to these self-structures.

5. CpoB_{Bd} plays a vital role in both HD and HI lifestyle, and its deletion is lethal.
6. As Δ PBP1B and PBP1B tag mcherry are not possible to follow by tagging, CpoB is a possible window onto studying and understanding the PBP1B activity.

8.13 Future work

1. In order to understand the network that supports function of CpoB_{Bd}, protein-protein interaction and bacterial two-hybrid assays are required, (using proteins with signal sequences removed to allow B2H study inside *E.coli*) to discover what other proteins play a role in making the patches plug in the prey cells.
2. To clarify what will happen to the fluorescent spot patches, an exit video is required to see the fate of the patches exactly as the prey bdelloplasts are lysed by newly formed *Bdellovibrio* progeny.
3. Pull-down experiments of C-terminally His-tagged CpoB_{Bd} is needed to identify any possible partners that may play a role in plug formation, as *Bdellovibrio* lack genes encode both LpoA and LpoB. If there are any other proteins playing that role, and they are still to be discovered.
4. Construct a N-terminal PBP1B fluorescence tag to see if it is viable and its location during both predatory and host-independent lifestyle.
5. Label cultures of prey being invaded by fluorescent *Bdellovibrio* CpoB_{Bd}-mCherry with fluorescent D-amino acids (FDAAs) as in Kuru et al. (2017) to see if the CpoB-containing spheres spots released after lysis the prey cell contain cell wall material or just cell membrane.
6. Membrane stain is required to visualise membrane regions that cover the fluorescent spherical plug patch, in the *E. coli* outer membrane to see if

they are bounded/surrounded/encircled by *E. coli* outer membrane only or by *Bdellovibrio* membrane only or by both *E. coli* and *Bdellovibrio* membranes

9 Chapter Nine: Overall Discussion

Pioneering work on the *B. bacteriovorus* diguanylyl cyclases (DGC) by Hobley et al. (2012) revealed that *Bdellovibrio* have four of the GGDEF domain proteins that they are part of c-di-GMP network that controls essential functions in bacteria and *B. bacteriovorus* HD100. Hobley and co-worker (2012) worked on *Bdellovibrio* GGDEF domains identifying that for three of these GGDEF domains when each one of them was deleted it resulted in phenotypic effects like lost predation ability for DgcB, lost gliding motility for DgcA or inability to convert from HD to HI lifestyle for DgcC. In contrast to what might be expected from this, the deletion of fourth GGDEF domain DgcD resulted in no obvious phenotype. Furthermore, when Hobley and co-workers tested the predation ability of Δ *dgcB* mutants, they found that this strain was unable to clear the prey cells from the culture after 24 hrs in comparison to the wild type strain of *Bdellovibrio* that was able to kill the prey (*E. coli* S7-1) within 24hrs.

An unexpected finding later, was that when they left cultures of Δ *dgcB* mutants that included prey cells (*E. coli* S17-1) for 72 hrs they found that Δ *dgcB* mutant apparently started to kill the prey cells and form bdelloplasts. Hobley and co-workers suggested that there might be genetic suppression happening in the genome of this mutant and they named the new strain that resulted from Δ *dgcB* mutant as a *Suppressor* strains. *Suppressor* strains that were able to kill the prey were sent for genome sequencing by Simon King and David Milner. As it was known at that time (2011) genome sequencing was not very efficient and the initial results of genome sequencing revealed that *Suppressor* strains still had the same *dgcB* mutation (the deletion *dgcB* was still there) but there were point mutations in another two genes *asKa* (*bd0134*) that encodes a key enzyme in DAP/Lysine pathway and *bd0467* gene has transposase homology. Consequently, my PhD project was drawn dependent on the results of initial genomic sequencing of this strain.

Thus my project consisted of six parts. First part: discovering what happened in the *Bdellovibrio* genome to produce the Δ *dgcB* suppressor strains and is the *Suppressor* strain (derived from predation upon *E. coli* S17-1) produced in all types of prey cells. Second part: testing the ability of *Suppressor* strains to prey on different types of prey and if they are producing more or less c-di-GMP. The third part: evaluating the effect of the point mutation in *bd0467* through deletion of this gene and see effect of this deletion on *Bdellovibrio* predation ability and plaque formation. The fourth and fifth part: evaluating the effect of the point mutation in *asKa* (*bd0134*) on predation ability and plaque formation, testing the effect of deletion of this gene on predatory lifecycle, testing the effect of addition extra copy of *asKa* (*bd0134*) on plaque formation ability of Δ *dgcB* mutant and *Suppressor* strain, Also, investigating some other genes of DAP/Lysine synthesis pathway whose products play roles in PG formation and asking if they have any role in DAP addition during invasion. The final additional part of this project was about the modification that occurs in prey cell wall during the entry of *Bdellovibrio* to form entry pore.

