Testing the effects of prey surface changes on *Bdellovibrio bacteriovorus* predation

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Abstract

Bdellovibrio bacteriovorus is an invasive bacterial predator of a wide range of Gram negative bacteria including many ESKAPE pathogens. It attaches to the outer membrane of the prey cell and invades into the periplasm of prey cells, ultimately killing the cell from the inside. The target for prey recognition on the outer membrane is currently unknown. *B. bacteriovorus* is predatory both *in vitro* and *in vivo* in zebrafish for several of these pathogens. However, some pathogens are known to adapt their membrane composition when stressed by host or other different environments. One such pathogen is *Acinetobacter baumannii*.

A. baumannii is frequently found to be multidrug resistant where resistance has developed to the drugs of last resorts namely the carbapenem and polymyxin classes of antibiotics. One way that *A. baumannii* does this is via the loss of lipooligosaccharide (LOS) from the outer membrane. LOS is a form of lipopolysaccharide (LPS) but without the O-antigen and a modified core oligosaccharide.

In a first part of the project, strains of *A. baumannii* with known outer membrane modifications from published work of the Trent lab were tested for changes in predation by *B. bacteriovorus*. It was previously hypothesised that the Lipid A component of LPS was a target for *Bdellovibrio* attachment, but surprisingly this work shows no significant change in predation in these LOS deficient mutants. These data therefore disproves Lipid A or the core oligosaccharide as the target for predation; future work should look at other conserved components on the Gram negative surface.

In the second part, *in vivo* studies in zebrafish were done via microinjection of a clinical strain of *A. baumannii* 11W359501 into the hindbrain. Previous work found that there was no *B. bacteriovorus* predation *in vivo* but occured *in vitro*. Therefore, tests for cell surface adaptation by the pathogen were undertaken, with RNA sequencing from samples of *A. baumannii* 11W359501 pre- and post-adaptation in host. Analysis of these RNA-seq data has elucidated the upregulation of genes encoding a potential type VI secretion system mechanism which may be responsible for killing *B. bacteriovorus in vivo*, abrogating its predatory effects. Future work could check the involvement of this system and further experiments are suggested.

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Thanks must also go to Prof Ian Connerton, who assembled the *A. baumannii* 11W359501 genome in record time for use in the project.

Finally, thanks to my parents for their support throughout this year.

Abbreviations

ABC	ATP binding cassette
ATCC	American Type Culture Collection
Bp	base pairs
CDC	Centre for Disease Control and Prevention
cfu/ml	Colony forming units per millilitre
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DHSC	Department of Health and Social Care (UK)
DNA	Deoxyribonucleic acid
g	grams
GFP	Green Fluorescent Protein
GPL	Glycerophospholipid
h	hours
IM	Inner membrane
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
M	Molar
Mbp	1,000,000 base pairs
min	minutes
ml	millilitre
OD	Optical Density
OM	Outer membrane
OMP	Outer membrane protein

PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PG	Peptidoglycan
PHE	Public Health England
PNAG	Poly-N-Acetyl Glucosamine
RNA	Ribonucleic acid
rpm	revolutions per minute
RTPCR	Reverse transcriptase polymerase chain reaction
s	seconds
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
WHO	World Health Organisation
WT	Wild type
°C	Degrees centigrade
µ	micro
n	nano
l	litre

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1.0 Introduction

1.01 Background – Antibiotic resistance

Resistance to antibiotics has become a widespread problem around the globe in the last few decades with many government and international programs attempting to curb the problem (DHSC, 2013, CDC, 2011). Bacteria have evolved to be multi-drug resistant via two general mechanisms – mutation and horizontal gene transfer (Martinez, 2014). The main transmission and dissemination of resistance genes throughout bacterial populations in the environment occurs through horizontal gene transfer and has been reported to spread through the following practices: the use of antibiotics as growth promotors in animal husbandry, discharge from antibiotic manufacturing and poorly treated sewage (Bengtsson-Palme *et al.*, 2018, Qiao *et al.*, 2018). This topic is especially important when looking at human pathogens as the ability to treat life threatening bacterial infections is diminished without antibiotics.

Of such pathogens, there are a group of bacteria that are of high clinical importance termed ESKAPE pathogens (Pendleton *et al.*, 2013). ESKAPE is an acronym of their bacterial names: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species. These pathogens have seen a rapid increase in antibiotic resistance due to the fact that they can escape the bactericidal effects of the antibiotics. Also, they can survive in the external environment outside the body where they meet other bacteria to acquire resistance genes from. Possessing multi-drug resistance genes, these pathogens are becoming increasingly difficult to treat and the fact that they are the top cause of nosocomial infections means that finding a way to combat these pathogens is a top priority.

My project investigates one of these ESKAPE pathogens – *Acinetobacter baumannii*.

1.02 *Acinetobacter baumannii* – The pathogen

1.021 Epidemiology

A. baumannii is a pleomorphic coccobacillus, Gram negative, opportunistic pathogen that is naturally found in soil and surface water (Howard *et al.*, 2012). It is depicted in Figure 1.021. It causes fatal pneumonia and bacteraemia with mortality rates ranging from 8-40% (Alsan and Klompas, 2010). Incidence of disease is more common in hospitalised patients, immunocompromised patients, people with open wounds or with medical devices such as catheters. Transmission is often via person-person contact or by contaminated surfaces (CDC, 2010).

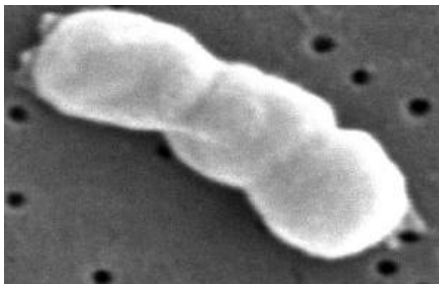


Fig 1.021. Electron micrograph of a chain of three *Acinetobacter baumannii*. Taken using a scanning electron microscope under a magnification of 2430x. These cells become spherical during stationary growth phase. Image from Center for Disease Control and Prevention, Public Health Image Library.

A 10 year *Acinetobacter* infections report in the UK determined between 1.3 to 1.6 instances of *Acinetobacter* related bacteraemia per 100,000 people from 2009 to 2018 (PHE, 2019). In the same report, *A. baumannii* was shown to be responsible for 18% of *Acinetobacter* bacteraemia cases with other notable species being *A. lwoffii* (33%) in 2018. Incidence of infection was higher in: working age (18-60 years old) compared to seniors (65+ years old); men compared to women; and a shorter hospitalisation period (>1 week but <1 month) (Vaze, 2013).

Treatment of *A. baumannii* infections is very difficult due to the pathogen's arsenal of antibiotic resistance mechanisms preventing the multiple drug actions from killing them (Section 1.023). Currently, treatment falls upon the carbapenem and polymyxins classes of antibiotics (Fishbain and Peleg, 2010). However, recent data have indicated resistance to have developed for these crucial antibiotics in some isolates of *A. baumannii* (Section 1.023)

As *Acinetobacter* is mainly spread via person-person contact, hand hygiene is an easy and important preventative measure to stop the spreads of infection. Disinfecting surfaces is also important as *A. baumannii* can survive for a long time in such environments (Section 1.022). Other methods of infection prevention include education of both healthcare workers and the general public and surveillance cultures of patients and environment during outbreak (Garnacho-Montero *et al.*, 2015).

1.022 Virulence factors

There are many virulence factors of *A. baumannii* which contribute to a range of functions, from host invasion to survival. The focus of this section is mainly on strategies *A. baumannii* employs to survive both *in vivo* and *ex vivo*. Figure 1.022.1 shows the *A. baumannii* cell surface and the different components and mechanisms in place which contribute to the virulence of the pathogen.

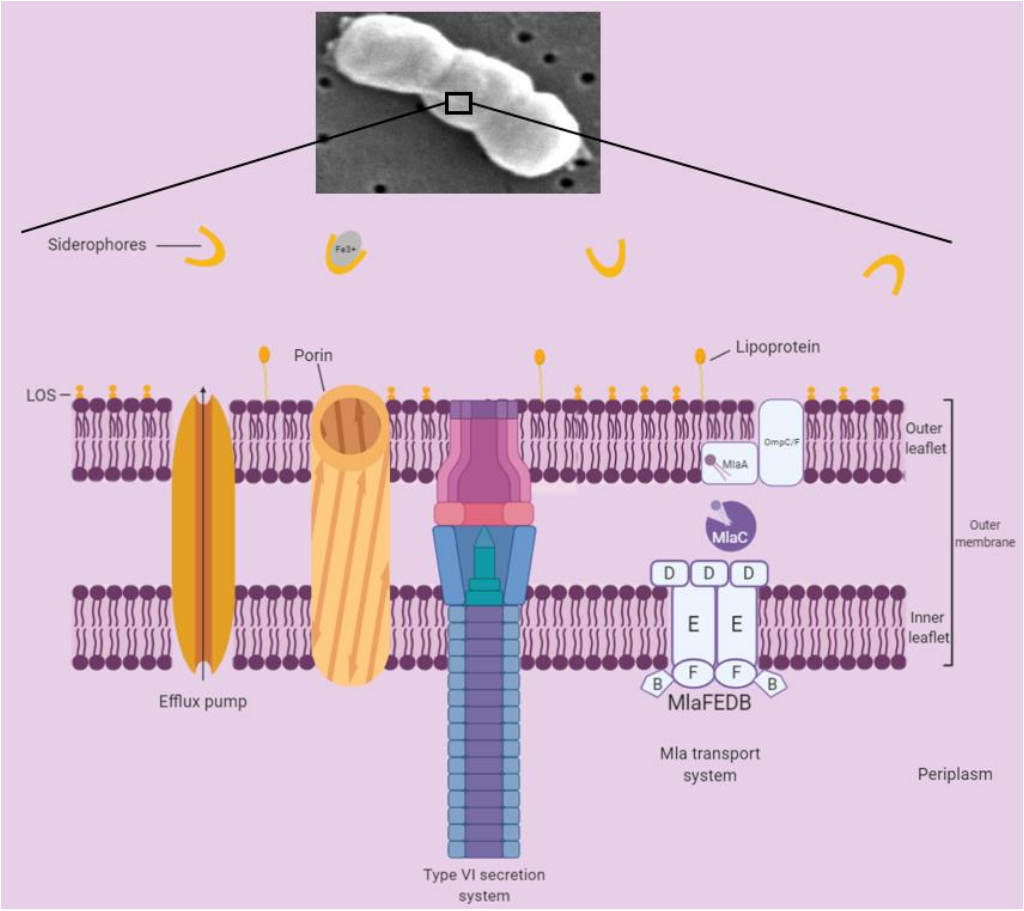


Fig 1.022.1 An overview of the *Acinetobacter baumannii* cell surface. Decorated on the outer leaflet of the outer membrane are lipooligosaccharide, lipoproteins, porins and efflux pumps. The type VI secretion system spans from the inner to outer membrane, which secrete effector proteins out of the cell. The Mla transport system plays a role in the replacement of glycerophospholipids.

Desiccation

A. baumannii, although a non-sporulating Gram negative bacterium, has a remarkable tolerance for desiccation with a number of mechanisms contributing to its survival. More recent studies have elucidated a protective role for exogenous trehalose on dry surfaces where *A. baumannii* cells could be detected after approximately one month (Zeidler and Müller, 2019). The two-component regulator BfmR has been reported to control expression of stress-related proteins such as the KatE catalase, which protects *A. baumannii* when dried as well as detoxifying hydrogen peroxide (Farrow *et al.*, 2018). The ability to survive for a prolonged amount of time on surfaces means that infections are mainly spread via the hands of healthcare workers and contaminated equipment (WHO, 2017).

Resistance to disinfectant

We have established that *A. baumannii* can persist in the environment for a very long time so cleaning and disinfecting surfaces that may be contaminated is very important. In hospitals, the disinfectant chlorhexidine is widely used to kill both Gram positive and Gram negative bacteria by disrupting their membranes. Some strains of *A. baumannii* have evolved to encodes the efflux pump AceI, which ejects chlorhexidine from the cell, promoting its persistence (Hassan *et al.*, 2013). Fortunately, there are other disinfectants tested such as propranolol and PVP-iodine, which are still effective against *A. baumannii* (Wisplinghoff *et al.*, 2007). These disinfectants however, may not have the wide range of effectiveness that chlorhexidine has on most other opportunistic bacteria in a hospital environment.

Siderophores

Iron is required in all living organisms as essential processes like respiration and DNA synthesis (Pantopoulos *et al.*, 2012). Bacteria secrete siderophores which are high affinity iron chelators which bind strongly to free iron ions to transport back through the bacterial cell membrane and used for the aforementioned processes. In the host environment iron is limited, therefore for pathogens to survive in such hostile conditions they must scavenge for it. Siderophores act as virulence factors for pathogens as they strip iron from host cells, which contributes to pathogenesis of the host. To combat this, host immune cells secrete lipocalin 2 which bind to siderophores to prevent the uptake of iron (Chakraborty *et al.*, 2012).

A. baumannii encodes a number of siderophores such as fimsbactin, baumannoferrin and most notably acinetobactin (Shapiro and Wencewicz, 2016). Acinetobactin is a siderophore which not only chelates iron but causes cell apoptosis and host killing (Gaddy *et al.*, 2012). Other studies have linked the role of siderophores and promotion of biofilm formation by ferric ions (Martinez, 2014). This shows that siderophores in *A. baumannii* aid not only in survival but are an integral part of the infection process.

Therapeutic targeting of siderophores has been suggested as an option due to their role in infection. These options include using therapeutic gallium to compete with iron and using engineered siderophores to enhance the uptake of antibiotics into the bacterial cell (Wilson *et al.*, 2016). The former acts as a competitive inhibitor to siderophores where they bind Ga^{3+} instead of Fe^{3+}. Gallium stops the iron dependent metabolic processes and kills the cell. However, the toxicity of this therapy would need to be taken into account, with mixed reports of low/high toxicity in animal models (Baldoni *et al.*, 2010, Antunes *et al.*, 2012). The second way conjugates antibiotics to the siderophores which is taken up by the pathogen. As most resistant bacteria stop uptake of antibiotics via the porin channel, this novel way of drug entry

through the siderophore-uptake system could potentially prove to be a therapeutic solution (Górska *et al.*, 2014).

Type VI secretion system

The type VI secretion system (T6SS) is a trans-periplasmic structure allowing bacteria to secrete protein effectors into, or puncture another bacterial or eukaryotic cell. This system is mainly associated with inter-bacterial competition but can also sometimes target eukaryotic cells (Hachani *et al.*, 2016). The T6SS can also act to bridge other virulence factors such as the uptake of resistance genes by natural competence (Veening and Blokesch, 2017).

The T6SS machinery comprises of three main sections: the membrane complex, the baseplate and the tube-sheath complex (Wang *et al.*, 2019). The membrane complex is made of TssJLM which spans the periplasm. TssJ is an outer membrane lipoprotein whereas TssLM are anchored to the inner membrane (Cherrak *et al.*, 2019). The membrane complex is suggested to undergo a conformational change to allow the passage of the VgrG spike and Hcp tube when the apparatus is triggered (Durand *et al.*, 2015). The baseplate is made of the VgrG spike and the wedge TssEFGK. VgrG is the tip of the needle apparatus which has a PAAR domain sequence at the very end used to dock effector proteins (Bondage *et al.*, 2016, Burkinshaw *et al.*, 2018). The tube-sheath complex is made of the Hcp tube, the TssBC sheath and the TssA cap. When the sheath contracts, the tube is pushed out through the cell envelope to deliver effectors out (Kudryashev *et al.*, 2015). A stopper TagA interacts with the TssA cap to stop polymerisation of the tail and supports the sheath after contraction (Santin *et al.*, 2018). After contraction, an ATPase ClpV disassembles the TssBC sheath by binding to a site on TssC which is only available in the contracted state (Douzi *et al.*, 2016). The whole structure of the T6SS machinery is depicted in Figure 1.022.2.

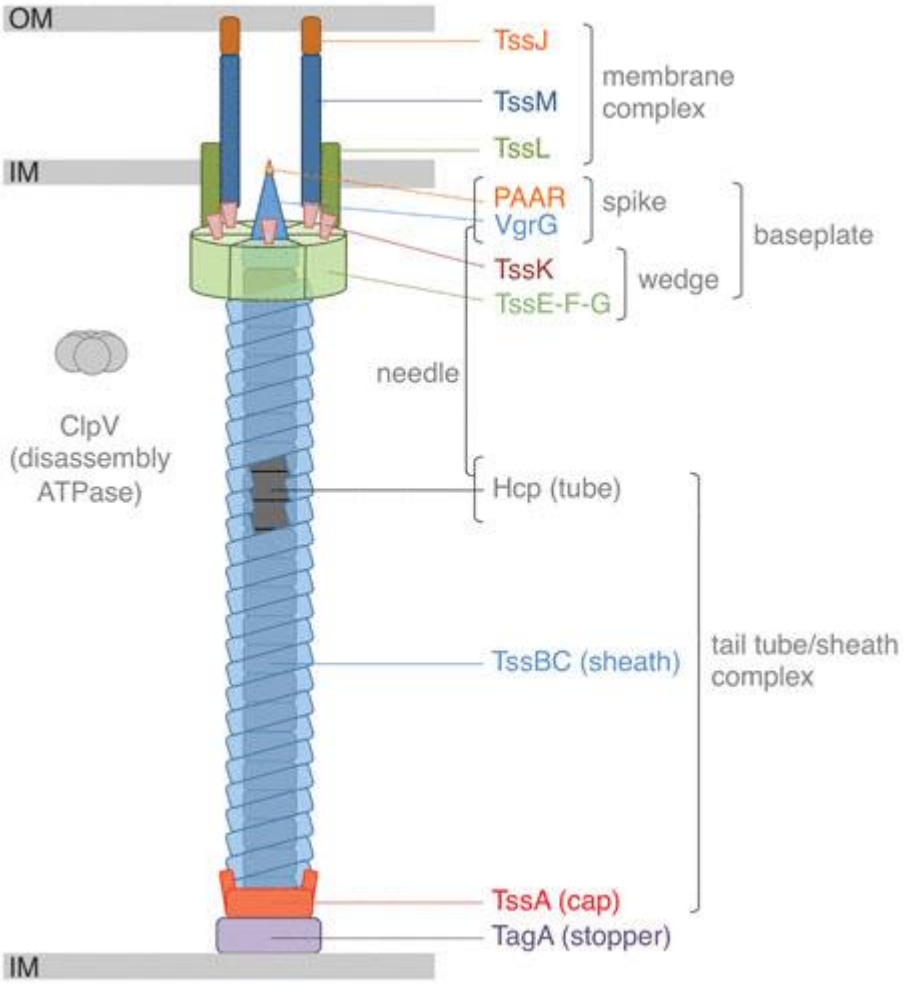


Fig 1.022.2 Structure of the T6SS apparatus. The membrane complex is made of three subunits TssJLM which spans from the outer to inner membranes. The baseplate is made of the wedge and the spike. The wedge has four subunits TssEFGK which attach to the membrane complex and the tail-sheath complex. The spike is made of VgrG which has a PAAR domain sequence at its tip. This holds effector proteins to be secreted when the T6SS contracts. The tail-sheath complex is made of three components: the sheath, TssBC with a hollow Hcp tube at its centre; the TssA cap which interacts with TagA to stop polymerisation. ClpV aids is disassembly of the machinery. (Figure from Cherrak *et al*., 2019)

1.023 Antibiotic resistance

A. baumannii has been reported to be resistant to most major antibiotics except for polymyxins (Gordon and Wareham, 2009). Still in use are the β-lactamase inhibitors, carbapenem and polymyxin classes of antibiotics which target the cell envelope components. However, resistance is beginning to develop against these drugs.

Carbapenems are β-lactam antibiotics which are very potent and have a broad range of activity against pathogens, hence called a drug of last resort (Nowak, 2016). Recently, it has been reported that more than 10 isolates of *A. baumannii* were resistant to carbapenems from 12 of 27 countries studied (WHO, 2017). These data show the scope of the problem and the need to solve this problem is increasingly important. *A. baumannii* uses many mechanisms to acquire resistance genes through the uptake of foreign DNA and mobile genetic elements. Through these mechanisms, several OXA-type β-lactamases have been discovered in the *A. baumannii* genome which confers carbapenem resistance via the production of hydrolysing oxacillinases (Pagano *et al.*, 2016, Héritier *et al.*, 2005).

Of the polymyxin class of antibiotics, colistin (polymyxin E) is the most frequently used to treat *A. baumannii* infection due to its potency on Gram negative bacteria and general chemical safety in humans (Liu *et al.*, 2014).

The positively charged polymyxin class allows interaction with the negatively charged lipopolysaccharide (LPS), specifically the hydrophobic Lipid A moiety (Falagas *et al.*, 2010). Calcium and magnesium ions (Ca^{2+}/Mg^{2+}) linking the phosphate groups of LPS stabilise the membrane (Brown and Tsang, 1978). Colistin displaces this therefore disrupts the outer membrane and causes leakage of the cell contents, killing it (Bialvaei and Samadi Kafil, 2015).

Resistance to colistin has been reported with approximately 10% of tested *A. baumannii* strains being resistant (Chang *et al.*, 2012, Muthusamy *et al.*, 2016). Figure 1.023 summarises the two main mechanisms of colistin resistance: firstly through the loss of lipooligosaccharide (LOS) on the outer membrane (OM) surface, preventing colistin from targeting Lipid A (Section 1.03); secondly, through Lipid A modification by the addition of amines, phosphoethanolamine (PEtN) and galactosamine (GalN) (Arroyo *et al.*, 2011) (Pelletier *et al.*, 2013). It has been suggested that these modifications reduce the binding affinity of colistin due to the alteration of the electrostatic charge on the OM.

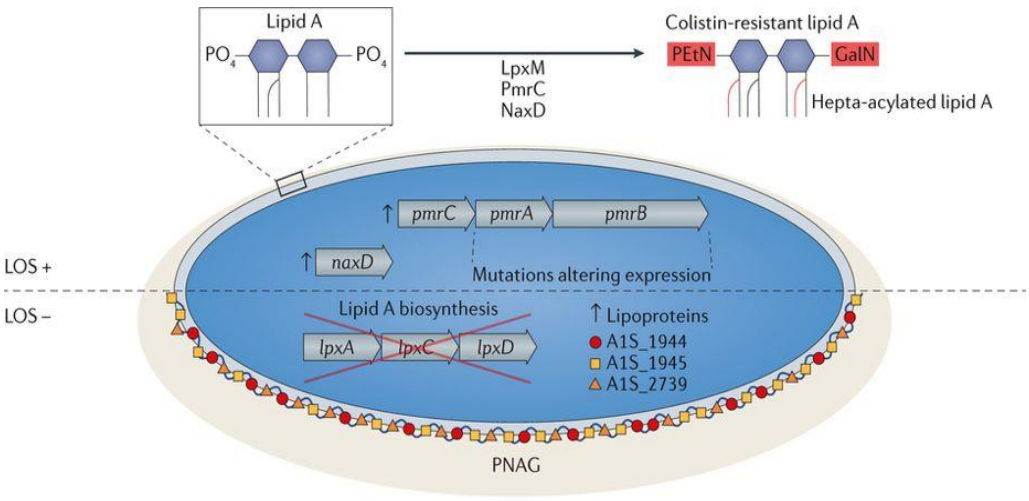


Fig 1.023 Mechanisms of colistin resistance. There are two main mechanisms of resistance: the loss of lipooligosaccharide (LOS) or the modification of Lipid A. The loss of LOS is due to mutations in the Lipid A biosynthesis machinery where mutations in *lpxA, lpxC* or *lpxD* cause biosynthesis to stop. Some lipoproteins are reported to be upregulated as a result. Modification of Lipid A by the addition of phosphoethanolamine (PEtN) and galactosamine (GalN) is mediated by *pmrC* and *NaxD* respectively. (From Harding *et al.*, 2017)

To overcome resistance, combination therapy with colistin and other antibiotics have been tested and used with high efficacy (Lee *et al.*, 2017). Combining antibiotics is not only able to treat multidrug resistant *A. baumannii* infections but will reduce the chance of further resistance developing. Although this is good news, antibiotics cannot be relied on as the only therapeutic option; research for alternative treatments are top priority (Section 1.05).

1.03 The *Acinetobacter baumannii* cell surface

The outer membrane (OM) is the hallmark of Gram-negative bacteria consisting of the asymmetrical inner leaflet containing phospholipids, and the outer leaflet containing lipopolysaccharide (LPS). LPS comprises of three main components: the Lipid A anchor, core polysaccharide and the repeating O-antigen. The O-antigen is a polysaccharide on the outer surface of LPS which functions as a major immunological feature of Gram negative bacteria, providing resistance to multiple human immune system responses *in vivo* (Kenyon *et al*., 2017). However, it has been discovered that its essentiality is not required in some organisms (Zhang *et al*., 2013). *A. baumannii* has an altered form which lacks the O-antigen and has other sugar core modifications called lipooligosaccharide (LOS). The structures of LPS and LOS are compared in Figure 1.031.

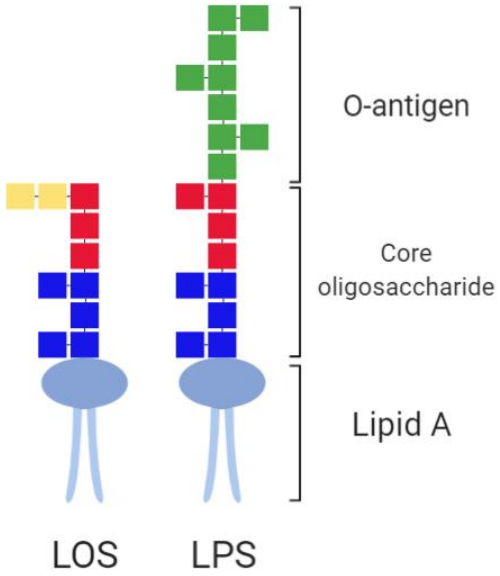


Fig 1.031 Structure of lipooligosaccharide (LOS) and lipopolysaccharide (LPS). LPS composes of a Lipid A moiety anchored to the outer membrane with an attached core oligosaccharide and a repeating O-antigen unit at the top. Generally, Gram negative bacteria express the LPS on the surface of their outer membrane but for certain bacteria like *Acinetobacter baumannii*, they lack the LPS and possess LOS. LOS lacks the O-antigen and has an extended core oligosaccharide (Adapted from Whitfield and Trent, 2014).

LOS deficient *A. baumannii* are selected for by the antibiotic colistin. Colistin targets the Lipid A biosynthetic pathway; however, mutations in this pathway (LpxA, LpxC, LpxD) do not produce Lipid A, yet create a completely viable LOS deficient phenotype in some strains of *A. baumannii* (Moffatt *et al.*, 2010, Boll *et al.*, 2016) (Fig. 1.023).

This is unusual as *A. baumannii* is one of only a few species of bacteria documented to survive without LPS/LOS on its cell surface, with the others being *Neisseria meningitidis* and *Moraxella catarrhalis* (Steeghs *et al.*, 1998, Peng *et al.*, 2005). Little is known about how this is possible but there are a few hypotheses on the potential mechanism. *A. baumannii* LOS mutants are shown to have upregulated lipoproteins (A1S_1944, A1S_1945, A1S_ 2739) and the capsular polysaccharide PNAG (Boll *et al.*, 2016, Henry *et al.*, 2012). It is speculated that these structures help decorate and strengthen the outer cell surface to compensate for the cellular instability to osmotic changes or physical insults caused by the loss of LOS (Fig 1.023).

Mla transport system

Another possible hypothesis includes increases in the expression of the Mla transport system which is normally responsible for exporting glycerophospholipids (GPLs). It was shown through an evolution experiment that in the absence of LOS, the fitness of these strains were improved by the disruption of two genes: a gene in the *mla* gene cluster and *pldA*, a phospholipase (Powers and Trent, 2018). These proteins encoded by these genes are usually involved in the maintenance of the asymmetry of the OM.

The Mla transport system transports GPLs but there is a divide in the literature of which way this transport system works: anterograde or retrograde. This transport system consists of 6 proteins: MlaEF, an ABC transporter complex; MlaB, a cytoplasmic accessory protein; MlaD, a

membrane anchored periplasmic protein; MlaC, a periplasmic chaperone; MlaA, an OM lipoprotein (Malinverni and Silhavy, 2009) (Fig. 1.032). MlaC interacts with both MlaA in the outer leaflet and MlaFEDB in the inner leaflet (Ekiert *et al.*, 2017). MlaA facilitates diffusion of GPLs through the OM (Abellón-Ruiz *et al.*, 2017), but whether it does so in a retrograde or anterograde manner is unknown. MlaFEDB forms a hexameric ABC transporter, where MlaD shuttles GPLs across the membrane by interaction with MlaC (Ekiert *et al.*, 2017).

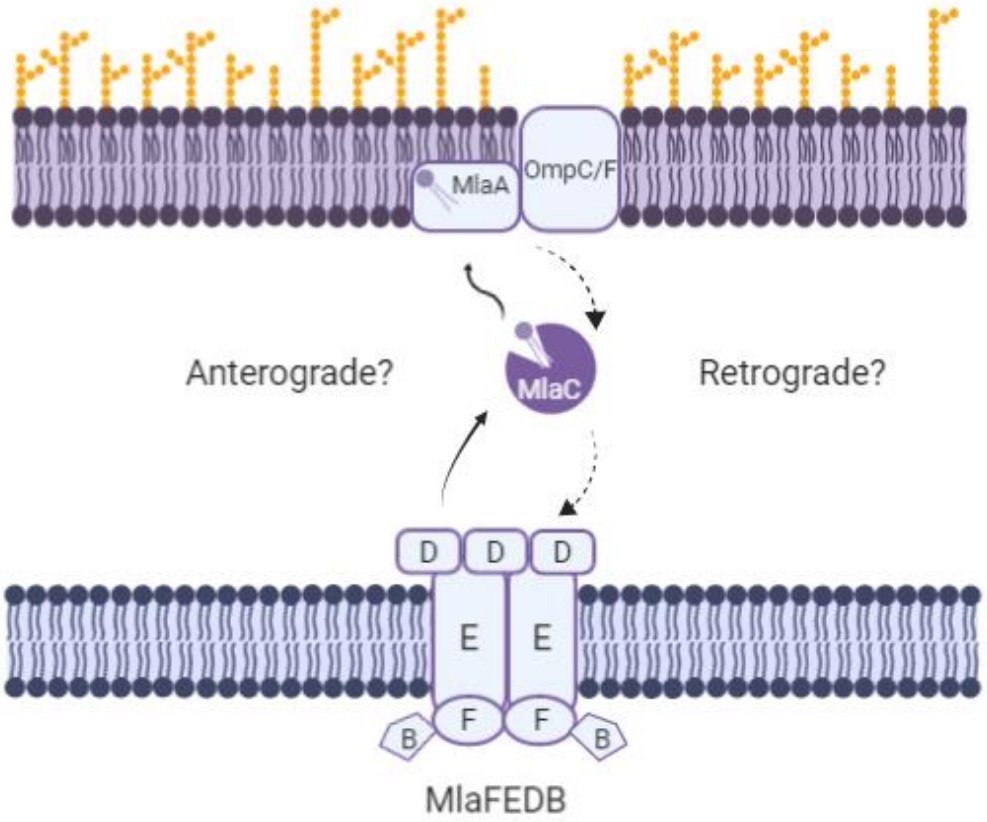


Fig 1.032 The Mla transport system is used for the transportation of glycerophospholipids (GPLs) either to or from the outer to inner leaflet. MlaFEDB forms the ABC transporter in the inner leaflet and MlaA is an outer membrane lipoprotein in the outer leaflet. MlaC is a periplasmic chaperone facilitating the transfer of GPLs across the membrane. It is not known which mode the Mla transport system works in presently.

The earliest study suggested a retrograde transport where the mutations in the Mla pathway resulted in an accumulation of GPLs in the outer leaflet of the OM (Malinverni and Silhavy, 2009). Other evidence for this mode of transport involves the role of the Tol-Pal complex in which the absence of this complex results in GPL accumulation in the outer leaflet (Shrivastava *et al.*, 2017).

On the contrary, it was recently shown that newly synthesised GPLs were accumulated in the inner membrane (IM) in *mla* mutants of *A. baumannii* (Kamischke *et al.*, 2019). Also, it was shown that GPLs move from the MlaFEDB complex to MlaC, from the IM to the periplasmic space in an ATP independent manner, down a concentration gradient, which supports the anterograde transport model (Hughes *et al.*, 2019).

The exact mode of transport for this system remains ambiguous at this moment, but recent work on the structure of the Mla components have been useful in piecing together how this system works.

1.04 *Bdellovibrio bacteriovorus*

Bdellovibrio bacteriovorus is a small Gram negative bacterium capable of invasive predation on other Gram negative bacteria. It invades the periplasm where it establishes itself, replicates and lyses the prey cell before finding a new prey to invade. This lifestyle is unique to *Bdellovibrio* spp. and requires a novel set of predatory proteins which lacks certain systems like the type VI systems for example. *B. bacteriovorus* was first discovered by Stolp and Starr where they recorded its predatory properties on *Pseudomonas* lawns (Stolp and Starr, 1963). It was isolated from soil samples but can also be readily found in many aquatic environments (Li and Williams, 2015, Williams *et al.*, 1995, Schoeffield and Williams, 1990). There are two lifestyles that *B. bacteriovorus* uses to grow and survive: host dependent and host independent. This thesis will focus only on the host dependent lifestyle of *B. bacteriovorus*.

The host dependent lifestyle is the more common of the two and is summarised in Figure 1.04. The first step of the cycle is for *B. bacteriovorus* to invade into a prey cell so that it can grow and replicate. To do this, *B. bacteriovorus* uses its flagellum and gliding motility apparatus to increase the chance of prey encounter and entry (Lambert *et al.*, 2011).

The prey target for predation on the prey cell surface is not well understood at the moment. No simple *B. bacteriovorus* resistant prey mutants lacking a gene for a prey surface element has been found, so this target may be essential to prey viability. Addressing this is one of the aims of this project and will be elaborated further in Section 1.06 and the discussion.

It was shown that type IV pili mediate initial attachment to the prey outer membrane component (Evans *et al.*, 2007, Mahmoud and Koval, 2010, Chanyi and Koval, 2014). Prey entry utilises type IVa pilus retraction (Evans *et al.*, 2007) which probably binds to the peptidoglycan cell wall and several *B. bacteriovorus* penicillin binding proteins (which include

DacBs, Bd0816 and Bd3459) encoding endopeptidases. Some penicillin binding proteins act on the peptide cross-links of the cell wall to form a pore, allowing entry inside into the inner periplasm of the prey (Lerner *et al.*, 2012). *B. bacteriovorus* is seen to deform as they enter this pore before closing through unknown mechanisms.

Once inside the prey, the prey cell wall is modified by genes Bd0886 and Bd1176 to strengthen it (Kuru *et al.*, 2017). After, the predator grows, replicates and develops before finally lysing the prey bdelloplast and escaping into the environment. It does this by using lytic enzymes to break through the cell wall.

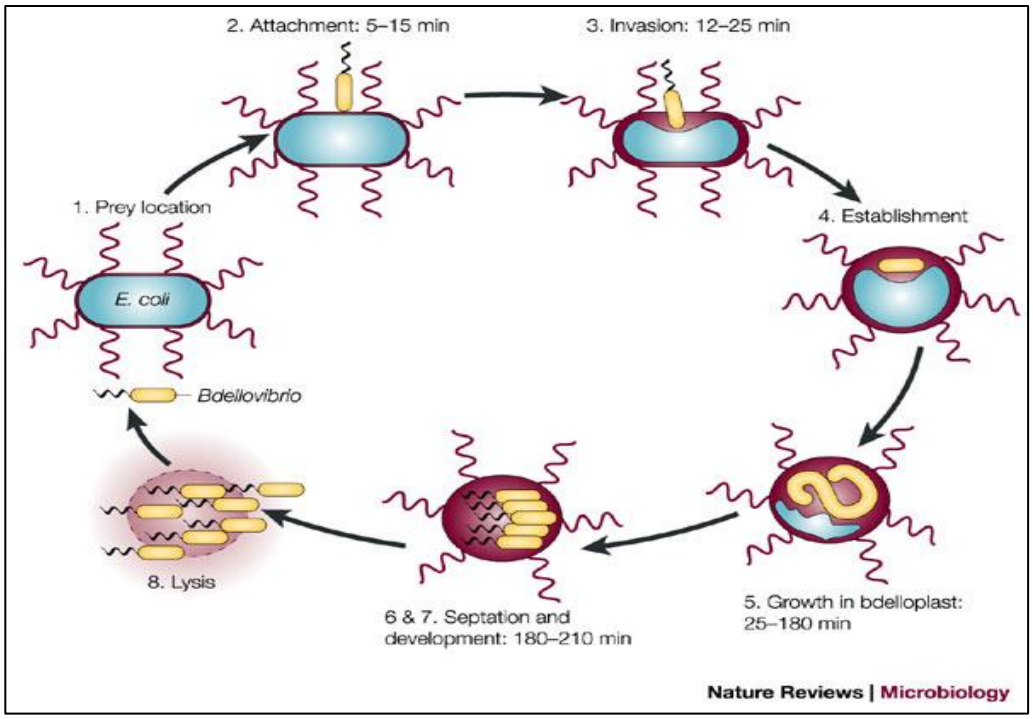


Fig 1.04 The host dependent cycle of *B. bacteriovorus*. 1) *B. bacteriovorus* locates the prey using its flagella and gliding motility. 2) *B. bacteriovorus* attaches to the prey cell using type IV pili on an unknown receptor. 3) Invasion to the inner periplasm beyond the prey wall is mediated by peptidoglycan hydrolases. 4) *B. bacteriovorus* establishes itself in the periplasm and forms the bdelloplast. 5) Growth and replication in the bdelloplast occurs from 25 to 180 minutes. 6&7) *B. bacterivorous* septates and develops a flagellum. 8) Lysis of the cell wall occurs through the production of lytic enzymes (From Sockett and Lambert, 2004).