9.1 DgcD point mutations are the real reason for the suppression in Δ *dgcB* mutant strain

Prior to this work, the predation ability of Δ *dgcB* mutant strain was not tested extensively and broadly after 72 hrs using different types of prey. The first part of this project has answered a lot of the aspects including can the suppressor emerge from long term incubation with all different types of prey (*E. coli*, *A. baumannii*, *P. putida*, *S. marcescens* and *S. typhimurium*), can the suppression emerge from long term incubation with all different types of prey (*E. coli*, *A. baumannii*, *P. putida*, *S. marcescens* and *S. typhimurium*) on lawn prey plates, how and when suppressors happen and dominate in the culture. Also, testing the possibility of occurrence of suppression in genome of Δ *dgcB* mutant strain on prey lawns plate through formation plaque.

What has been demonstrated in this project that DgcB is essential for predation and my present findings are consistent with what Hobley and co-workers found previously. There was no predation happening during the first 24 hrs with different types of prey. It is interesting to note that the attachment ability of Δ *dgcB* mutant strain has now been tested by me and they were able to attach to prey cells but in lower percentage than control strain. This indicated that the deletion of *dgcB* did not affect the attachment ability of this mutant. Furthermore, the finding from this study was reported that the suppression in genome of Δ *dgcB* mutant strain took place after 48 hrs and some bdelloplast (rounded prey cells) were starting to appear in cultures of the mutant and the culture become mostly full with bdelloplasts at 72 hrs and by 96 hrs culture was mostly full with *Bdellovibrio* with few remaining bdelloplasts with most prey lysed.

Due to improvement in depth of genetic sequencing from era of Hobley and co-worker up to date, all *Suppressor* strains that resulted from prolonged incubation period (from my project and previous Hobley and co-worker strains) were sent for extensive genome sequencing. One of the remarkable findings in genome sequencing data that all suppressor strains (including previous send strains that apparently with only *askA* (*bd0134*) and *bd0467* mutations) had point mutations in the same gene that is *dgcD* (one of the GGDEF domain gene and the only one that did not produce any phenotype when deleted from *Bdellovibrio* genome), the mutation were in coiled coil and transmembrane domain regions but affected different amino acids. In addition, all *Suppressor* strain had point mutations in *bd0467* gene that has transposase homology. Also, some *Suppressor* strains (Simon King's strains) had additional point mutations in *askA* (*bd0134*) the key enzymes in DAP/Lysine that is involved in cell wall synthesis.

The results of the genome sequencing raised the question: Do all the point mutations in the genome sequencing data have a role in emerging the *Suppressor* strains or does one of them have the role. Therefore, this study tested validity of

the genome sequencing data results through testing these point mutations and asking if they are the real cause that led the *Suppressor* strains to lyse some prey types or not. My project confirmed that the point mutations in the *dgcD* gene that were found in the *Suppressor* strains were the real reason that led to lysis of *E. coli* S17-1 and *A. baumannii*. Additionally, these point mutation produced a DgcD that synthesised more c-di-GMP that affected the swarm motility of *E. coli* MG1655.

9.2 DgcD point mutations occurred in some prey types (*E. coli* and *A. baumannii*) not in all types make *Bdellovibrio* selective predator

The predation ability of *B. bacteriovorus* HD100 was tested a lot previously and it has the ability to kill a broad range of Gram- negative bacteria (Willis et al. 2016, Baker et al. 2017). One unanticipated finding from my project was that the suppression in the genome of Δ *dgcB* by mutations in *dgcD* gene were occurred with specific types of prey (*E. coli* S17-1 and *A. baumannii*) but not with other prey types (*P. putida*, *S. marcescens* and *S. typhimurium*) after prolonged incubation period (72 hr or more).

Notably, the *Suppressor* strains also able to kill *E. coli* S17-1 but not with *P. putida*. This meant that the suppression when occurred allowed *Bdellovibrio* to become a selective predator. Taking all these observations together, it is possible that the *dgcD* point mutations happened in specific types of prey cells due to a specific content in the cell envelope of that type of prey which not found in the other or some types of the prey cells secreted specific enzymes that prevent the *dgcD* point mutations from being selected for and become dominant.

9.3 The effect of the *dgcD* point mutation specific for Δ *dgcB* mutant not for other diguanylyl cyclases mutants

All aforementioned evidences above demonstrate that the point mutations in *dgcD* arose several times independently, in the absence of *dgcB* gene in *Bdellovibrio*, in attack phase in the presence of prey. These mutations were required for predation in the absence of *dgcB*.