1.05 *Bdellovibrio bacteriovorus* therapy

A treatment for the future could incorporate the use of the predatory bacterium *Bdellovibrio bacteriovorus*, which preys upon Gram negative bacteria. Unlike other alternative treatments currently researched such as phage therapy, *B. bacteriovorus* therapy has not been tested for human use so far but animal studies have determined a potentially great clinical efficacy for other ESKAPE pathogens but not yet for *A. baumannii*. There are multiple processes used to attach and invade the prey cells therefore resistance to this cannot develop simply.

Recorded instance of 'resistance' to *B. bacteriovorus* in the literature has been very rare. The first was described by Varon, who isolated a dark mutant strain of *Photobacterium leiognathi* which was much more resistant to predation by *B. bacteriovorus* than the original strain (Varon, 1979). This is predicted to be due to the presence of an S-layer which was shown to be the cause of the resistance in *Aquaspirillum serpens*, *Aquaspirillum sinuosum* and *Aeromonas salmonicida*. The removal of the S-layer of *Caulobacter crescentus* restored the ability for *B. bacteriovorus* to prey on these cells (Koval and Hynes, 1991).

B. bacteriovorus was tested in several animal models including: young chicks, which resulted in a lowering of *Salmonella* numbers of the cecal contents as well as a reduction in cecal inflammation (Atterbury *et al.*, 2011); zebrafish larvae, to treat *Shigella flexneri*, which showed a 30-40% increase in survival compared to a control (Willis *et al.*, 2016); mice to treat *Klebsiella*, which showed a reduction of prey burden in the lungs but not the blood (Shatzkes *et al.*, 2017); rats, to see the effect *B. bacteriovorus* has on the host immune system and its ability to treat blood infections (Shatzkes *et al.*, 2015). All of the studies lacked an induction extensive inflammation against *B. bacteriovorus*.

Studies in cell lines have shown similar results. In five different human cell lines studied, no cytotoxicity or inflammatory response was observed by the addition of *B. bacteriovorus* (Gupta *et al.*, 2016). There were no reported negative effects in macrophages when *B.*

bacteriovorus was engulfed. The predator persists within the macrophage before phagolysosomal degradation over the course of 24 to 48 hours (Raghunathan *et al.*, 2019). All these data from the animal and cell lines studied suggest *B. bacteriovorus* to be a safe potential therapy to combat multidrug resistant bacteria such as *A. baumannii* in the future.

1.06 Possible outer prey surface targets for *B. bacteriovorus* predation

It is unknown what surface element *B. bacteriovorus* attaches to when preying on Gram negative bacteria. To investigate this direct interaction, *A. baumannii* was chosen due to its high cell variability which highlights its protective nature against both antibiotics and the innate immune system (Geisinger *et al.*, 2019). It was thought that *B. bacteriovorus* targeted the LPS because this is a specialised structure on Gram negative cell surfaces. LPS itself is modified in *B. bacteriovorus* by mannosylation which prevents the predator from recognising each other as prey (Hobley *et al.*, 2012b). This implies that LPS may be modified to prevent autopredation, highlighting LPS to be a potential target of recognition (Schwudke *et al.*, 2003). LPS consists of three main components: the O-antigen, core polysaccharide and Lipid A.

The O-antigen is shown to be non-essential in protecting prey cells against *B. bacteriovorus* predation. The absence of the O-antigen is reported to make the prey cell more accessible to *B. bacteriovorus* predation (Schelling and Conti, 1986). This has been described as a ‘mask’ for attachment sites and has since been supported by a recent study where *B. bacteriovorus* attaches more rapidly on O-antigen deficient mutants of *Vibrio cholerae* (Duncan *et al.*, 2018). These studies prove that the O-antigen cannot be the target for predation.

Prey mutants with core polysaccharide defects have shown reduced attachment of *B. bacteriovorus*. Varon and Shilo showed *B. bacteriovorus* predation on core polysaccharide mutants lacking: 1. glucosamine, 2. glucosamine and galactose, 3. All hexose and heptoses (Varon and Shilo, 1969). With more sugars missing, the attachment time of the predator to the surface increased, suggesting it may be a sugar component that *B. bacteriovorus* binds to. However, the prey range of *B. bacteriovorus* is broad, so the sugar must be conserved across Gram negative bacteria.

There are not many studies previously done that specifically target Lipid A in predator interaction. But because the other components of LPS were proven not to be the target of *B. bacteriovorus* attachment, it was assumed that Lipid A was the logical target. However, this project has shown that this is not the case. Predation on LOS deficient strains from Boll *et al*. has shown that these mutants are preyed upon (Boll *et al.*, 2016) (Section 4.04). If none of the components of the LPS are the target for predation then there must be another component that is exclusive to the Gram negative surface that *B. bacteriovorus* attaches to. Some possible targets may be β-barrel structures such as porins, lipoproteins or glycerophospholipids; these targets are discussed in Section 5.022.

2.0 Aims

The initial aim of this project was to answer the question: 'Why can *B. bacteriovorus* prey on *A. baumannii in vitro* but cannot *in vivo*?'

This led to the main initial aim:-

1) **Testing transcription of *A. baumannii* when adapted to zebrafish hosts to probe for any cell surface associated changes which might prevent predation**

In *Klebsiella pneumoniae*, it was shown that Lipid A was modified when passaged *in vivo* through mice lung tissue with an LpxO-dependent Lipid A modification which mediates resistance to colistin (Llobet *et al.*, 2015). We wondered if host adaptation in a zebrafish host would induce changes on the cell surface in *A. baumannii* as it did for *K. pneumoniae* in this study. As *B. bacteriovorus* encounters the outer surface of the prey cell prior to invasion, such changes could be responsible for the *in vivo* predation resistance.

To investigate this, RNA was extracted from *A. baumannii* at different timepoints when adapting inside a zebrafish host. The RNA was sent for sequencing to compare regulation of genes pre- and post-adaptation. Interesting differentially regulated genes would be tested to see how they affect *B. bacteriovorus* predation.

However, due to the RNA-seq being delayed for several months because of a change in commercial reagents available to the RNA-seq provider Vertis, it was impossible to get transcriptional changes in time to construct mutants to test for predation associated cell surface changes. Therefore, a second aim using *in vitro* predation experiments with defined *A. baumannii* LOS- mutant strains from (Boll *et al.*, 2016, Powers and Trent, 2018) was added instead. These ancestor strains from Boll *et al*. lacked LOS whereas the evolved strains from Powers and Trent displayed improved fitness from the LOS- strains due to deleted *mla* and *pldA* genes.

In this second section of the project, the aim was:-

2) **Does the absence of LOS from the *A. baumannii* outer membrane have any effect on *B. bacteriovorus* predation *in vitro*?**

When the RNA-seq data was analysed, results from both sections were brought together to investigate the importance of the differentially regulated genes that have surface effects.

For Aim 1: We found transcriptional evidence for a potential mechanism by which *A. baumannii* avoids *B. bacteriovorus* predation *in vivo* via the T6SS. Future work may investigate the T6SS mechanism in *A. baumannii* more thoroughly to determine if this is the cause of the lack of predation *in vivo*.

For Aim 2: We found that the absence of LOS did not affect *in vitro* predation by *B. bacteriovorus*.

Both experimental streams are discussed in the thesis.

3.0 Materials and Methods

3.01 Tables of bacterial strains, relevant *A. baumannii* genes and PCR primers used in this study

The bacterial strains used in this study are listed in Table 3.011, the relevant *A. baumannii genes* for this study are listed in Table 3.012 and PCR primers that are used are listed in Table 3.013.

Bacterial strain	Genotype/Phenotype	Reference
A. baumannii 11W359501 (clinical isolate #36)	Ampicillin and Tetracycline resistant	Clinical Isolate from Dr Matthew Diggle (2015), EMPATH NHS Diagnostics NU Hospital UK
A. baumannii 5075 (Ancestor)	Wild-type	(Jacobs *et al.*, 2014)
A. baumannii 5075, LOS^- (Ancestor)	*lpxC* SNP	(Boll *et al.*, 2016)
A. baumannii 5075, LOS^+ (Evolved)	*pckG* insertion, *smr/mutS2* SNP	(Powers and Trent, 2018)
A. baumannii 5075, LOS^- (Evolved)	*mlaE*$^-$, *smr/mutS2* SNP, *pldA* replacement, *lpxC* insertion	(Powers and Trent, 2018)
A. baumannii ATCC 19606 (Ancestor)	Wild-type	(Bouvet, 1986)
A. baumannii ATCC 19606, LOS^- (Ancestor)	*lpxC* SNP	(Boll *et al.*, 2016)
A. baumannii ATCC 19606, LOS^+ (Evolved)	Wild-type	(Powers and Trent, 2018)
A. baumannii ATCC 19606, LOS^- (Evolved)	*lpxA* SNP	(Powers and Trent, 2018)
B. bacteriovorus HD100	Wild-type	(Stolp and Starr, 1963)
E. coli S17-1	*thi*, *pro*, *hsdR*$^-$, *hsdM*$^-$, *recA*; integrated plasmid RP4-Tc::Mu-Kn::Tn7	(Simon *et al.*, 1983)

Table 3.011: *Acinetobacter baumannii*, *Bdellovibrio bacteriovorus* and *Escherichia coli* strains used in this study.

Gene name	Base pairs (bp)	Description of gene	Reference
lpxA	102	Lipid A biosynthesis	(Boll *et al.*, 2016)
lpxC	131	Lipid A biosynthesis	(Boll *et al.*, 2016)
lpxD	160	Lipid A biosynthesis	(Boll *et al.*, 2016)
lpxL	100	Lipid A biosynthesis	(Boll *et al.*, 2015)
lpxM	126	Lipid A biosynthesis	(Boll *et al.*, 2015)
omp33-36	172	Outer membrane porin	(Smani *et al.*, 2013)
pldA	106	Remove PLs from OM	(Thi Khanh Nhu *et al.*, 2016)
bamA	104	OMP	(Singh *et al.*, 2017)
a1s_0269	115	Secretion pathway	(Jacobs *et al.*, 2012)
eptA	116	Adds pEtN to Lipid A	(Potron *et al.*, 2019)
recA	144	DNA repair and general recombination	(Gomes *et al.*, 2018)
rho	109	Involved in transcriptional termination	(Gomes *et al.*, 2018)
gyrB	100	DNA gyrase – unwinds doubled stranded DNA	(Rumbo-Feal *et al.*, 2013)

Table 3.012: *Acinetobacter baumannii* outer membrane associated and housekeeping control genes. Table shows the base pair length of each gene with a basic description of what the gene is involved in.

Primer name	Sequence
***Acinetobacter baumannii* relevant genes**	
LpxA_U_F	5-tgcaagtgttggcgaagttt-3
LpxA_U_R	5-tgtaagctgcaatgttcgcg-3
LpxC_U_F	5-cgtgtggtaaaagcgagtgg-3
LpxC_U_R	5-gcaggaatatcgacaggtgga-3
LpxD_U_F	5-gggcgcttacattgttaccg-3
LpxD_U_R	5-tggatgaatttgggcagtgc-3
LpxL_U_F	5-tacgcccggtgagtttcaat-3
LpxL_U_R	5-tgcccacggtaaaatagcca-3
LpxM_U_F	5-acgtgggaagtcatgaatgca-3
LpxM_U_R	5-gagattcgcttgttcacggc-3
Omp33-36_36_F	5-gcaacatcgatggcactaaca-3
Omp33-36_36_R	5-ccaccccaaacatggtcgta-3
Omp33-36_19606_F	5-cggttttgaagctgctggtg-3
Omp33-36_19606_R	5-caagcttggcggaattcacc-3
PldA_U_F	5-tggtcgtttgcttggggtaa-3
PldA_U_R	5-accgcgttctaaacccacat-3
BamA_U_F	5-gcacttgttagcgctatggc-3
BamA_U_R	5-tttgcaggggtaagacggac-3
A1S_0269_U_F	5-acgctttgctgcagatgttg-3
A1S_0269_U_R	5-tggagcaatctggccattca-3
EptA_U_F	5-atcatggcgaatcaaccgga-3
EptA_U_R	5-tttccaactttcagagaaccaca-3
Housekeeping control genes	
RecA_U_F	5-agcaaagcattacaagccgc-3
RecA_U_R	5-accaatccccaatgcgatgt-3
Rho_U_F	5-agaaatctggcacacgtcgt-3
Rho_U_R	5-tccatagccgccaactcatc-3
gyrB_U_F	5-gactacagccaagtttgcgc-3
gyrB_U_R	5-acagttacacgtggccagta-3

Table 3.013: All PCR primers used in this study.

3.02 Growth of bacterial cultures

3.021 Growth of *E. coli* S17-1 and *A. baumannii* strains

E. coli was only used as prey for *B. bacteriovorus* in this study. All *A. baumannii* strains were grown according to this methodology.

Frozen stocks of *E. coli* S17-1/*A. baumannii* were streaked onto YT agar plates (Appendix A) and incubated statically at 37°C overnight. For liquid cultures, individual colonies were sub-cultured into 50 ml (*E. coli* S17-1) or 20 ml (*A. baumannii*) YT broth (Appendix A) and incubated at 37°C, 200 rpm overnight.

3.022 Growth of *B. bacteriovorus* HD100

B. bacteriovorus was cultured in liquid culture in CaHEPES buffer (Appendix A) as previously described in Lambert *et al*., 2006. B. bacteriovorus plaque assays were performed on YPSC agar as previously described in Stolp and Starr, 1963.

3.03 Preparation of frozen stocks

All frozen stocks were made with 700 µl bacterial culture from 16h overnight incubation and 150 µl 80% sterile glycerol inside a 2 ml screw-capped tube. The samples were snap-frozen in liquid nitrogen before transferred to -80°C for storage.

Ethics Statement

Animal experiments namely zebrafish breeding were performed according to the Animals (Scientific Procedures) Act, 1986. Breeding is covered under a site Home Office licence 30/3378 to Martin Gering at University of Nottingham aquarium & BSD, and by personal licence I119079C3 to Dr Chris Moore who bred zebrafish, provided embryos at 2 days post fertilisation and supervised the zebrafish experimentation directly.

3.04 Zebrafish husbandry and preparation for injection

To obtain zebrafish embryos for this study, *D. rerio* WT mating pairs were used by Dr Chris Moore. Embryos were obtained following the methods described by Westerfield, 2007. Embryos were raised as described in Willis *et al*., 2016. Zebrafish were developmentally staged according to Kimmel *et al.*, 1995.

3.05 Preparations for zebrafish injection

3.051 Preparing bacteria as injection suspension

A single colony of *A. baumannii* 11W359501 was inoculated into YT-broth and incubated shaking overnight at 37°C, 200 rpm. The following day, 10 ml of YT was inoculated with 200 µl of overnight broth and grown until an OD_{600} reading of 0.5 was obtained. The culture was centrifuged at 5100 rpm for 10 minutes in a Sigma 4K15 centrifuge. The supernatant was discarded and washed twice using 1 ml PBS by centrifugation at 12,000 rpm for 2 minutes (VMR Microstar 12). After washing, the inoculant was resuspended in 25 µl PBS per ml of original culture harvested and kept at room temperature. The bacteria were used within 1 hour from preparation. These enumerations were done and described in Section 3.07.

3.052 Preparing injection moulds

2% agarose solution in 0.5% E2 buffer (Appendix A) was used to make the mould in which zebrafish were placed for injection. E2 agarose solution was poured very thinly at the bottom of the petri dish to form a base layer and left to set. A plastic ridged former (TU-1, adaptivesciencetools.com) was wet with E2 buffer and placed ridge-side down above the base layer. Another layer of E2 agarose was poured to submerge the former and left to set. The former was removed and the petri dish was sealed to prevent drying.

3.06 *A. baumannii* injections into zebrafish hindbrain

Zebrafish larvae 2 dpf were transferred into a 2% E2 agarose mould (Appendix A) supplemented with 200 µg/ml Tricaine (Sigma-Aldrich) to anaesthetise for injection. Injection needles (Harvard Apparatus) were pulled using the P-97 Flaming/Brown Micropipette Puller (Sutter Instruments) and loaded with 1 nl *A. baumannii* suspension which was injected into the hindbrain of the anaesthetised larvae. Injections were done with the IM300 Microinjector (Narishige). Larvae were incubated at 28.5°C post-injection. The larvae then underwent one of three fates: bacterial enumerations (Section 3.07), RNA extraction (Section 3.08) or killed humanely. To humanely kill the zebrafish larvae, an excess of tricaine was added to the petri dish containing zebrafish and neat Distel was added to kill the *A. baumannii*. The dish was placed in the -20°C freezer overnight.

3.07 *A. baumannii* enumerations for *in vivo* growth in zebrafish

To enumerate the *A. baumannii* in zebrafish, larvae were terminally anaesthetised in 10 ml of PBS with 1 ml of tricaine added before being rapidly transferred to a 1.5 ml microcentrifuge tube containing 250 µl of PBS, carrying over as little liquid as possible. A battery operated micropestle was used immediately to homogenise the larva. 100 µl of the homogenate was added to 900 µl of PBS for serial dilution. Dilutions were done from N to 10^{-4}. The start and end inocula were also enumerated. This was taken by injecting 1 nl twice into 1 ml of PBS before and after injections into the zebrafish hindbrain. To plate, 50 µl of the diluent was pipetted onto LB agar and spread using a spreader. Enumerations were taken for 0, 2, 4, 6 and 24 hours. Following spreading, plates were incubated at 37°C overnight. Colonies were counted and recorded the following day.

3.08 RNA extractions of *A. baumannii* from zebrafish

RNA extraction was performed using the RNeasy Micro Kit (Qiagen). Some of the protocol given in the kit (https://www.qiagen.com/us/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en) has been altered and is described below. To extract RNA, 20 larvae were washed twice in PBS and transferred into a 1.5 ml microcentrifuge tube with approximately 1 ml PBS. An excess of tricaine was added to anaesthetise the fish (150 µl in 1 ml). Following anaesthesia, the PBS solution was removed and 200 µl Trizol was added. The larvae were carefully homogenised using a battery operated micropestle before an additional 800 µl Trizol was added in a fume cupboard. For the control, 100x 1 nl injections were performed in 1 ml PBS and Trizol was added before homogenisation as above. The sample was shaken vigorously and incubated at room temperature for 5 minutes. Following incubation, the sample can be stored at -80°C for RNA extraction at a later date. When ready for extraction, 200 µl of chloroform was added and shaken vigorously for 15 seconds and incubated for a further 3 minutes. The sample was centrifuged at 12,000 x *g* for 15 minutes at 4°C. After centrifugation, the sample separates into different phases: a lower, red phenol/chloroform phase, a middle interphase and an upper RNA containing colourless aqueous phase. Only the upper colourless aqueous phase was transferred to an RNase-free tube. 600 µl of 70% ethanol was added to the tube and the following steps were performed as stated in the RNeasy Micro Kit protocol. It is important that the 80% ethanol used in the kit is prepared the day of use to avoid evaporation. Also, 20 µl of RNA was eluted instead of 14 µl from the manufacturer's instructions. Following elution, the RNA was quantified using a spectrophotometer (BMG Labtech FLUOstar Omega) and stored at -80°C.