These findings led to ask a vital question that needs to be answered: Is the hidden relationship that found between DgcD and restoration of predatory invasion for a Δ *dgcB* mutant strain also seen with phenotypes of other *Bdellovibrio* diguanylyl cyclase mutants? Therefore, the point mutated* and wild type forms of *dgcD* were conjugated as extra copies into both the *dgcA* mutant and *dgcC* mutant strains. The results observed that extra copies of the wild type *dgcD* and Δ *dgcB*-suppressing point mutations in *dgcD* were able to rescue these mutants from the phenotype that results from deletion of *dgcA* or *dgcC* genes. This suggests that making more c-di-GMP generally could compensate for the specific deletion of a *dgc* genes. However the wild type *dgcD* extra copies worked alone in the physiological conditions that the Δ *dgcC* mutant and Δ *dgcA* mutant strains were growing (Figure 9-1). This did not work for the Δ *dgcB* mutant strain physiological conditions. This suggests that there may be a hidden cross-talk between growth phase physiology and DgcD activity. It seems possible that a signal which activates and dimerizes the GGDEF domains of DgcD is not present in the growth conditions of *Bdellovibrio* with prey added (where the Δ *dgcB* mutant strain was in the selection for suppressors) and so the DgcD * changes were selected for in those conditions. (Unpublished genome sequencing by Sockett laboratory of a single Δ *dgcC* suppressor shows no mutations in *dgcD* gene)

9.4 Effect of addition extra copy of *bd0134* to Δ *dgcB* mutant and Suppressor strains

The ability of plaque formation for Δ *dgcB* mutant strain was tested in this project also to see if suppression can occur on solid surface or occur only in liquid culture. The results showed that the Δ *dgcB* mutant strain had lost their ability to produce plaques and my present findings are matched with what Hobley and co-workers found previously even use different types of prey and prolonged incubation period. This mean that suppression do not occur on solid surface but only in liquid culture. Furthermore, the plaque formation ability for *Suppressor* strain was tested and the unexpected finding that the suppressor strain which did now lyse *E. coli* S17-1 in liquid culture was unable to form plaque on solid media (containing an *E. coli* S17-1 in prey lawn) even though there was prolonged incubation of the plate. There are two possible explanations for these finding either osmolarity on solid surface higher than in liquid culture so there was no selection for *dgcD* point mutations like what happen in liquid culture, or deletion of the *dgcB* made these strains loss gliding ability on solid surface which is important for *Bdellovibrio* to escape and exit from the prey cell to infect a new prey cells in lawn plate.

Surprisingly, the addition of an extra copy of *bd0134* to these strains resulted in the unanticipated finding where both *dgcB mutant* and *Suppressor* strains started to form plaques in a shorter time period. This result suggests a strong link was exist between *asKa* (*bd0134*) and plaque formation in the plates. This effect is contributed to by overexpression of *asKa* (*bd0134*) that results from addition of the extra copy of *asKa* (*bd0134*).