3.081 Removal of genomic contaminants from RNA samples

To remove the genomic contamination from RNA samples, the DNAse treatment was repeated following the RNeasy Micro Kit protocol from Section 3.08. If contamination still persisted in the sample, the SV Total RNA Isolation System (Promega) was used following the manufacturer's protocol (https://www.promega.co.uk/-/media/files/resources/protocols/technical-manuals/0/sv-total-rna-isolation-system-protocol.pdf?la=en).

3.09 DNA manipulation techniques

3.091 Genomic DNA extraction from bacteria

To extract genomic DNA from a bacterial culture, the culture was grown according to Section 3.02. The GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) was used to extract the genomic DNA.

3.092 PCR amplification

All PCR and RTPCRs were performed using the VWR Doppio Gradient PCR machine, within 0.2 ml flat capped reaction tubes. Primers for the amplified genes are described in Table 3.013, which were designed using the Primer3 program and ordered from Sigma-Aldrich.

For standard DNA PCRs, DreamTaq DNA polymerase (ThermoFisher) was used to verify the presence or absence of a gene as an amplified fragment. Table 3.092.1 shows the reagents and volumes used. 10X DreamTaq buffer contains KCl and $(NH4)_2SO_4$ to enhance specificity of primer binding in the $MgCl_2$ buffer. All genes in Table 3.013 were PCR amplified under the conditions in Table 3.092.3.

For RTPCRs, the one-step RTPCR kit (Qiagen) was used to convert extracted RNA into DNA for confirmation tests and quantification on gels. This was done as previously describes in Lambert *et al*., 2016. Table 3.092.2 shows the reagents and volumes used. Table 3.092.3 shows the conditions used for the RTPCR.

Both PCR and RTPCR were performed according to the manufacturer's instructions. All PCR products were analysed by agarose gel electrophoresis.

Reagent	Volume (µl)
10X DreamTaq Buffer	2.5
2 mM dNTP stock	2.5
10 mM Forward primer (100 pmol/µl)	0.5
10 mM Reverse primer (100 pmol/µl)	0.5
DreamTaq polymerase	0.5
Template DNA (approximately 200 ng/µl)	1
Sterile distilled water	17.5
Total	**25**

Table 3.092.1: Reagents and volumes used for genomic PCR amplification.

Reagent	Volume (µl)
QIAGEN RTPCR 5X buffer (contains 12.5 mM $MgCl_2$)	5
5X Q solution	5
10 mM dNTP stock	1
10 mM Forward primer (100 pmol/µl)	0.15
10 mM Reverse primer (100 pmol/µl)	0.15
QIAGEN RTPCR Enzyme mix (contains the QIAGEN products Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase, and HotStarTaq® DNA Polymerase)	1
Template RNA (approximately 100-300 ng/µl)	0.5
RNase-free water	12.2
Total	**25**

Table 3.092.2: Reagents and volumes used for RTPCR.

(RT)PCR conditions	PCR		RTPCR	
	Temp (°C)	Time	Temp (°C)	Time
Lid Heating	To 105	-	To 105	-
Reverse transcription			50	30 mins
Initial denaturation	95	2 mins	95	15 mins
For 30 3-step cycles:				
Denaturation	95	30 secs	95	30 secs
Annealing	58	30 secs	58	30 secs
Extension	72	1 min	72	1 min
Final extension	72	5 mins	72	5 mins
Storage	4	Hold	4	Hold

Table 3.092.3: PCR and RTPCR conditions set in a VWR Doppio Gradient thermocycler. The table shows the times and temperatures of each step of a (RT)/PCR. 30 cycles were performed for the denaturation, annealing and extension steps.

3.093 Agarose gel electrophoresis for DNA and RNA analyses

0.8 – 2% agarose gels were made with TBE buffer with ddH_2O water (Appendix A), supplemented with 0.1 µg ml^{-1} of ethidium bromide. 3X loading buffer (Appendix A) was added to samples which do not already have loading buffer in them. 10 µl of 50 bp DNA ladder was used as the DNA marker. 15 µl of samples were loaded into the gel topped with TBE buffer. The electrophoresis was run using 100 V for 1 hour. 0.8% agarose gels were used for all DNA separation assays whereas 2% agarose gels were used for DNA RTPCR electrophoresis from small RNA templates. After electrophoresis, the gel was visualised on a BIO-RAD Gel Doc XR+ System using the Quantity One 1-D Analysis software.

3.10 Agilent Bioanalyser

The Agilent 2100 Bioanalyser was used to detect RNA concentrations within samples as described in Lambert *et al*., 2016.

3.11 Predation assay preparation and enumerations

3.111 Preparing *B. bacteriovorus* and *A. baumannii* prey bacteria for predation assays

Predation assay of *B. bacteriovorus* HD100 on *A. baumannii* 11W359501 were performed in CaHEPES buffer. Two days prior to this experiment, *B. bacteriovorus* cultures were prepared (Section 3.022). One day prior, *A. baumannii* cultures were prepared (Section 3.021) and 50 ml *B. bacteriovorus* was prepared using 50 ml CaHEPES, 3 ml *E. coli* S17-1 and 1 ml of the previous prey lysate culture from the day before and incubated shaking at 29°C, 200 rpm.

The day of the experiment, *A. baumannii* prey was prepared by centrifugation (Sigma 4K15) at 5525 x *g* for 10 minutes. The supernatant was removed, and the pellet resuspended in 2 ml CaHEPES. The OD_{600} was measured in a spectrophotometer (Biochrom WPA CO8000 Cell density meter) and the concentrated culture was diluted to OD 1.0 to give approximately 10^8 to 10^9 cfu/ml *A. baumannii*. *B. bacteriovorus* was checked for contamination (Section 3.022) and single filtered through a 0.45 µm filter to remove previous *E. coli* S17-1 prey cells.

3.112 Enumerations of *A. baumannii* strains

Enumerations were carried out using the Miles and Misra method of plating (Hedges, 2001). This method offers a quick and accurate mode of plating. 20 µl of culture was spotted in triplicate onto pre-dried LB agar and left to dry. The plates were incubated statically overnight at 37°C and colonies were counted the following day.

3.12 Preliminary predation assay using *B. bacteriovorus* and *A. baumannii* 11W359501

For the preliminary predation assay, the $10^8/10^9$ cfu/ml *A. baumannii* was serially diluted 10^3 to get $10^5/10^6$ cfu/ml. The 50 ml *B. bacteriovorus* culture was centrifuged at 5525 x *g* for 20 minutes and the pellet resuspended in 1 ml CaHEPES (50x concentrated). 500 µl of 50x *B. bacteriovorus* was added to 5 ml of the *A. baumannii* prey at 0 hours in parallel with a CaHEPES control and incubated shaking at 37°C, 200 rpm. *A. baumannii* enumerations were carried out at timepoints 0, 2, 6, 24 and 48 hours (Section 3.112).

3.13 Persistence experiments on *A. baumannii* ATCC 19606 and 5075 mutant strains

Preparation of these strains for the persistence experiment was done following the steps for *A. baumannii* preparation in Section 3.111. *B. bacteriovorus* was not prepared for this experiment. The $10^8/10^9$ cfu/ml *A. baumannii* was serially diluted 10^3 to get $10^5/10^6$ cfu/ml using CaHEPES. 5 ml of each *A. baumannii* strain was incubated shaking at 37°C, 200 rpm. *A. baumannii* enumerations were taken at timepoints 0, 2, 4, 6 and 24 hours (Section 3.112).

3.14 Predation assay with *B. bacteriovorus* HD100 on *A. baumannii* ATCC 19606 and 5075 strains

The *A. baumannii* ATCC 19606 (Ancestor & Evolved LOS+/-), *A. baumannii* 5075 (Ancestor & Evolved LOS+/-) and *B. bacteriovorus* HD100 (WT) strains were prepared following the methods in Section 3.111. In addition to the 0.45 µm filtered *B. bacteriovorus* predator culture, a conditioned media control was used in parallel. This was made by filtering the *B. bacteriovorus* overnight culture through a 0.22 µm filter once to remove all bacterial cells but the cell-free prey lysate was kept. 5 ml of 10^8 to 10^9 cfu/ml of each *A. baumannii* strain was incubated shaking at 37°C, 200 rpm. 500 µl of *B. bacteriovorus* and the conditioned media control was added to the *A. baumannii* culture at 6

hours into the experiment. *A. baumannii* enumerations were taken at timepoints 0, 2, 4, 6, 24 and 30 hours (Section 3.112).

3.15 Low level *B. bacteriovorus* predation assays

3.151 *A. baumannii* ATCC 19606 Ancestor LOS+/- strains enumerations under low level *B. bacteriovorus* predation

The *A. baumannii* ATCC 19606 Ancestor LOS+/- and *B. bacteriovorus* HD100 strains were prepared following the methods in Section 3.11. A conditioned media control was made using a 0.22 µm filter three times on overnight *B. bacteriovorus* to prevent smaller bacteria to pass through the filter. The low levels of *B. bacteriovorus* (10^2, 10^4, 10^6) was made by serial dilution of the 10^8 *overnight B. bacteriovorus* culture. The different concentrations of *B. bacteriovorus* and the conditioned media control was added to the *A. baumannii* at 6 hours. Besides these changes, the same conditions and enumerations were kept as in Section 3.14.

3.16 Microscopy

Microscopy was done on the Nikon Eclipse Ti-E Phase Contrast fluorescent Wide Field Microscope to visualise *A. baumannii* cell morphology and *B. bacteriovorus* predation. For live-dead staining of the bacteria, LIVE/DEAD BacLight Bacterial Viability and Counting Kit (ThermoFisher) was used. The manufacturer's protocol was followed to stain the bacteria (www.assets.thermofisher.com/TFS-Assets/LSG/manuals/mp34856.
pdf). Samples of the 10 µl bacterial mix was spotted onto 0.8% agarose and 100x oil emulsion was used to visualise the bacteria.

3.17 Analysis of RNA-seq data

To analyse the transcription, the genome of *A. baumannii* 11W359501 had to be assembled from multiple sequences of contigs to create the complete genome. Sequencing of the genomic DNA was done using Illumina Hi-seq (MicrobesNG), and sequence assembly was performed using CLC Genomics Workbench version 11.0.1 (Qiagen, Aarhus, Denmark) by Prof Ian Connerton. The complete genome was annotated using the prokaryotic genome annotation software, Prokka (done with Biolinux 8 Ubuntu 1.12). The genbank file was converted to a ptt. format using the gb2ptt program with default parameters (done with Biolinux 8 Ubuntu 1.12).

Following zebrafish larvae injections (Sections 3.05-3.06) and total RNA extraction (Section 3.08). the RNA samples were sent to Vertis for sequencing but there was a delay in the processing due to a change in reagent supply as the Illumina RiboZero, a ribosomal RNA depletion kit was discontinued.

The RNA-seq FASTQ files obtained from Vertis were merged together using Galaxy to form single files to input into the bacterial RNA-seq data analysis program Rockhopper (Tjaden, 2015, McClure *et al.*, 2013). Rockhopper generated the transcriptome data from the RNA-seq which had been quantified. Data from the three timepoints chosen (Ab only representing 0 h; 1.5 h; 5 h) were calculated for fold change (5 h value / 1.5 h value = the fold change from 1.5 h to 5 h to represent the change in gene expression induced by adaptation to host).

As not many *A. baumannii* genomes have been fully annotated, manual annotations were done to elucidate the product of some hypothetical proteins. The transcriptional start and stop locations (located by Rockhopper) were copied into Artemis Sanger (Carver *et al.*, 2012), a program which allowed the analysis of the *A. baumannii* 11W359501 genome. The amino acid FASTA sequences were exported from Artemis into NCBI Protein Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi), which was mapped to general *A. baumannii* genomes to see what

annotated genes had the highest homology to the hypothetical ones. Because *B. bacteriovorus* interacts at the outer surface of prey bacteria, prey surface expressed gene products were highlighted and tabulated in Appendix F.

4.0 Results

4.01 *Bdellovibrio bacteriovorus* predation assays on *Acinetobacter baumannii* strains show Lipid A is not targeted in predation

As previous literature has not definitively confirmed a specific receptor for *B. bacteriovorus* attachment, it was assumed that the Lipid A component of the LPS in Gram negative bacteria was the target (Section 1.06). In the first section of this study, predation was performed on multiple strains of *A. baumannii* (Table 3.01). The main focus of the strains is primarily on the *A. baumannii* ATCC 19606 Ancestor and Evolved strains, with comparisons on the *A. baumannii* 11W359501 used in the zebrafish microinjection assays (Section 4.06). Some work was done on *A. baumannii* 5075 but was stopped due to technical difficulties (Section 4.042).

The Ancestor and Evolved strains refer to the strains published from the Trent lab (Boll *et al*., 2016; Powers and Trent, 2018). The Ancestor strain from Boll *et al*. are the original WT strains whereas the Evolved strains from Powers and Trent have undergone an evolution experiment which possess mutations in the mla transport system and PldA. In each of the Ancestor and Evolved categories are further LOS+ WT strains and LOS- mutants which are generated by SNPs in *lpxA* or *lpxC* genes. As Lipid A is a component of LOS, predation was performed on each of these Ancestor LOS+/- and Evolved LOS+/- strains to verify whether predation occurs in the absence of Lipid A.

4.02 Preliminary predation assay on *A. baumannii* 11W359501

Predation was carried out on *A. baumannii* 11W359501 using *B. bacteriovorus* HD100 to confirm that *A. baumannii* 11W359501 was susceptible to predation *in vitro*. This also set some groundwork for the optimum conditions that would be used in the next experiments using mutant strains. For this experiment, 1:10 50x *B. bacteriovorus* was added to approximately 10^6 *A. baumannii* 11W359501 suspended in CaHEPES buffer (Section 3.12). This was done because it was

suspected that WT *A. baumannii* strain took approximately 6 hours to adapt to the CaHEPES buffer due to the viable counts for the buffer suspended control remaining stable from 6 hours onwards to the end of the experiment but dropping within the first few hours. 10^8 predatory *B. bacteriovorus* was added immediately at 0 hours and viable counts were taken at 24 and 48 hours. The cfu/ml of *A. baumannii* dropped from $1.8x10^5 \pm 2.9x10^4$ at 0 hours to $3.3x10^3$ at 24 hours. Growback after predatory treatment was observed from 24 to 48 hours, with the final cfu/ml being $3.5x10^6 \pm 3.2x10^6$ at 48 hours.

Figure 4.02 shows the graph of *A. baumannii* 11W359501 survival in CaHEPES and under predation by *B. bacteriovorus*. The significant growback could be attributed to death of *B. bacteriovorus* and the release of their proteins or bdelloplast remains in the environment. *A. baumannii* could use these as energy sources for growth (Hobley *et al*., 2006).

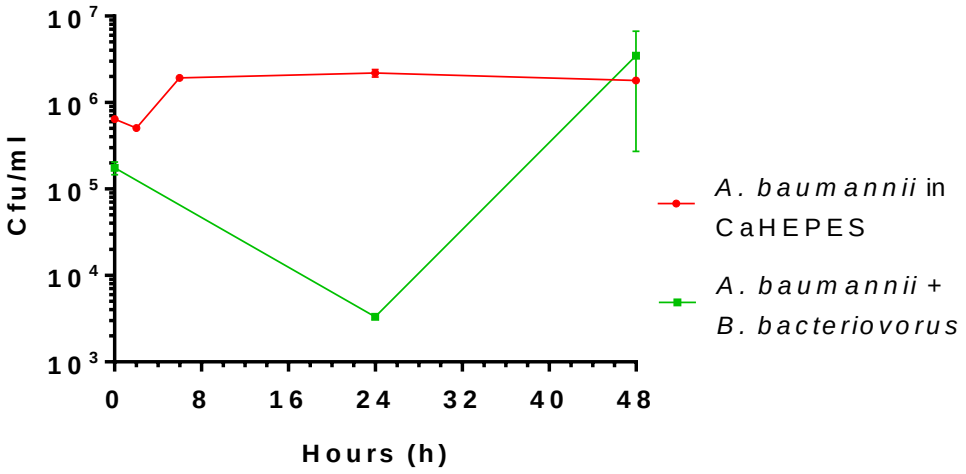


Fig 4.02: Survival curve of *A. baumannii* 11W359501 in CaHEPES buffer and under predation by *B. bacteriovorus* HD100. The buffer control (red) shows an adaptation phase from 0 to 6 hours before counts become stable until 48 hours. The predation curve (green) showed *A. baumannii* cfu/ml fall after *B. bacteriovorus* HD100 was added at 0 hours until 24 hours, where significant growback was observed until 48 hours where the ending cfu/ml was similar to the control. Two independent repeats were performed in duplicate for this experiment.

4.03 Testing the persistence of *A. baumannii* mutant strains in CaHEPES

Two strains of *A. baumannii* with different phenotypes were requested and sent from S. Trent's lab to see if predation was possible on these OM mutants. These strains were *A. baumannii* ATCC 19606 and *A. baumannii* 5075-derived (Table 3.011) Ancestor and Evolved strains, with and without lipooligosaccharide (LOS+/LOS-) in their outer membrane (Section 4.01). However, before predation assays were performed, these mutant strains were tested for survival in CaHEPES, a minimal buffer that supports *B. bacteriovorus* growth predatorily on prey. This ensures that *B. bacteriovorus* predation could be tested without the *A. baumannii* dying due to other environmental factors instead of predation.

The data for this experiment is shown in Appendix B. All strains were shown to be able to grow and survive in CaHEPES. However, it took approximately 6 hours for all strains to become adapted to this environment, therefore the 6 hour timepoint was chosen to add *B. bacteriovorus* for predation assays in subsequent experiments.

4.04 *B. bacteriovorus* HD100 predation on *A. baumannii*

Having established that the *A. baumannii* mutant strains can survive well in CaHEPES after a 6 hour adaptation, predation assays were set up to determine whether *B. bacteriovorus* can prey on the mutant LOS- strains of *A. baumannii.* This would allow isolation of the potential sites of attachment for *B. bacteriovorus* predation. Unlike the persistence experiment, the initial *A. baumannii* culture was not diluted to $10^4/10^5$ cfu/ml and instead kept at 10^8. It was established that there was a 4 to 6 hour adaptation period of the *A. baumannii* in CaHEPES, therefore the prey was left to adapt for 6 hours before adding *B. bacteriovorus* to the culture. These steps are described in Section 3.14. The *B. bacteriovorus* conditioned media and predator inoculum were

enumerated as a control both for any presence of *B. bacteriovorus* in the control sample and the number of *B. bacteriovorus* in the inoculum (data not shown).

4.041 Predation on *A. baumannii* ATCC 19606

The *A. baumannii* ATCC 19606 Ancestor and Evolved strains were preyed upon in separate experiments, each with three independent repeats. Fig 4.041A shows predation on the Ancestor strains, Fig 4.041B shows predation on the Evolved strains.

Initial predation for the Ancestor LOS+/- strains was similar from 0 to 24 hours. From 24 to 30 hours, only the cfu/ml of the LOS+ control dropped ($5.3x10^8 \pm 1.7x10^8$ to $1x10^7 \pm 6.2x10^6$). The LOS- control remained constant throughout the experiment. This is interesting as there is a significant difference between 24 and 30 hours only in the LOS+ control compared to the LOS- control counterpart. Predation on the Ancestor LOS+/- strains showed a similar decrease in cfu/ml from $6x10^8$ at 6 hours to $6.8x10^5$ and $1.9x10^7$ for LOS+ and LOS- respectively at 30 hours. Growback was observed from 24 to 30 hours from $4.8x10^6$ to $1.9x10^7$ cfu/ml for the Ancestor LOS- HD100 preyed strain only.

For the Evolved strains, the LOS+/- controls remained similar, returning to approximately $2\text{-}4x10^8$ cfu/ml over the course of the 30 hours. Predation on these strains caused the cfu/ml to drop from $3x10^8$ at 6 hours to $1\text{-}3x10^6$ at 30 hours with both the Evolved LOS+ and LOS- strains decreasing at a similar rate. No growback was observed for the Evolved LOS- HD100 preyed strain when compared to its Ancestor counterpart. The major difference between the Ancestor and Evolved strains observed was the Ancestor LOS+ control which dropped significantly from 24 to 30 hours only, compared to the Evolved LOS+ control which remained constant throughout the experiment.

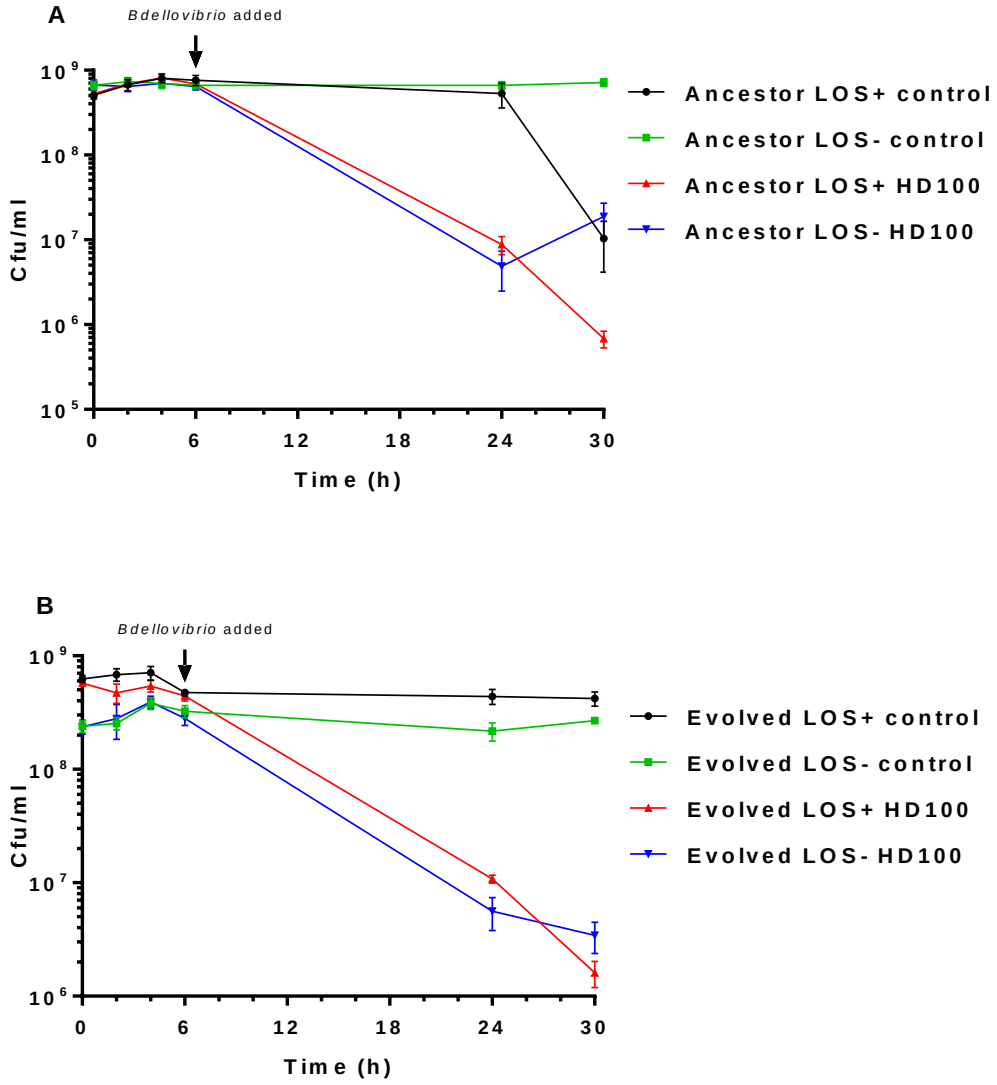


Fig 4.041: *A. baumannii* ATCC 19606 Ancestor and Evolved, LOS+ and LOS- strains preyed upon by *B. bacteriovorus* HD100 over the course of 30 hours. The *A. baumannii* strains were left to grow and adapt to the minimal conditions of CaHEPES for 6 hours before *B. bacteriovorus* was added (arrowed). The black and green controls do not have *B. bacteriovorus* added. The red and blue assays are supplemented with *B. bacteriovorus* at 6 hours. *A. baumannii* enumerations were taken at 0, 2, 4, 6, 24 and 30 hours. A) *A. baumannii* ATCC 19606 Ancestor strains. B) *A. baumannii* ATCC 19606 Evolved strains. Three independent repeats were done in duplicate for this experiment. The mean and SEM were calculated for each point.

4.042 Predation assay tests on *A. baumannii* 5075 led to the end of experimentation on this strain for practical reasons

Like the *A. baumannii* ATCC 19606 strains, the same protocol was used for the predation of the 5075 strains (Section 3.14). Both Ancestor and Evolved strains were preyed upon in the same manner however, the colonies produced for some strains were very difficult to count due to the irregular colonies and sizes. This made the results very variable, therefore experimentation on this strain was stopped. Figure 4.042 shows images of *A. baumannii* 5075 Ancestor and Evolved LOS+/- colonies.

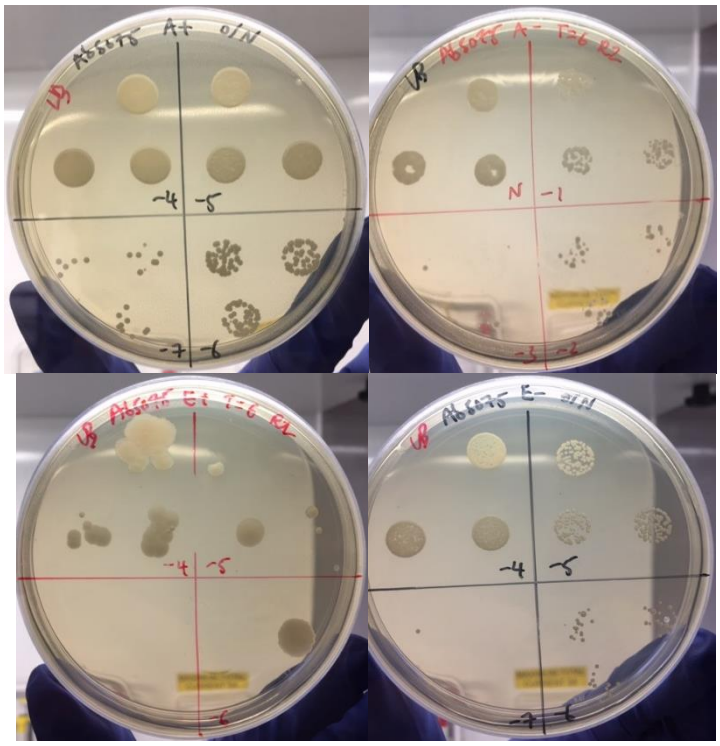


Fig 4.042: Images of *A. baumannii* 5075 Ancestor and Evolved LOS+/- strains. Ancestor LOS+ (A+, top left) and Evolved LOS- (E-, bottom right) colonies appear to have a normal morphology. However, Ancestor LOS- (A-, top right) has a broken colony morphology where multiple colonies are joined together with variable sizes within each 20 µl Miles and Misra drop. Evolved LOS+ (E+, bottom left) has a large, wide colony morphology where the colonies have overlapped with each other, making them difficult to count and therefore inaccurate.

4.05 Low level predation assays

Following the predation assay done for Section 4.041, a few plaque colonies were found in the enumeration controls for the *B. bacteriovorus* conditioned media. This meant that there were possibly low levels of *B. bacteriovorus* in the *A. baumannii* LOS+/- controls. Analysing the data from Section 4.041 where the Ancestor LOS+ control strain began to fall after 24 hours, it was possible that a low level of *B. bacteriovorus* may have been killing the *A. baumannii*. Therefore, a low level *B. bacteriovorus* experiment was designed where 10^2, 10^4 and 10^6 *B. bacteriovorus* was used to prey on a high 10^8 *A. baumannii* culture. This time, the conditioned media was filtered through a 0.22 µm filter thrice as opposed to once in Section 4.041.

4.051 Low level *B. bacteriovorus* HD100 predation on *A. baumannii* ATCC 19606 mutants

The conditions for this experiment were kept the same as in Section 4.041 as controls so that only the amount of *B. bacteriovorus* was changed as below. *B. bacteriovorus* enumerations showed no sign of plaques in the conditioned media. Only the *A. baumannii* Ancestor strains were tested in this experiment as the Evolved LOS+ strain did not drop in Section 4.041. These data showed no significant difference in predation by 10^2, 10^4 and 10^6 cfu/ml of *B. bacteriovorus* to the control which indicates that low levels of *B. bacteriovorus* were not affecting predation at all. All 3 independent repeats showed that all LOS- strains grouped above their LOS+ counterpart, which suggests that LOS- strains survive in the experimental buffer conditions slightly better than their LOS+ counterparts. This reinforces the hypothesis that the drop in cfu/ml of Ancestor LOS+ in Section 4.041 was not caused by potential low levels of *B. bacteriovorus* predation in the conditioned media, but of the properties of the LOS+ Ancestor strain itself. Figure 4.051 shows 10^8 *A. baumannii* ATCC 19606 Ancestor LOS+/- strains preyed upon by *B. bacteriovorus* at 10^2, 10^4 and 10^6 cfu/ml.

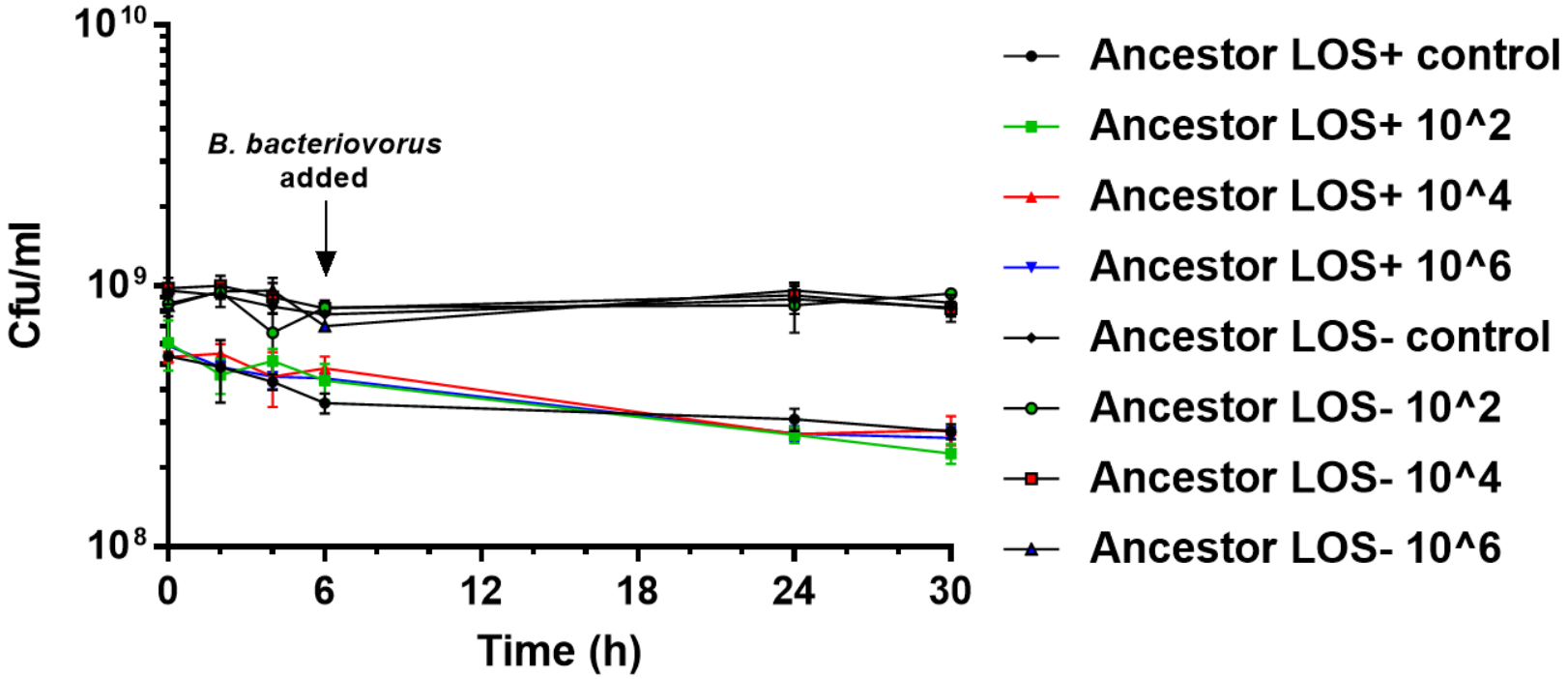


Fig 4.051: Low level (10^2, 10^4 and 10^6 cfu/ml) *B. bacteriovorus* HD100 predation on high level (10^8 cfu/ml) *A. baumannii* ATCC 19606 Ancestor LOS+/- strains. Enumerations of *A. baumannii* were taken at timepoints 0, 2, 4, 6, 24 and 30. From 0 to 6 hours, the *A. baumannii* was left to adapt to the minimal CaHEPES conditions before 10^2 – 10^6 *B. bacteriovorus* were added at 6 hours. LOS+ strains are displayed without a black border whereas the LOS- strains do. Each point has been assigned a colour which matches to their LOS+/- counterpart conditions. No significant difference was observed between *A. baumannii* + predator and the control. Three independent repeats were done for each *A. baumannii* Ancestor strain.

4.052 Low and high cell number comparison predation assay with *B. bacteriovorus* enumerations

The experiment done in Section 4.051 showed that 10^2 to 10^6 cfu/ml of *B. bacteriovorus* did not significantly kill any of the *A. baumannii* strains compared to the control. To see what happened to the *B. bacteriovorus* numbers at low levels and confirm that 10^8 predator cfu/ml could kill the *A. baumannii* as in Section 4.041, *A. baumannii* enumerations were taken using the same method as in Section 4.051 and *B. bacteriovorus* enumerations were done (Section 3.152).

For this experiment, predation using 10^6 and 10^8 cfu/ml of *B. bacteriovorus* on both *A. baumannii* ATCC 19606 Ancestor LOS+/- strains was compared. Control was done alongside the predation, as well as *B. bacteriovorus* enumerations on their own. Different timepoints for enumerations were chosen for this experiment: 0, 6, 8, 24 and 30 hours, omitting the 2 and 4 hours done in previous experiments. *B. bacteriovorus* was added to the culture at 6 hours. It was established that approximately 6 hours was needed for *A. baumannii* adaptation in CaHEPES buffer. A later timepoint (8 hours) was chosen to monitor any early stage predation that may happen in this experiment. Figure 4.052 shows the enumerations of differing concentrations of *B. bacteriovorus* predation on 10^9 *A. baumannii* and *B. bacteriovorus* on its own.

These results show that all *A. baumannii* and *B. bacteriovorus* controls remain fairly constant. Predation using only 10^6 *B. bacteriovorus* did not show a drop in cfu/ml of 10^9 *A. baumannii* LOS+ or LOS- cultures, suggesting 10^6 predator had no effect on the *A. baumannii*. Predation on these *A. baumannii* cultures using 10^8 *B. bacteriovorus* resulted in a 10^3 cfu/ml drop in both LOS+ and LOS- strains from 10^9 to 10^5/10^6 cfu/ml from 6 to 24 hours. Growback after predatory treatment was observed for both LOS+ and LOS- treatments from 24 to 30 hours.

Enumerations of 10^6 cfu/ml *B. bacteriovorus* showed a decrease in predators when added to the *A. baumannii* LOS- prey, with cfu/ml

dropping from 10^5 to 10^3 from 6 to 30 hours. This was the expected result as there was no sign of *A. baumannii* cfu/ml decrease in their enumerations. However, the 10^6 cfu/ml *B. bacteriovorus* enumerations added to the LOS+ strain showed some stability, from ~10^5 to 10^6 cfu/ml over the length of the experiment. Growth of *B. bacteriovorus* (green dotted) increased slowly from 6 to 24 hours but became quicker from 24 to 30 hours. No significant drop in cfu/ml was observed for the *A. baumannii* enumerations, which is unexpected. Both 10^8 cfu/ml *B. bacteriovorus* on the *A. baumannii* prey showed an increase from 10^7 to 10^9 cfu/ml from 6 to 30 hours as expected with *A. baumannii* numbers falling.

These data confirm that a concentration >10^6 *B. bacteriovorus* is needed to prey upon 10^8/10^9 *A. baumannii* LOS+/- strains. Due to the conflicting results on the 10^6 *B. bacteriovorus* enumerations, it is not certain if *A. baumannii* is killing the *B. bacteriovorus* in culture, but these data suggest this might be happening. Possible explanations for why this occurs are discussed in Section 5.04.