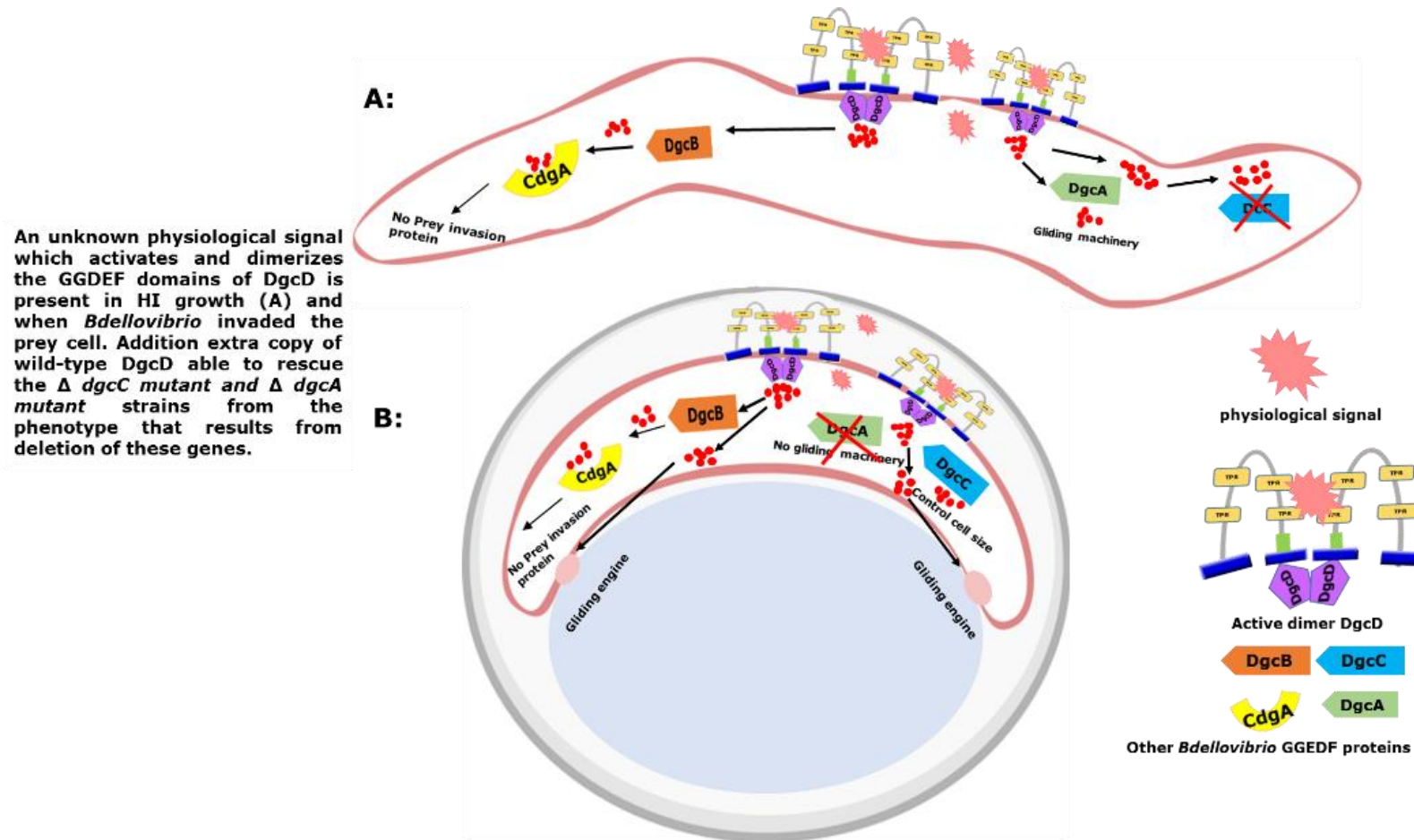


Figure 9. 1: The effect of addition of wild type (WT) version of DgcD to, A: $\Delta dgcC$ mutant strain that previously unable to grow as HI culture and addition of extra copy of WT DgcD enables this strain to grow as HI culture B: $\Delta dgcA$ mutant strain that previously unable to escape from prey cell and exit to finish the predatory cycle and addition the extra copy of WT DgcD enables this strain to escape and exit from prey cell. This because the growth conditions here activate a physiological signal that may dimerise and activate WT DgcD (via TPR domains probably). The outer membrane was not drawn in these cells for simplicity.

The possible explanation for all findings that obtained from *dgcD* point mutations is the following: DgcD is inactive monomer protein in attack phase and it might become activated DgcD dimer when growth occurred naturally for *Bdellovibrio* during predatory growth inside bdelloplasts or HI growth, but we have never detected this experimentally. This may involve natural activation-dimerization of TPR domains of DgcD caused by a physiological signal not present in attack phase. When the suppression of Δ *dgcA* mutant and Δ *dgcC* mutant strains were tested in both growth phases (trying to grow as an HI or escaping prey after growing in bdelloplast). When testing Δ *dgcB* suppression, the *Bdellovibrio* were in non-growing attack phase attached to prey and trying to invade- but not growing.

However, in Δ *dgcB* mutant strain, the DgcD requires conformation-changing point mutations to form active GGDEF domain dimers and make more c-di-GMP as an alternative pathway. In different physiological conditions a "signal" may naturally switch on DgcD probably dimerizing it naturally via its TPR domains and then this changing the conformations of transmembrane domain/coiled coil regions but the point mutations cause the GGDEF dimerization artificially. This is why extra copies of wild type DgcD alone works in the Δ *dgcA* mutant and Δ *dgcC* mutant strains because the signal is present in their growth conditions but not in attack phase where the Δ *dgcB* mutant strain is "stuck" by its mutation. Only the Bd3766* changes can "save" Δ *dgcB* mutant strain as the physiological signal to dimerise and activate them is not present in attack phase. The signal might not be a protein- DgcD came into *Bdellovibrio* possibly by horizontal transfer as alone gene without another protein coding gene in an operon with it (Figure 7-1). DgcD may sense a natural metabolite associated with growth at its TPR domains.

In the *E.coli* motility assay, DgcD is alone in the *E.coli* and they do not have the same "signal" as the *Bdellovibrio* growth physiology while they are in swarm plates, so the DgcD wild type makes less c-di-GMP (or none) compared to the

DgcD* mutants in *E.coli* and the swarm is bigger for DgcD WT versus DgcD* mutants.

Loss of the plaques formation also resulted from the deletion of *dgcB* but the *dgcD* point mutations do not restore the plaque formation. The restoration of the plaque formation happened through adding the extra copy of *asKa* (*bd0134*). The possible explanation for this could be more products of the DAP/Lysine pathway were formed and that effect on restoring the plaque formation, possibly by stabilising the cell wall for some osmotic condition in plates and not liquid culture.

The empirical findings from this project provide a new understanding of how one diguanylyl cyclase gene can compensate the absence of another diguanylyl cyclases and cover the phenotype that results from that absence.