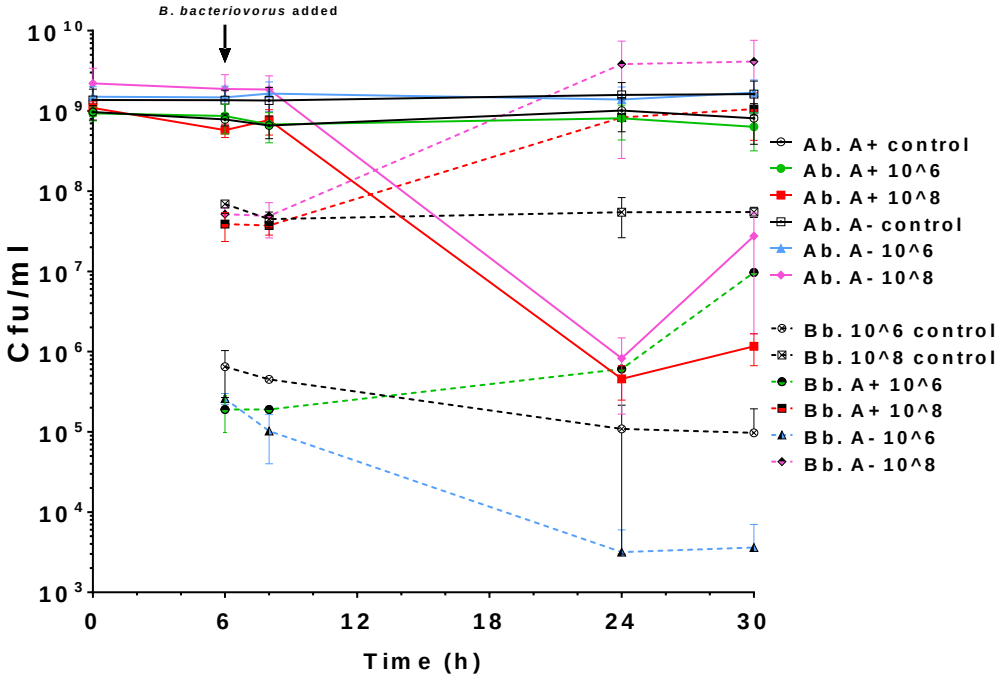


Fig 4.052: Comparison of high (10^8) and low (10^6) concentrations of *B. bacteriovorus* HD100 predation on *A. baumannii* ATCC 19606 Ancestor LOS+/- strains over 30 hours and enumerations of *B. bacteriovorus*. The *A. baumannii* strains were left to grow and adapt to CaHEPES for 6 hours before *B. bacteriovorus* was added. *A. baumannii* and *B. bacteriovorus* enumerations were taken at 0, 6, 8, 24 and 30 hours. Two independent repeats in duplicate were done for this experiment. The SEM is shown for each result. Legend: Ab. = *A. baumannii*; Bb. = *B. bacteriovorus*; A+ = Ancestor LOS+; A- = Ancestor LOS-; *B. bacteriovorus* enumerations are shown with a black border and connecting lines are dotted; *A. baumannii* enumerations are unbordered with filled connecting lines; shapes and colours from *A. baumannii* and *B. bacteriovorus* enumerations match their counterparts; all controls are in black.

4.053 Microscopy of low level predation

Microscopy was done to visualise differences in predation between *A. baumannii* Ancestor LOS+ and LOS- strains and what happens in low level (10^6 cfu/ml) of *B. bacteriovorus* predation. Both *A. baumannii* and *B. bacteriovorus* were stained with LIVE/DEAD stain (Section 3.16) which distinguishes live and dead bacteria by fluorescently staining intact and damaged cell membranes. This allowed the visualisation of the smaller *B. bacteriovorus* attaching and killing *A. baumannii* cells so speculation of what happens in the low level *B. bacteriovorus* predation can be attempted.

The timepoints for microscopy were taken at 6, 24 and 30 hours. *B. bacteriovorus* was added to the prey at 6 hours. Fluorescent microscopy with LIVE/DEAD stain of *B. bacteriovorus* predation on *A. baumannii* at different timepoints is shown in Appendix C. Statistical tests and quantification of live and dead cells were not performed due to a time constraint.

For A+ 10^6, little death of *A. baumannii* was observed for the 3 timepoints and very few *B. bacteriovorus* were observed at these timepoints. It is unclear whether this is due to *B. bacteriovorus* death or entering inside the *A. baumannii* cells, masking the stain. For A- 10^6, very little *A. baumannii* death was observed but more *B. bacteriovorus* were seen by 30 hours. For the A+ and A- 10^8 images, some *B. bacteriovorus* could be seen attaching to prey at 6 hours. At 24 hours, most *A. baumannii* prey had been preyed upon with many bdelloplasts seen in both A+ and A- prey. At 30 hours, not many bdelloplasts can be seen compared to 24 hours for both A+ and A- as the *B. bacteriovorus* have burst out of the cells. Remains of the bdelloplasts can be seen in the background.

By looking at both the live-dead microscopy (Appendix C) and the viable counts (Figure 4.051), both these data sets suggest that there is some mechanism behind *A. baumannii* killing the *B. bacteriovorus* when the predator is added at low levels.

4.06 Zebrafish hindbrain injections with *A. baumannii* 11W359501

Initial zebrafish hindbrain injection studies first began in 2016 where Willis *et al*. injected both *Shigella flexneri* and *Bdellovibrio bacteriovorus* HD100 into the hindbrain of the zebrafish and discovered that *B. bacteriovorus* HD100 could successfully prey upon and clear the *Shigella* infection. Subsequently, Dr Chris Moore from the Sockett lab found that *B. bacteriovorus* HD100 could not clear an *Acinetobacter baumannii* infection when injected into the zebrafish hindbrain. What is interesting is that in an *in vitro* environment, *A. baumannii* was found to be susceptible. Therefore, it was hypothesised that this was due to a host adaptation process of *A. baumannii*, so the initial experiments were to determine the growth of the *A. baumannii* and see at what timepoint the adaptation process in the host began.

A. baumannii 11W359501 is a host adapted clinical isolate isolated from a patient. This was the strain previously used by Dr Chris Moore to show sensitivity to predation *in vitro* but not in zebrafish hosts. The strain was studied on this strain because it is a good representation of a host adaptable *A. baumannii* isolate capable of causing disease nosocomially. By studying predation on this strain, it may be possible to simulate problems that may arise in host adaptation in a clinical setting and this work could help in elucidating whether predation is affected by host adaptation of the prey.

The *A. baumannii* 11W359501 genome was sequenced from genomic DNA prepared by Dr David Negus and the genome was annotated by Prof Ian Connerton on the Biosciences department at the University of Nottingham. It is 4.13 Mbp in size with 3,888 proteins found (Genbank RefSeq: NZ_CP041035.1). This was compared to a scaffold genome of *A. baumannii* ATCC type strain 19606, another strain that was used frequently in this study, and also by other researchers. Unfortunately, a complete genome could not be found for this strain as the sequencing had not been published online (Appendix G). This *A. baumannii* ATCC 19606 scaffold genome is 3.98 Mbp in size with 3,683 proteins found

(GenBank RefSeq: NZ_MJHA00000000.1). By comparison the *A. baumannii* 11W359501 genome used is larger, which can be explained by the gene acquisition systems like conjugative systems for horizontal gene transfer, or the expression of transferred genes for resistance to antibiotics or disinfectants, changes in osmolarity, temperature or drying, especially in a nosocomial environment.

The *A. baumannii* 11W359501 inoculum was prepared as in Section 3.051. 1 nl of inoculum containing approximately 17500 cfu/ml *A. baumannii* was injected into the zebrafish hindbrain and incubated at 28.5°C for 24 hours. The start and end inocula were taken to confirm the number of bacteria in the needle before and after the zebrafish were injected. Injections were completed within 12 minutes of starting. Zebrafish were sacrificed and enumerations were taken at 2, 4, 6 and 24 hours post-injection. Figure 4.06 depicts the adaptation (from 0 – 6 hours) and growth (after 6 hours) of *A. baumannii* for 24 hours within the zebrafish hindbrain compartment. The start and end inocula were used for 0 hours to simulate the number of bacteria prior to injection as *in vivo* counts could not be taken at 0 hours. Bacterial growth appears to be triphasic, with a lag phase (0 – 2 hours), a faster log phase (2 – 6 hours) and a continuous log phase (6 – 24 hours). However, it can be debated that stationary phase is reached between 6 – 24 hours but this is unconfirmed due to a lack of an additional timepoint. It is hypothesised that adaptation to host occurs during the lag phase.

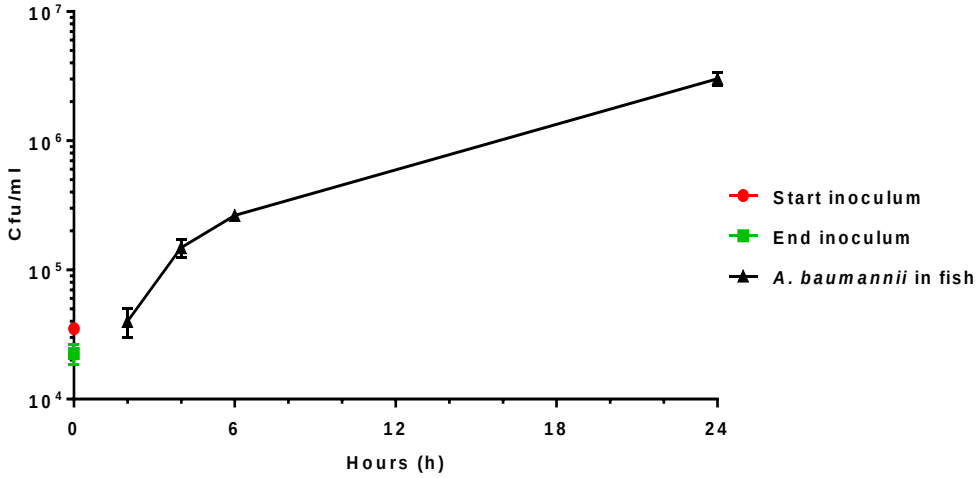


Fig 4.06: Growth curve of *A. baumannii* 11W359501 in zebrafish hindbrain during 24 hours incubation at 28.5°C. At each corresponding timepoint, four zebrafish were sacrificed. The *A. baumannii* cfu/ml steadily increased during the incubation period from $3.5x10^4 \pm 2.7x10^3$ cfu/ml starting inoculum to $3x10^6 \pm 3.3x10^5$ cfu/ml at 24 hours. The starting and ending inocula have been recorded as a guideline to show how much inoculum was in one injection. These data were obtained based on 4 independent repeats of fish enumerations.

4.07 Testing for *A. baumannii* genes that are important in *in vivo* adaptation

4.071 Isolation of potential up/downregulated genes by DNA PCR

A list of potential up or downregulated genes from when *A. baumannii* adapts from an *in vitro* environment to *in vivo* was made based on previous work from S. Trent's lab and others (Table 3.012). Genes in this list are predicted to change in expression levels when adaptation occurs, therefore it was hypothesised that they are associated with the cell surface (outer membrane proteins, porins or Lipid A biogenesis) when *B. bacteriovorus* preys or encounters prey. The *A. baumannii* 11W359501 strain was compared to the *A. baumannii* ATCC 19606 strain in Prof S. Trent's published work. Universal primers for both *A. baumannii* strains were designed and PCRs were run to isolate these genes (Table 3.013). Of these genes, only the Omp33-36 gene did not share a sufficiently similar base sequence between the two *A. baumannii* strains for universal primers to be designed (75.9% DNA identity), therefore primers were designed for each strain separately. The similarity between strains was 75.9% which was much lower than other genes that were compared (data not shown). Figure 4.071 shows the successful isolation of these potentially differentially regulated genes and their corresponding base pair lengths can be found in Table 3.012.

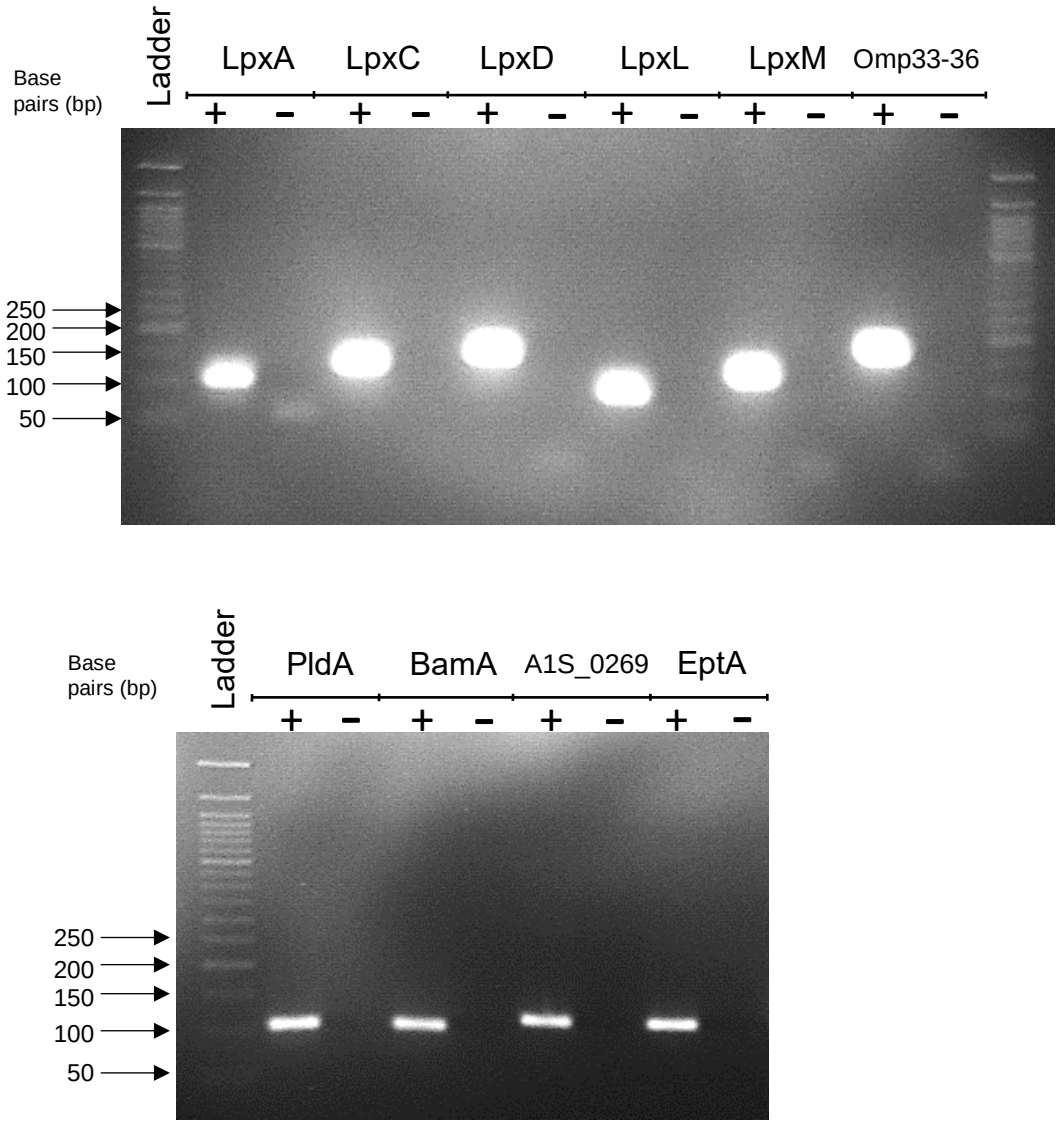


Fig 4.071: PCR products of potentially up or downregulated genes when adapting from *in vitro* to *in vivo* in *A. baumannii*. *The A. baumannii* 11W359501 genomic DNA was the template used to isolate these potential genes. Positive and negative controls were done for each gene to minimise errors. No contaminants were seen in each negative control. The PCR products were cross-referenced back with the product sizes in Table 3.012. All products were of the correct length.

4.072 Isolation of potential control housekeeping genes by DNA PCR

Potential housekeeping genes were found from the literature (Table 3.012) to be used for comparison with the differentially regulated genes above. They were chosen due to their expression levels being consistent during *in vivo* adaptation. Comparison with the expression levels of differentially regulated genes provides a benchmark to see changes in regulation of the potential genes. *recA*, *rho* and *gyrB* were chosen because these genes are constitutively expressed at all times. Their roles are: *recA* – DNA repair; *rho* – transcriptional termination; *gyrB* – DNA gyrase activity (Table 3.012). For subsequent experiments, recA was chosen because the product seemed to be the brightest.

Universal primers were designed for these genes and were PCR amplified from the genome of *A. baumannii* 11W359501 (Section 3.092). Figure 4.072 shows the successful isolation of these housekeeping genes and their corresponding base pair lengths can be found in Table 3.012.

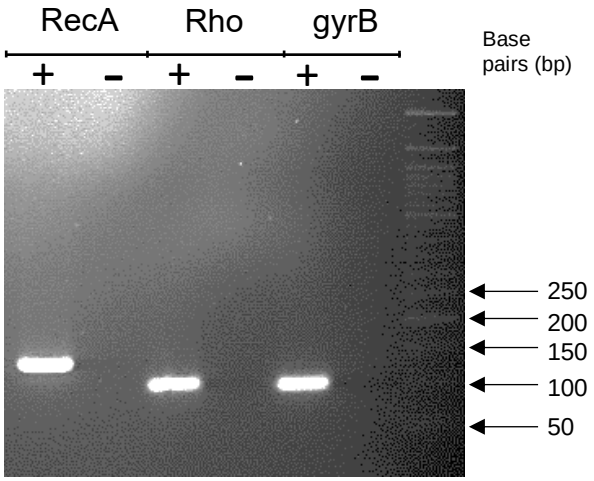


Fig 4.072: PCR products of potential housekeeping genes in *A. baumannii*. The *A. baumannii* 11W359501 genomic DNA was the template used to isolate these genes. Positive and negative controls were done for each gene to minimise errors. No contaminants were seen in each negative control. The PCR products were cross-referenced back with the product sizes in Table 3.012. All products were of the correct length.

4.08 Quality control of RNA samples sent for RNA-seq

In order to find out what genes are important in *in vivo* adaptation of *A. baumannii*, total RNA extraction was done at different timepoints to compare what genes were differentially regulated in the transcriptome. The 0, 1.5 and 5 hour timepoints were chosen after studying the growth rate in Figure 4.06, to represent pre-adaptation, adapting and post-adaptation stages of *A. baumannii* respectively. Extracting the total RNA yields both *A. baumannii* and zebrafish RNA, which needs to be separated so only *A. baumannii* RNA is read in the analysis (Section 3.08; Section 4.09).

Multiple steps were taken for quality assurance of the RNA samples. Genomic contamination tests ensure that only RNA was present in the samples and all DNA was depleted. RTPCRs were performed to check concentrations of RNA in the samples. RNA degradation was checked by Agilent RNA bioanalyser. The in depth details of this process is described in Appendix D.

4.09 Important genes found in RNA sequencing analysis

A. baumannii transcriptional analysis via RNA-seq of 1.5 hour and 5 hour post-injected *A. baumannii* into zebrafish hindbrain was done to identify potential differentially regulated genes in the pathogen. Searches for genes associated with surface changes that might alter *B. bacteriovorus* attachment and those responsible for the “resistance” to predation *in vivo* were done. These gene products may possibly be that are detrimental to *B. bacteriovorus* in their interaction with *A. baumannii*. The data was mapped to the *A. baumannii* 11W359501 genome to see differentially regulated genes.

The RNA-seq data was analysed and genes of interest were sorted into groups of a particular function. The compiled data can be found in Appendix F. It was expected that OM surface modifications would be present, so membrane proteins and lipoprotein genes were checked but there were not many majorly interesting genes found. The most interesting seems to be type VI secretion system related genes where 11/14 genes that were identified are upregulated at least 2-fold from 1.5 to 5 hours. Analysing these data, it was hypothesised that the type VI secretion system may be involved as a mechanism to help *A. baumannii* establish itself by reducing competition and as a side effect, protect from *B. bacteriovorus* predation. This may explain why low level *B. bacteriovorus* does not have a clear effect on *A. baumannii* and why *A. baumannii* is not preyed on *in vivo*.

Besides the type VI secretion system, many siderophore genes were found to be downregulated from 1.5 to 5 hours. This was not expected as nutrient acquisition genes should be upregulated when *A. baumannii* is in a host environment. Some *mla* genes were found which encodes glycerophospholipid transport either to or from the OM. However, none of these genes were upregulated >2-fold. These genes are discussed further in (Section 5.03). There was some increase in toxin genes such as *cvaA* (colicin V secretion protein) and *prtN* (pyocin activator protein) which might perhaps contribute to the killing of *B. bacteriovorus in vivo*.

5.0 Discussion

5.01 Discussion background

This project was based on the work of Dr Chris Moore (unpublished work) where zebrafish larvae hindbrain were injected with multiple relevant pathogens to see the clearance effect of *Bdellovibrio bacteriovorus* predation in an *in vivo* environment. These pathogens include *Serratia marcescens*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* which are all susceptible to predation *in vitro*. Drops in cfu/ml were observed for *S. marcescens* and *K. pneumoniae* when injected into zebrafish hindbrain but what is interesting about *A. baumannii* is that no drop in cfu/ml was observed. This led to a few questions: What are the targets of *B. bacteriovorus* attachment? When and how does a pathogen resist *B. bacteriovorus* predation *in vivo*? To answer this, the project was split into two parts.

The first part consisted of an *in vitro* approach to look at *B. bacteriovorus* predation on cell surface mutants. As *B. bacteriovorus* can only prey on Gram negative bacteria, logically the target for attachment would be somewhere on the outer membrane as it is uniquely found on Gram negative bacteria. It was previously hypothesised that Lipid A was the site of *B. bacteriovorus* attachment because previous experiments have shown that other main LPS components: O-antigen, core polysaccharide were not the attachment targets (Varon and Shilo, 1969) (Section 1.06). Many Gram negative bacteria die without outer membrane LPS but *A. baumannii* does not. Hence, predation was done on *A. baumannii* mutant strains lacking the lipooligosaccharide (LOS) to verify this.

The second part of the project attempts to answer: why does *B. bacteriovorus* prey on *A. baumannii in vitro* but cannot *in vivo*? It was hypothesised that *A. baumannii* was adapting to host environments resulting in a changed cell surface which also had the side-effect of preventing *B. bacteriovorus* predation due to different receptors that *B. bacteriovorus* is unable to bind to for attachment. Hence, RNA

sequencing of the *A. baumannii* adaptation process to host environments was done to try and elucidate what happens here.

5.02 What are the targets for *B. bacteriovorus* predation?

5.021 Lipid A is not the *B. bacteriovorus* attachment target

LOS is LPS with a modified core polysaccharide and lacking Lipid A. Predation on LOS- strains were expected to fail as it was hypothesised that Lipid A was the target for *B. bacteriovorus* predation. These LOS- strains were shown by the Trent lab (Boll *et al.*, 2016) to lack Lipid A.

These data show that both LOS+ and LOS- strains of the Ancestor and Evolved phenotypes of *A. baumannii* ATCC 19606 were equally preyed upon with no significant difference in predation was observed (Section 4.04). This rejects the hypothesis that Lipid A is the target for *B. bacteriovorus* predation is untrue as both LOS+ and LOS- strains – with and without Lipid A – are preyed upon by *B. bacteriovorus* HD100.

There is not much speculation in the literature as to what the target for attachment could be. A general outer membrane composes of glycerophospholipids (GPLs), LPS and OM proteins including lipoproteins and porins (Koebnik *et al.*, 2000). The possibilities of predation on these targets are explored further.

5.022 Potential targets for *B. bacteriovorus* attachment

5.0221 Glycerophospholipids

GPLs are present in both Gram negative and Gram positive bacteria but for the latter, the GPLs are shielded by a thick wall of peptidoglycan (PG). If GPLs are the target for *B. bacteriovorus* predation, PG in the Gram positive cell wall may be the reason why predation does not work. Therefore, it may be relevant and possible to test whether GPLs are the target in Gram positive bacteria lacking PG. Much work has been done on the so-called L-form bacteria which completely lack the cell wall (Errington, 2017). The lack of the cell wall makes these L-forms very unstable but are still able to grow in an osmoprotective medium (Mercier *et al.*, 2014). Predation on L-forms may be difficult as the weakened cell membrane could immediately break upon the predator coming into contact with the prey. GPL transport and its relationship with other outer membrane proteins will be discussed further in Section 5.03.

It is possible that there could be no predation at all in these L-forms as *B. bacteriovorus* has been shown to produce enzymes which digest prey peptidoglycan which is implicated to release essential signals for the crucial for invasion (Hobley *et al.*, 2012a). Without these signals, predation may not be possible.

5.0222 Lipoproteins

Of the main OM proteins, the lipoproteins and porins are abundantly found in the normal Gram negative bacterial OM. The general structure and composition of lipoproteins are similar between classes (Jonas, 2002), making lipoproteins a potential general recognition site for *B. bacteriovorus* attachment. Lipoproteins are essential components of the Gram negative cell envelope, providing a variety of functions such as stabilising the cell envelope (Suzuki H, 1978), iron uptake (Ostberg *et al.*, 2013) and attachment for

pathogenesis (Jin *et al.*, 2001). This may prove difficult to test as Gram negative bacteria naturally have lipoproteins embedded in their outer membrane. There has not been an example of a totally lipoprotein-deficient mutant that can survive from previous literature.

The lipoprotein trafficking pathway LolABCDE could possibly be explored in future predation experiments. Previous work has attempted to remove lipoproteins from the cell surface of *E. coli* by the deletion of *lolB* which prevents lipoprotein incorporation into the outer membrane (Tanaka *et al.*, 2001). This resulted in the toxic buildup of lipoproteins in the IM and was fatal to the cell. However, the overproduction of LolB in the outer membrane has been attempted (Yokota *et al.*, 1999). This study showed that LolB overproducing cells stimulated more membrane incorporation of lipoproteins LolB, Pal and NlpB. This can potentially be used to create an outer membrane with a high lipoprotein content and testing for increased predation rates between these mutants and WT could be carried out to see if lipoproteins are sites of attachment for *B. bacteriovorus*.

5.0223 Porins

The other major OM protein group are the porins – outer membrane channel proteins present in all Gram negative bacteria involved in the uptake of small molecules by diffusion (Benz, 1988). Porins generally have a β-barrel structure which is made of antiparallel β-sheets with hydrophobic amino acids on the outside and hydrophilic amino acids lining the inside (Fernández and Hancock, 2012). An alternate hypothesis is that *B. bacteriovorus* recognises β-barrel structures for attachment to diverse prey.

The evolution of β-barrel proteins have been suggested to come from ancestral ββ hairpins (Remmert *et al.*, 2010). This study identified sequence repeats in a majority of OM β-barrel proteins. A previous study has shown amino acid sequence alignments of porin genes from different classes of bacteria (Nikaido, 2003). Replication with the porin

genes obtained from the RNA-seq analysis to try and look at the potential binding sites of the OM surface exposed side of the porin was unsuccessful. When the amino acid sequences were aligned, there was very little homology between the 6 gene products (Appendix F). In addition, the similarities were not clustered together in a particular part of the gene which does not match the alignments from Nikaido.

My analysis of the expressed-in-host RNA-seq data from Section 4.09 has elucidated 6 differentially expressed porins in total (Appendix F). Of the 6 porin genes found, only one is very highly differentially regulated (upregulated) – the DcaP porin. This porin was first discovered by Parke *et al.* where they predicted a role in dicarboxylic acid trafficking into the cell (Parke *et al.*, 2001). Subsequent work has shown that *dcaP* is the most abundant upregulated gene during *A. baumannii* infection in rodent models (Bhamidimarri *et al.*, 2019). This study confirms the role of DcaP in carboxylic acid uptake which could contribute to *A. baumannii* pathogenesis. However, the fact that DcaP is upregulated in a rodent model infection suggests that this protein is generally upregulated in host pathogenesis and has no connection with *B. bacteriovorus* predation. It is possible that upregulation of DcaP might have affected the downregulation of a *B. bacteriovorus* recognised specific porin, without effects on the bacterial growth. However, there was no significant evidence of a such downregulated porin.

Future work

Given sufficient time, beyond this MRes study, generation of porin deletion strains of *A. baumannii* by mutagenesis could further test the recognition by the predator. Novel methods such as CRISPR and RecET-mediated recombineering could be used to induce specific gene deletions in *A. baumannii* (Karlapudi *et al.*, 2018, Tucker *et al.*, 2019).

Porins have been shown to affect growth and cell viability of other Gram negative bacteria, as shown in an $OmpA^-$ $OmpC^-$ $OmpF^-$ triple knockout mutant in *E. coli* (Choi and Lee, 2019). Although this mutant strain exhibits growth defects, their colony morphology seems normal. In addition, Meuskens *et al*. has produces a quadruple knockout of $OmpA^-$ $LamB^-$ $OmpC^-$ $OmpF^-$ in *E. coli* BL21 which displays a similar phenotype as the strain produced by Choi and Lee (Meuskens *et al.*, 2017). This quadruple mutant (BL21ΔABCF) has been shown to improve expression of recombinant OMPs, which could be useful in generating a strain with a lone OMP.

Conversely, the overexpression of porins on the OM could allow us to do a comparative predation susceptibility experiment. Ghosh *et al*. shows porins can be overexpressed to ~80% total membrane protein in *E. coli* by using pBluescript-derived vectors (Ghosh *et al.*, 1998). Measuring the time of predation on high and low % OM porin content bacteria could help us elucidate whether porins are the attachment sites.

Porin manipulation in the prey membrane could also be attempted. This could be done by deleting porins in a prey bacterium that naturally has few porins (e.g. *E. coli*) and replacing those genes with porins from *B. bacteriovorus*. In theory, it would be expected that the predator porins are distinct from prey porins so *B. bacteriovorus* does not prey on itself. Replacing all known porin genes in the prey would allow the investigation of whether the porins are the site for attachment. If attachment does still occur, that would suggest porins are not the site of *B. bacteriovorus* attachment.

Another way to test for porin involvement in *B. bacteriovorus* attachment could be the use of intracellular pathogens which are hypothesised to have fewer porins as they are adapted to the host environment. This could be done with bacteria such as *Salmonella typhimurium* or *Neisseria meningitidis*. *In vivo* predation assays in zebrafish hindbrain could be designed with comparisons to bacteria with normal porin expressions.

Since complete resistance to *B. bacteriovorus* has not been recorded, it can be assumed that it attaches to an essential-for-life general structure where the removal of one gene will not stop predation. Both lipoprotein and porins are abundant in the membrane, with many of the protein structures under each class of OM protein being very similar. Therefore, it is likely *B. bacteriovorus* attaches to many different proteins of similar structure on the surface – the next step would be to find out what this structure may be.

5.03 The Mla transport system: anterograde vs retrograde

The question of how *A. baumannii* can survive in the absence of LOS remains to be answered. There is conflicting evidence whether the Mla transport system transports GPLs in a retrograde or anterograde manner (Section 1.03). Powers and Trent showed 7/10 *mla* genes had mutated in a 120 generation evolution experiment and suggested the Mla transport system acts in a retrograde manner by the removal of GPLs from the outer leaflet of the OM (Powers and Trent, 2018). Clean deletions of *mlaE* and *pldA* showed an increased fitness in LOS- *A. baumannii* where it is thought that GPLs are not removed from the OM, therefore appear fitter. Powers and Trent argues that if the Mla transport system were anterograde, upregulation of *mla* genes would beneficial to the bacteria as the incorporation of GPLs to the OM would stabilise the membrane.

In contrast to this, Kamischke *et al*. argue that the transport system is anterograde, where GPLs are trafficked from the IM to the OM. In their study, mutants show a reduction of GPLs in the OM and their accumulation can be seen in the IM (Kamischke *et al.*, 2019). This paper suggests that GPLs are removed from the OM via vesicle formation or phospholipase activity. Kamischke *et al*. critiques Powers and Trent's hypothesis citing they should have looked at GPL content in the OM of the *mla* and *pldA* mutants for confirmation. They point out that the Mla transport system should be anterograde as the LOS would need to be replaced in LOS deficient strains – most likely by GPLs by this system.

A possible way to determine the mode of transport could be via transmission electron microscopy using a negative stain (Golding *et al.*, 2016). Comparison of the cell surface between the Evolved LOS+ and LOS- strains from Powers and Trent could show possible dilution of features within the OM. An anterograde transport system may give the LOS- strain a more dilute membrane that looks less healthy due to

fewer GPLs trafficked to the membrane. Retrograde transport may give a membrane that resembles the LOS+ counterpart.

The RNA-seq analysis shows 7 *mla*-associated genes that have been identified (Appendix F). Of these 7 genes, the 4 normal genes are <2 fold differentially regulated therefore there is no drastic up/down regulation when adapting to a host environment. The other 3 genes are antisense which suggests a role in blocking the expression of the complementary *mla* gene. Only the *mlaC* antisense RNA is upregulated >2 fold which means *mlaC* is repressed when wild-type *A. baumannii* is adapting to host. MlaC is a periplasmic protein which aids in the transport of GPLs to or from the OM (depending on antero/retrograde system). The repression of this system when *A. baumannii* is adapting to the host suggests GPLs cannot be replaced into or out of the membrane. Either way, this seems counter intuitive as such a constitutive process should not be repressed in *A. baumannii* adaptation. One hypothesis could be that *A. baumannii* stops this process to divert energy for the upregulation of genes for host pathogenesis and defence. More work will need to be done for confirmation.

Although *mla* regulation is shown to be altered in LOS- strains (Powers and Trent, 2018), it is not certain if these antisense mechanisms are employed in their study. Examination of expression levels of the antisense genes in LOS- mutants should be explored to show whether this system is active in the absence of LOS.

Regardless of whether the Mla system is antero/retrograde, an important question to answer is whether or not the absence of LOS affects predation. My predation results indicate little difference in predation in strains with or without LOS, except for when Ancestor LOS+ drops at 30 hours but Ancestor LOS- does not (Section 4.041). The LOS- strain is shown to be fitter than the LOS+ in this regard which may be attributed to the energy requirement to synthesise the nine carbon-containing molecules in LOS, and the cost of transporting and

incorporating it into the outer membrane. One possible continuation to consider could simply be to take longer timepoints at 48 and 72 hours to see if the cfu/ml of the LOS- strain begins to fall due to nutrient deprivation. A recent study reported that *A. baumannii* LOS mutant strains exhibit a similar growth rate to the WT when grown in minimal media (Nagy *et al.*, 2019). This could be due to the rate of outer membrane biogenesis because of phospholipid flipping and not nutrient availability. Coupling this hypothesis to the data from this study, it would explain why the Ancestor LOS- strain growing significantly better than the LOS+ counterpart as the LOS+ does not utilise the phospholipid flipping mechanism, therefore would be subject to nutrient deficiency quicker than the LOS- mutants (Section 4.041).

5.04 A potential two way killing mechanism between *A. baumannii* and *B. bacteriovorus*

In the series of low level *B. bacteriovorus* predation experiments (Section 4.05), it was found that a level of ≤10^6 cfu/ml *B. bacteriovorus* could not clear 10^8 cfu/ml *A. baumannii* by *in vitro* experimentation in CaHEPES buffer. This was surprising as 10^6 cfu/ml is still a high number of *B. bacteriovorus* so some level of predation was expected. The absence of predation suggests that *A. baumannii* may possess some defence mechanism to kill the *B. bacteriovorus*, which can be interpreted as the prey not being susceptible to predation in host.

Looking at the RNA-seq data, the most interesting thing from that analysis was the upregulation of 11 out of 14 type VI secretion system (T6SS) genes by >2 fold. The upregulation of these genes suggest that it is not receptor change of the *A. baumannii* that confers a lack of predation but active defence mechanisms of the pathogen against the predator which does so. Therefore, there could exist a two way killing mechanism between *B. bacteriovorus* predation and *A. baumannii* T6SS mediated killing. This hypothesis could be further supported by

the *in vivo* zebrafish injection experiments where *A. baumannii* could not be cleared by *B. bacteriovorus*.

From the *in vitro* data, it is established that 10^8 cfu/ml *B. bacteriovorus* can kill *A. baumannii* so something must occur *in vivo* to stop predation from happening. A possibility is that the zebrafish immune system attempts to clear foreign material in the hindbrain, killing some of the *B. bacteriovorus* alongside the *A. baumannii* resulting in a lower cfu/ml of *B. bacteriovorus* remaining in the hindbrain. Then the model follows the *in vitro* assay where a lower *B. bacteriovorus* cfu/ml cannot clear the *A. baumannii* infection.

This leads to the belief that a low level *B. bacteriovorus* cannot kill the *A. baumannii* fast enough therefore *A. baumannii* upregulates the gene expression for the T6SS components to kill the *B. bacteriovorus* before they can kill it. A high enough cfu/ml will not give the *A. baumannii* enough time to alter the gene expression, therefore the predation phenotype can be seen.

Perhaps at a high cfu/ml of *B. bacteriovorus*, multiple predators attach to the *A. baumannii* prey and invade simultaneously but the upregulation of the *A. baumannii* T6SS can only kill one predator, allowing the other to enter. This may not happen at a low cfu/ml given simultaneous invasion would be less likely and *A. baumannii* T6SS acts much faster.

5.05 Future work exploring the T6SS and its effect *on B. bacteriovorus* predation

As explained in the Aims (Section 3.17), a change in the commercial supply of reagents used by the RNA-seq company Vertis caused the RNA-seq data to be delayed by several months, so confirmation of the hypotheses by gene deletion was not possible. However, future work has been suggested.

5.051 T6SS mechanics

A possible way to determine whether the T6SS is killing the *B. bacteriovorus* could be to do RNA-seq on *in vitro A. baumannii* that has been adapted in low level *B. bacteriovorus* (maybe 10^2 – 10^4) conditions to see the differentially regulated genes. We would expect the T6SS genes to be upregulated following this hypothesis.

An alternate hypothesis independent of the immune system is the accidental killing of *B. bacteriovorus* due to the upregulation of the *A. baumannii* T6SS as a defence mechanism in response to eukaryotic host cells. Studies have mentioned the ability for the T6SS to target other bacteria when coinfecting a eukaryotic host (Ho *et al.*, 2014, Schwarz *et al.*, 2010). Ho *et al*. also reports that *Acinetobacter* species activate their T6SS and fires in an unregulated manner, killing all neighbours indiscriminately. This would fit the hypothesis as it would reduce the predator burden to the point where no predation can be seen.