9.5 Model of the point mutation in DgcD that control the predation of *Bdellovibrio* Suppressor strain using data from this study

1. *Bdellovibrio* normally invade and grow inside other bacteria and form plaques. In attack phase, DgcD is an inactive monomer and does not make the c-di-GMP. Deletion of *dgcB* led to *Bdellovibrio* grow as HI and lost its ability to invade the prey cells or form plaques on the plates of prey lawn after long period of incubation (Figure 9-2).
2. When Δ *dgcB* mutant strain was left in attack phase in liquid cultures for more than 48 hrs *Bdellovibrio* started to invade the prey and form bdelloplast. This happened due to point mutation in *dgcD* that causing change in Bd3766 coiled coil region (in green region *) or transmembrane domain region (in blue region *) that forced a dimer of GGDEF domains to form in all growth conditions even without the presence of signal interact with TPR and makes an active dimer of GGDEF domains and this makes more c-di-GMP in predatory condition.

This compensates for lack of c-di-GMP from deletion of *dgcB* in suppressor strain and the predation resumes (Figure 9-3).

3. Addition extra copy of *askA* in suppressor strain results in restore the plaques. This might be due to more product of DAP/Lysine pathway that effect on the plaque formation ability possibly by strengthening cell wall of *Bdellovibrio* or the prey that it work on (by adding DAP) (Figure 9-4).

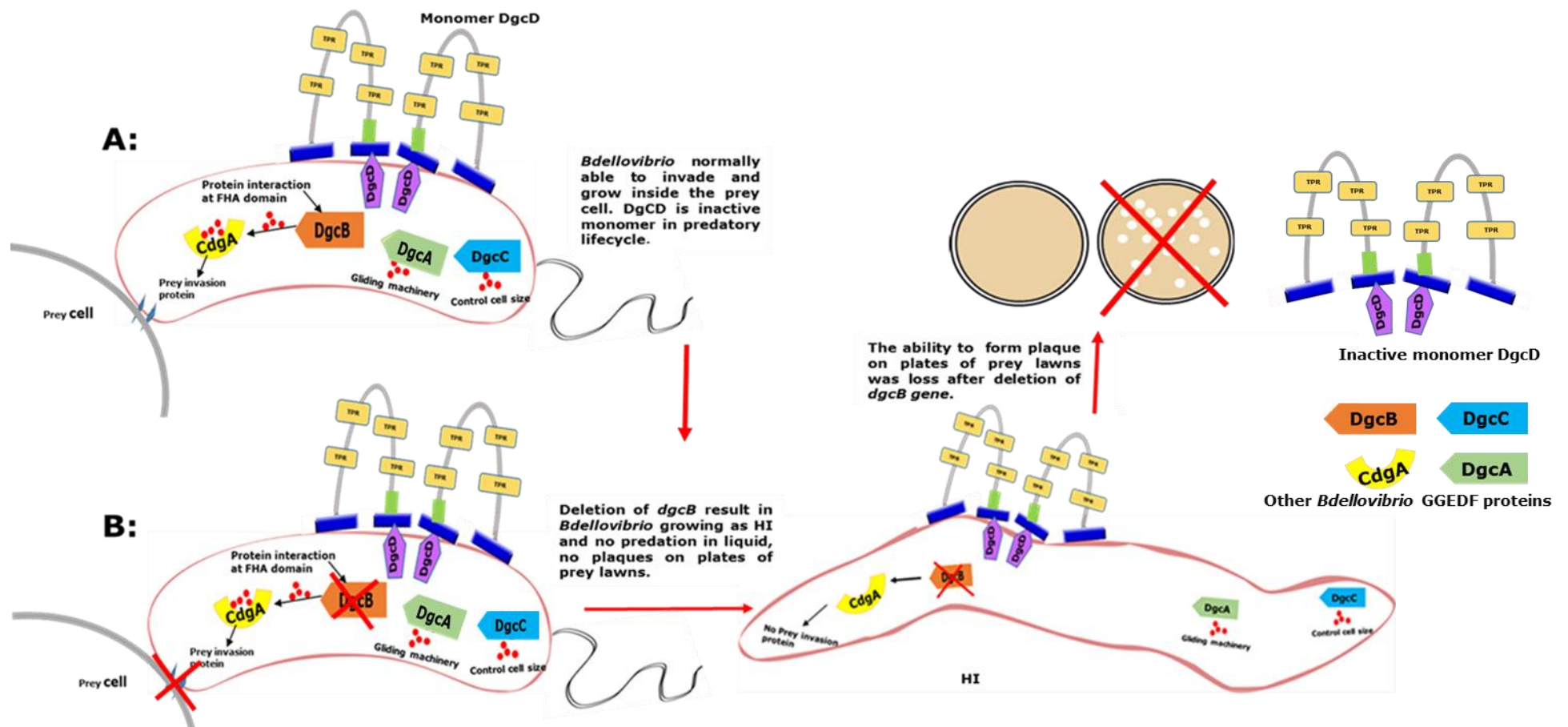


Figure 9. 2: The effect of deletion of *dgcB* gene on the predatory lifecycle and plaque formation. A: *Bdellovibrio* able to grow and divide inside the prey cell and also able to form plaque on the prey plate lawns. B: Deletion of *dgcB* led to *Bdellovibrio* unable to grow as HI and lost the predation ability with loss of plaque formation ability on the prey plate lawns. The outer membrane was not drawn in these cells for simplicity.

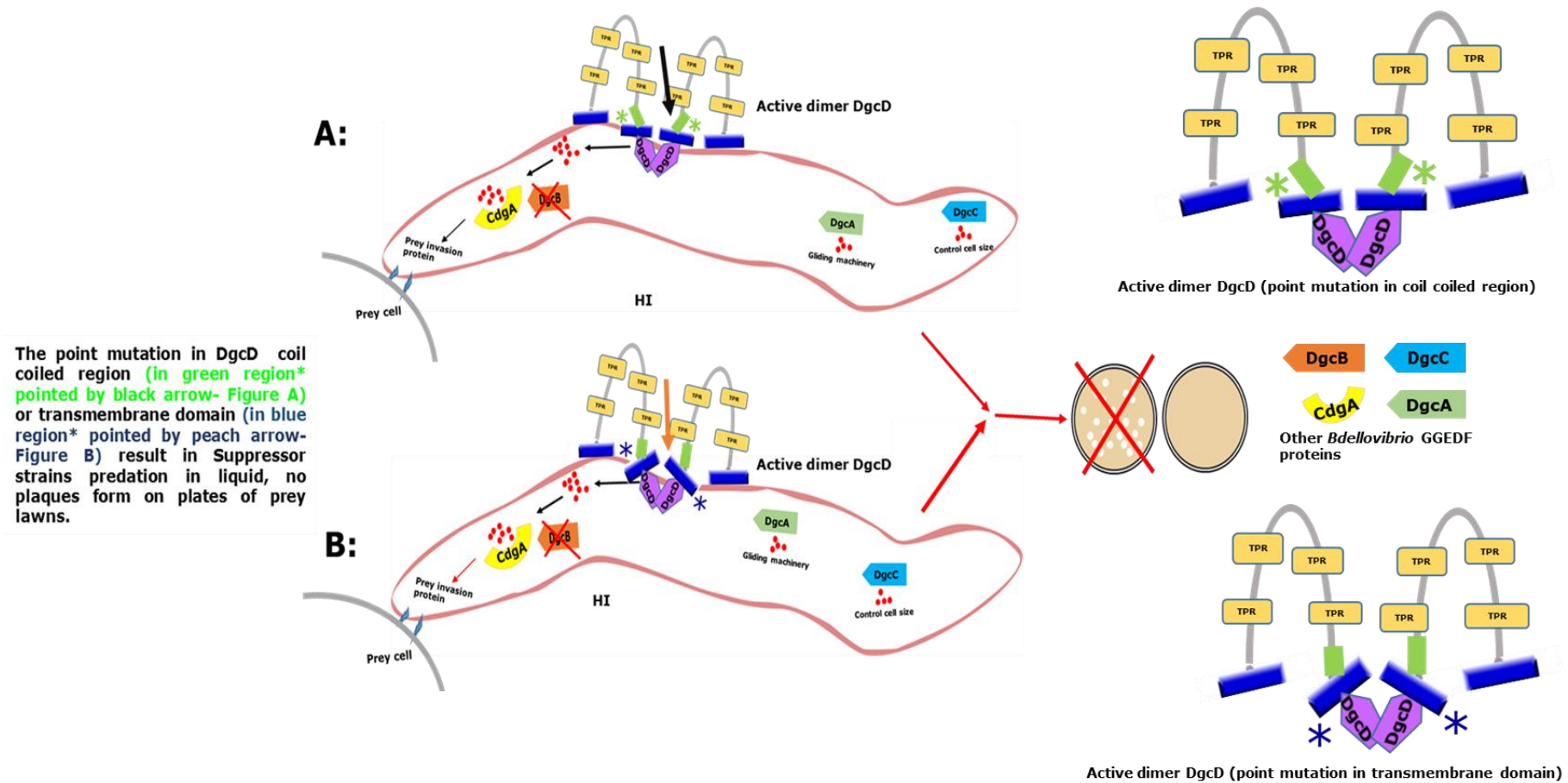


Figure 9. 3: The effect of point mutation in *dgcD* on Suppressor strains predation ability and plaque formation. A and B: The point mutations affected in DgcD coiled coil region (in green region* pointed by black arrow- Figure A) or transmembrane domain (in blue region* pointed by peach arrow- Figure B). The point mutations affecting DgcD play roles in regaining the predation ability of *Bdellovibrio* that was lost by deletion of *dgcB* gene. The outer membrane was not drawn in these cells for simplicity.

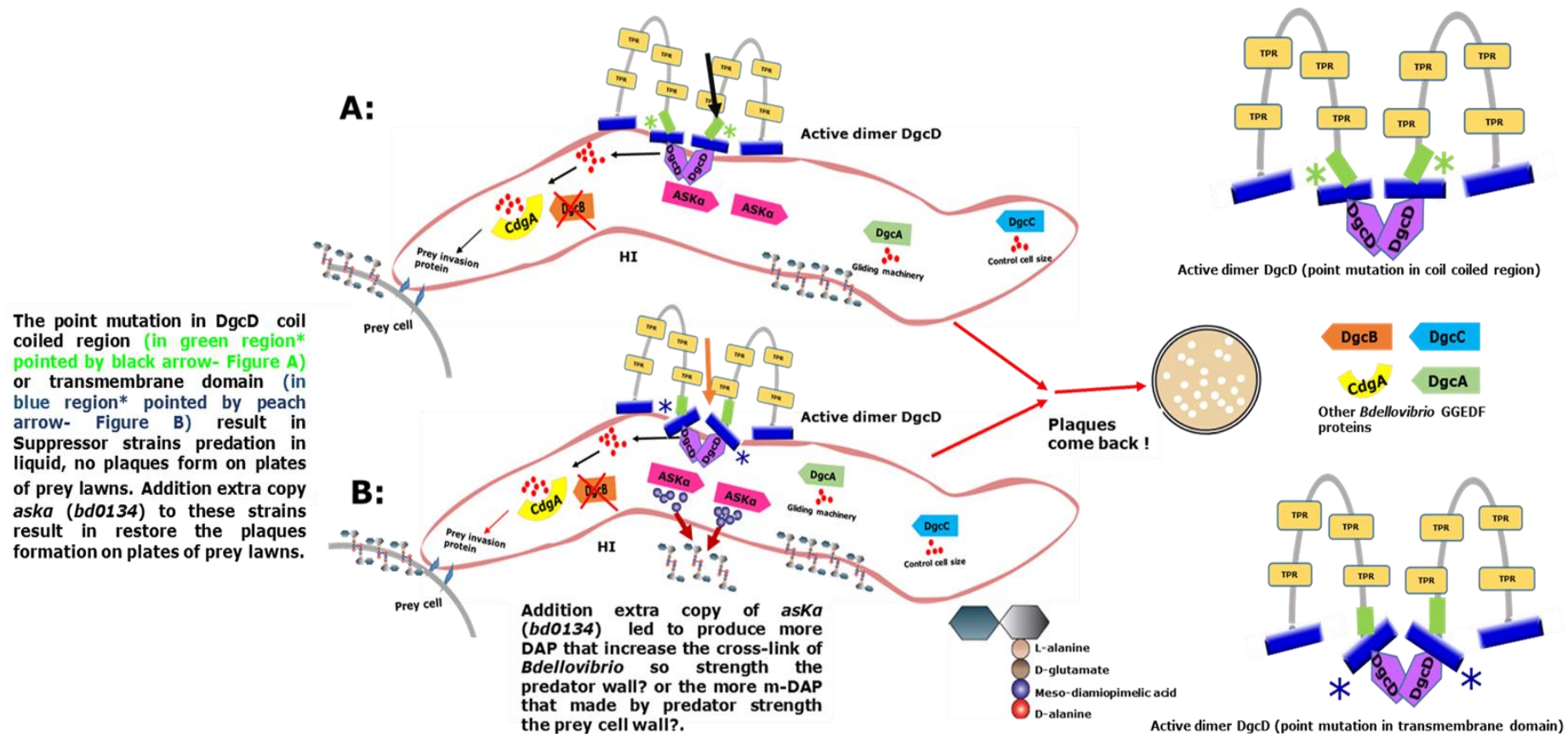


Figure 9. 4: The effect of adding extra copy of *Aska* to *Suppressor* strain (that contain Δ *dgcB* suppressing point mutations in *dgcD*) on the plaque formation of these strains on plates. A and B: The point mutation occurred in DgcD coiled coil region (in green region* pointed by black arrow- Figure A) or transmembrane domain (in blue region* pointed by peach arrow- Figure B). After adding extra copy of *aska* (*bd0134*) led to restoration of the plaque formation ability. The outer membrane was not drawn in these cells for simplicity.

The other aspect that touches on this project were evaluating the effect of the other point mutations in *bd0467* and *askA (bd0134)* found in results of the genome sequencing data. The point mutations in *bd0467* and *askA (bd0134)* were tested by deletion of *bd0467* and introduction of double cross over of point mutations in *askA (bd0134)*. The resultant strains still predator and able to produce plaques normally. Comparing the two results, it can be seen that these point mutations are phenotypically silent (without the *dgcD suppressor* mutations) and these mutations do not showed any potential suppressive effect on the predation or plaques formation of *Bdellovibrio*. Therefore, these point mutations seems do not have any role in delay or loss of predation ability that happened in *Δ dgcB mutant* or *742 Suppressor* strains.

Finally, in spite of much new knowledge about the role of DAP/Lysine synthesis pathway in non- predatory bacteria but till now no one has a close look for this pathway in predatory bacteria like *Bdellovibrio* to find out if this pathway participate only in maintaining the *Bdellovibrio* wall a lone or a role in predation for prey wall changes or both *Bdellovibrio*- prey walls. Four genes in DAP/Lysine synthesis were studied in this project (duplicated the key enzymes *aska* and *askβ* at start of pathway and one in the middle *dapB* of the pathway and one *dapE* before end of DAP/ lysine synthesis). The whole genome sequences of *B. bacteriovorus HD100* revealed that there are one pair of candidate genes: *aska (bd0134)* and *askβ (bd0528)* that their product shown role in predatory lifecycle in this study. The duplication in the ask genes were not found only in this *Bdellovibrio* pathway, duplication in the genes found with other genes that regulate important function in *Bdellovibrio* like flagella (Lambert et al. 2006), and DD-endopeptidase (Lerner et al. 2012) and GGDEF domains (Hobley et al. 2006). The duplication in some genes in *Bdellovibrio* may have a protective evolutionary effect for predatory cycle to continue in regular way, so no loss or inactivation of one gene can interrupt the cycle and or it might be that the presence of a gene

duplication in the *Bdellovibrio* genome can cover the absence of the other gene that are duplicated. This means that one is dominating housekeeping or another role but substitute in predation and the other is extra copy for predation. *aska* (*bd0134*) was obtained by *Bdellovibrio* by lateral gene transfer and *askβ* was ancestral gene. Both *aska* and *askβ* were studied in detailed and it were found that RT-PCR analysis *aska* and *askβ* showed the differing patterns of transcription. This project found that *aska* is not essential gene and its deletion delay a little bit the entry *Bdellovibrio* to prey cell. The deletion of this gene did not affects the HI life cycle. It is appeared that there was a cross- talk and correlation between *aska* deletion and *askβ* expression that proved through RT-PCR profile for *askβ* which dropped when *Bdellovibrio* was outside the prey cell using RNA isolated from *aska* mutant strain and this profile matched to RT-PCR profile of *aska*. In contrast *askβ* was essential and it was not deletable. It was evident that aspartokinase of *Bdellovibrio* might not play a role in invasion and they might have a role in maintenance in self or prey cell wall after invasion.

This project also was carried out to determine the role of *dapB* and *dapE* in both predatory and HI lifestyle of *Bdellovibrio*. These findings from *dapB* and *dapE* indicated both of them is expressed in both lifestyle of *Bdellovibrio* but what exciting was the location of fluorescent tag in DapB that had a central location in both HD and HI lifecycle. Additional to that, *dapE* is essential for *Bdellovibrio* like in non-predatory bacteria and its deletion was lethal. These findings from this study propose that *dapB* and *dapE* have a role in maintaining *Bdellovibrio* cell wall and this is associated with the central localization of *dapB* preparing DAP while for *dapE* its location is near the poles.

It is important to bear in mind that even though the advance level of knowledge that science reach till now but it is very difficult to see exactly what happened in DAP/Lysine pathway of *Bdellovibrio* as tiny bacteria like *Bdellovibrio* grow inside

other bacteria. Where products like DAP and c-di-GMP come from two bacterial cell types predator and prey.

Until recently, there has been not enough reliable evidence that about how the entry pore is formed in the prey cell to start the *Bdellovibrio* predatory cycle. Moreover, little is known about gene (s) that involved in entry pore and it is not clear who the PG modified in this situation. Therefore, a final purposes of my study is to shine new light on the entry pore formation through an examination one of the candidate proteins, CpoB that might be involved in this process. The results of my study show that CpoB plays a role at least in filling and rebuilding the entry hole of the *Bdellovibrio* used to enter the prey cell to start the predatory lifecycle, CpoB-mCherry labelled vesicle structure appears as a bright fluorescent patch at the place where the pore for entry is formed in prey cell. What is more, the deletion of *cpoB* was lethal for both lifestyles of *Bdellovibrio*.

These findings indicated that the CpoB participates either alone or more likely with help of other gene products such as CpoB to form the structures that close the entry hole. The deletion of *cpoB* was lethal which mean that this gene is essential and this is because that fact that the predatory lifecycle cannot continue without sealing the entry hole.

However, the exact mechanism by which the entry hole is formed is still not entirely understood but my work gives a new insight about the some genes that might participate in this complex process that need more investigation by following laboratory members.

Last but not least, my project also unveil about the autobdelloplast theory which is a possible mode of *Bdellovibrio* HI growth. Autobdelloplast theory is that a subpopulation of HI cells appear to round up at one pole, sometimes rounding up completely and resemble bdelloplasts with a filament inside. Sockett laboratory hypothesise (based on unpublished observations labelling these HI cells with fluorescent D-amino acids, showing active cell wall modification of these filaments) that these represent a novel means of cell division in these cells, mimicking filament division in bdelloplasts.

More than one gene participate in this theory as my projected revealed the participation of DapB, DapE, Bd0467 and CpoB additional to other genes that still not investigated but Sockett laboratory work on this project now .

After years of passionate work with *Bdellovibrio* to see what will be gained from this study, now I harvest my precious crops that I will give with all love and care to the next researchers in the laboratory.

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