5.052 Fluorescent tagging of T6SS for confirmation by microscopy

Fluorescently tagging a T6SS component and visualising the interaction between *A. baumannii* and a low level *B. bacteriovorus* could be another viable strategy. Previous work has shown that various subunits of the T6SS could be fluorescently tagged – these being TssB (VipA), a sheath protein which forms tube structures (Basler *et al.*, 2012) and TssA, which initiates tail sheath polymerisation (Zoued *et al.*, 2016). Both of these subunits seem to be able to function properly with the inserted tag. With the fluorescently labelled T6SS of *A. baumannii*, a range of predation assays can be designed visualising *in vitro* and *in vivo* zebrafish predation under a fluorescent microscope.

Unfortunately, there are no reported fluorescently tagged VgrG spike component studies in the literature. Labelling the VgrG subunit could potentially allow the visualisation of the spike mechanism entering and killing the *B. bacteriovorus*. It may be possible to use the same technique used by previous studies – tagging a superfolder GFP in the C-terminus of the gene (Basler *et al.*, 2012). However, it is not clear whether the tagged VgrG will be completely functional. If possible, one of two scenarios would be expected: 1) *B. bacteriovorus* randomly collides with the T6SS, killing it – this could be shown by live-dead staining of the *B. bacteriovorus*; 2) The T6SS spike is released at the point of *B. bacteriovorus* attachment, after it has attached. This would give good evidence that the T6SS kills the *B. bacteriovorus* during predation.

5.053 Comparing T6SS of other bacteria

As previously mentioned, Dr Chris Moore's experiments of *B. bacteriovorus* predation on *K. pneumoniae* and *S. marcescens* has been successful whilst predation on *A. baumannii* was not. The lack of "resistance" to *B. bacteriovorus* predation in these pathogens challenges the idea of whether the T6SS of *A. baumannii* is the cause of the "resistance" *in vivo*.

This raises the question of whether the former two pathogens encode the T6SS responsible for killing the *B. bacteriovorus* predator. Interestingly both *K. pneumoniae* and *S. marcescens* possess the T6SS machinery. However, it seems only specific strains encode the T6SS for both these species.

A computational study analysing T6SS genes in the genomes of three *K. pneumoniae* species has shown a differential number between strains (Barbosa and Lery, 2019). The predictive inconsistency of the presence of these T6SS genes suggests that *K. pneumoniae* only expresses these genes in specific situations. *S. marcescens* Db10 has been reported to be highly antibacterial and its T6SS is required to persist in a mixed culture (Murdoch *et al.*, 2011). This strain can fire its T6SS without cell contact and has been described previously for *Acinetobacter* species (Gerc *et al.*, 2015). A sequence comparison of T6SS genes between *A. baumannii* 11W359501 and *S. marcescens* Db10 has shown a low percentage of identity (highest 44%) between the main T6SS apparatus (Appendix E). This shows that the T6SS is not highly conserved between these two species therefore the T6SS of *S. marcescens* may not be acting or regulated in the same way as *A. baumannii*, hence does not get upregulated in host and *S. marcescens* killing is observed. Perhaps for future experiments, low level predation can be tested on already well established T6SS expressing bacteria like *Vibrio cholerae* to determine whether the T6SS will kill the low level *B. bacteriovorus* as with *A. baumannii*.

5.06 Consideration of other potentially important genes that were differentially regulated in RNA-seq data

5.061 Siderophores

A lot of different siderophore genes were found in the RNA-seq analysis, many of which are involved in the chelation of iron. These include the subunits of: acinetobactin, ferric anguibactin, enterobactin, bacterioferritin, aerobactin, ferrichrome outer membrane transporters and other general iron uptake genes (Appendix F). Interestingly, the general trend of these genes is downregulation which is unexpected as iron scavenging should be increased to gather the essential element especially in a limited host environment.

Of all the siderophores found, only 2 genes were found for acinetobactin subunits where both were severely downregulated, contradicting the current knowledge. In previous studies, it was shown that acinetobactin was isolated under low iron conditions (Yamamoto *et al.*, 1994) and suggested that it plays a role over the course of an infection (Shapiro and Wencewicz, 2016). Some of the ferric anguibactin genes are also downregulated – these siderophores have a similar structure to acinetobactin (Wuest *et al.*, 2009).

There is only one previous study exploring the downregulation of siderophores in bacteria but it is not applicable as it investigates interspecies competition between bacteria and does not involve a host infection system (Harrison *et al.*, 2007). These data would need to be studied further to find out why this downregulation occurs.

B. bacteriovorus itself does express an aerobactin-like siderophore system Bd1575-1576 and Bd1392 (Capeness, unpublished), so it would not be powerless to compete for iron if adapted to predation in the presence of a siderophore producing prey.

5.062 Toxins and T2SS

Of the 7 toxin related genes found in the analysis, colicin V and pyocin activator protein were upregulated >2 fold whereas zonula occludens toxin was downregulated >2 fold (Appendix F). Secreted colicin and pyocin may play some role in killing *B. bacteriovorus in vivo*. These genes are not majorly upregulated in the host, so it is assumed that they may be subsidiary to another mechanism which kills the predator (eg. T6SS).

There were also 6 type II secretion system (T2SS) genes found in the RNA-seq data. Of these genes, 1 was upregulated >2 fold – this was the *gspJ* gene, which encodes for a pseudopilin component. Furthermore, 2 genes were downregulated >2 fold, these being the hypothetical proteins E and F (Appendix F). The T2SS is known to secrete a variety of proteins out of bacterial cells including toxins, proteases, lipases and phosphatases (Korotkov *et al.*, 2012). This could be the mechanism by which the upregulated toxins are exported to damage the zebrafish cells *in vivo*. However, there do not seem to be as many genes for this system that is found in the transcriptome to fully cover the T2SS components, suggesting this may not be a crucial mechanism that is activated in these experiments. This is mentioned as it could play a small role in the overall picture of *A. baumannii in vivo* mechanisms.

5.07 Summary

What does *B. bacteriovorus* recognise on the surface of prey?

The work done in this project focusses predation of *A. baumannii* mutant LOS strains with *B. bacteriovorus*. These series of experiments has proven the long-speculated hypothesis wrong, that Lipid A was the target for *B. bacteriovorus* attachment in predation. Disproving this target as the attachment site indicates that the most probable structure of the Gram negative cell surface is not the receptor. By using the LOS-strains in this project, there is further evidence that gives support that the core oligosaccharide is not the target due to the presence of predation in these mutant strains.

‘Why can *B. bacteriovorus* prey on *A. baumannii in vitro* but cannot *in vivo*?’

The other half of this project has determined some interesting genes that may contribute to the virulence *A. baumannii* in host. Most notably, the type VI secretion system seems to be a critical mechanism for the prevention of *B. bacteriovorus* predation. Confirming the role of this mechanism will allow the formation of countermeasures to any obstacles that may prevent *B. bacteriovorus* from becoming a therapeutic tool, especially for multidrug resistant bacteria such as *A. baumannii*.

Therefore, future work should continue to look at possible targets of *B. bacteriovorus* attachment – a good starting point would be other Gram negative cell surface exclusive genes, namely porins and lipoproteins. Discovering this receptor would allow a more complete understanding of *B. bacteriovorus* predation mechanics, which could allow the generation of novel ways of overcoming fatal diseases.

6.0 Bibliography

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7.0 Appendices

7.01 Appendix A

Growth media and buffer solutions:

All media were made with ddH_2O and autoclaved prior to use.

The following ingredients were used to make up to a final volume of 1 litre.

YT broth/agar – for *E. coli/A. baumannii* growth

5 g NaCl (Fisher Scientific)

5 g Yeast extract (Difco Laboratories)

8 g Tryptone (Difco Laboratories)

For agar – 10 g Select Agar (Sigma-Aldrich)

Adjusted to pH 7.5 using 2M NaOH

YPSC (top and bottom) – for plague plating of *B. bacteriovorus*

1 g Yeast extract (Difco Laboratories)

1 g Peptone (Difco Laboratories)

0.5 g Sodium acetate anhydrous (Fisher Scientific)

0.25 g $MgSO_4.7H_2O$ (Fisher Scientific)

For YPSC top – 6 g Select Agar (Sigma Aldrich)

For YPSC bottom – 10 g Select Agar (Sigma Aldrich)

Adjusted to pH 7.6 using 2M NaOH

Sterile 0.17M $CaCl_2$ added to YPSC top after autoclaving to give 0.25 gL^{-1}

CaHEPES buffer – for predation cultures

5.94 g HEPES (Sigma Aldrich)

0.294 g $CaCl_2.2H_2O$ (Fisher Scientific)

Adjusted to pH 7.6 using 2M NaOH

Zebrafish experiment solutions

0.5x E2 buffer – for use with zebrafish

There are multiple steps in making this medium. Media and solutions are autoclaved before use.

20x E2 stock (to make 2 litres)

35 g NaCl (Fisher Scientific)

1.5 g KCl (Fisher Scientific)

0.82 g KH_2PO_4 (VWR)

4.8 g $MgSO_4$ (Fisher Scientific)

0.24 g Na_2HPO_4 (Sigma Aldrich

2000 ml ddH_2O

0.5M $CaCl_2$

14.6 g $CaCl_2.2H_2O$

200 ml ddH_2O

350mM $NaHCO_3$

5.9g $NaHCO_3$

200 ml ddH_2O

0.5x E2 buffer (to make 1 litre)

25 ml 20x E2 stock

1 ml 0.5M $CaCl_2$

1 ml $NaHCO_3$

1000 ml ddH_2O

1 ml 1% Methylene blue

20x Tricaine stock – for anaesthetising zebrafish

49 ml H_2O

1050 µl 1M Tris base (pH 9)

Autoclave

Add 200 mg Tricaine powder (MS-222, Sigma Aldrich) before use.

Can be stored for one month at 4°C. Must be protected from light.

2% Fish agarose –creates the mould to immobilise zebrafish

2 g agarose (Sigma Aldrich)

100 ml 0.5x E2 buffer

Melt in microwave.

Agarose gel electrophoresis solutions for DNA/RNA gels

TBE buffer

108 g Tris-HCl

55 g Boric acid

40 ml 0.5 M EDTA (pH 0.8)

10x loading buffer stock

25% w/v Ficoll400

0.4% w/v Orange G dye

3x loading buffer

900 µl 10X loading buffer stock

2.1 ml 20% w/v glycerol

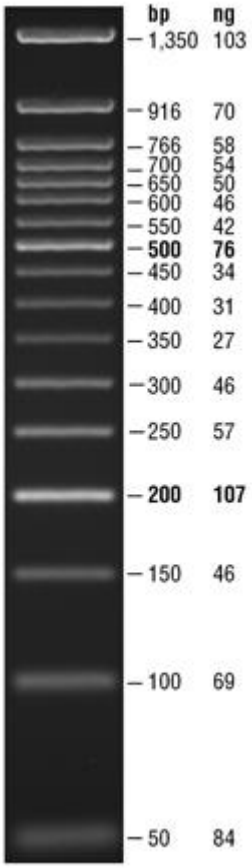
50 bp DNA marker

20 µl 50 bp marker (NEB)

120 µl 3x loading buffer

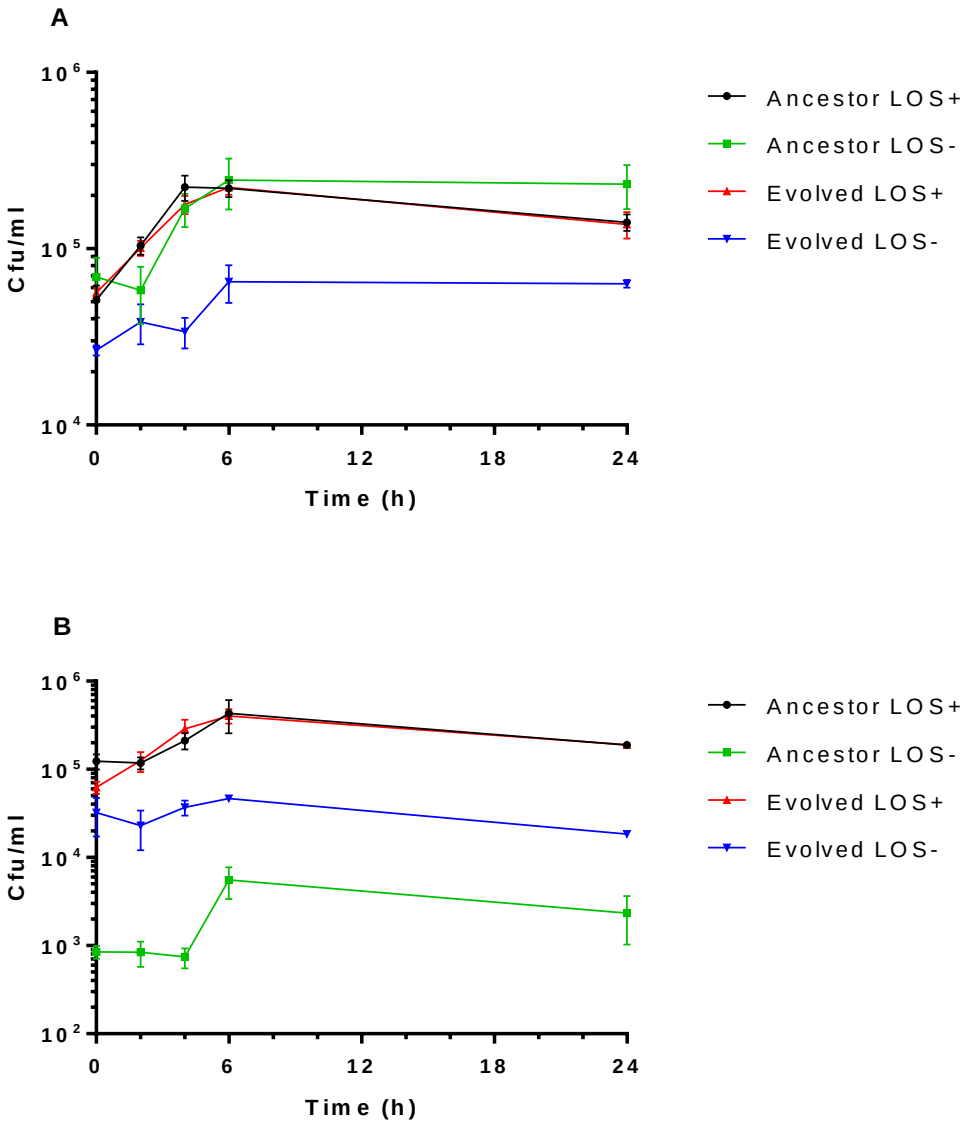
160 µl TE (10 mM Tris-HCl, 2 mM EDTA at pH 8.0)

100 µl 20% w/v glycerol



New England Biolabs 50 bp DNA marker used in this study.

7.02 Appendix B



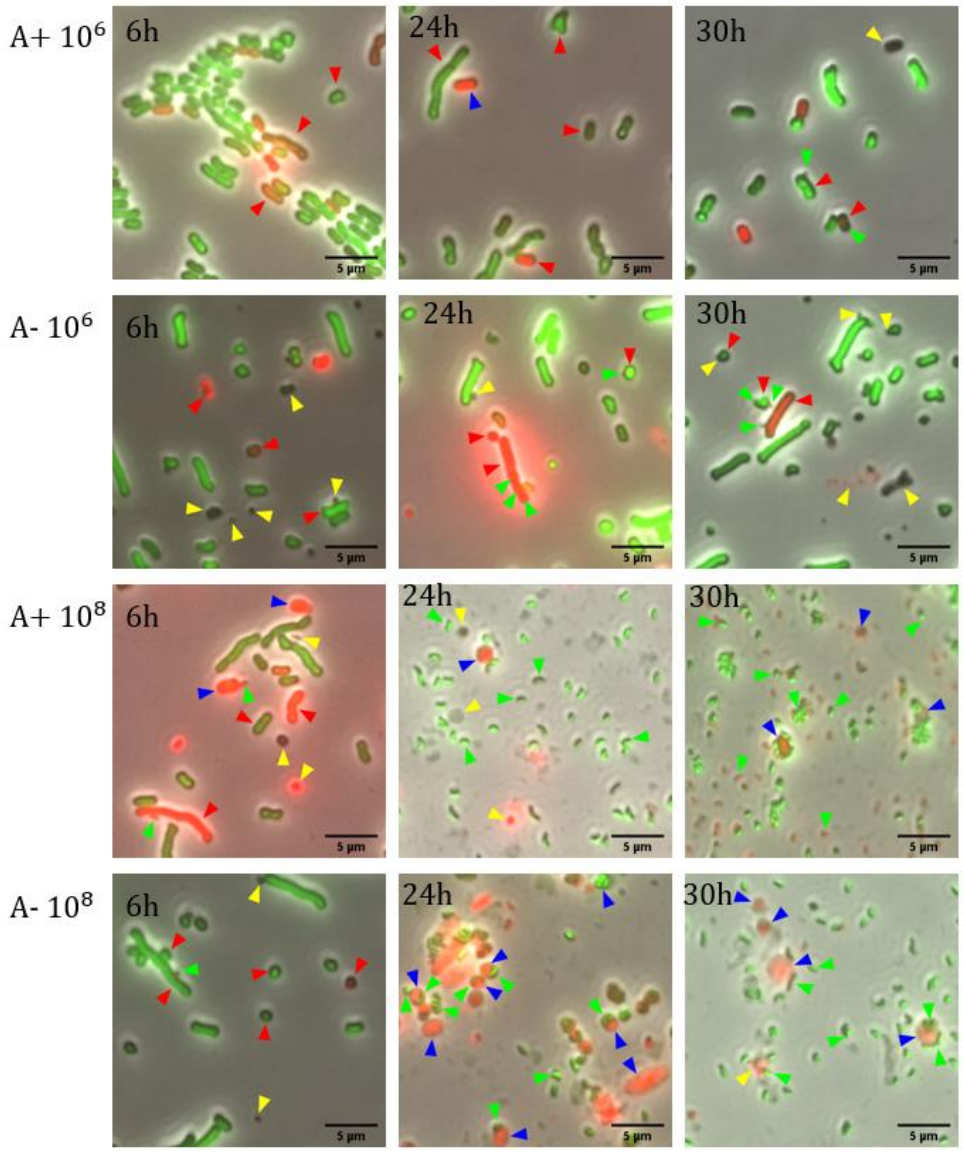
Viable count plot showing persistence of *A. baumannii* ATCC 19606 and *A. baumannii* 5075 Ancestor and Evolved LOS+/- mutants for 24 hours in CaHEPES at 37°C. At timepoints 0, 2, 4, 6 and 24 hours enumerations were taken by Miles and Misra. A) *A. baumannii* ATCC 19606 mutant strains. B) *A. baumannii* 5075 mutant strains.

In *A. baumannii* ATCC 19606 mutant strains, the bacterial viability for the Ancestor LOS+ strain seems to have stabilised at 4 hours whereas for the other mutants (Ancestor LOS-, Evolved LOS+/-) stabilisation occurred at 6 hours. The cfu/ml of LOS+ strains increased from 0 to 6

hours but the cfu/ml of the LOS- strains dropped from 0 to 2 hours (Ancestor LOS-) and 2 to 4 hours (Evolved LOS-) but increased overall by 6 hours. From 6 to 24 hours, the cfu/ml of all strains decreased.

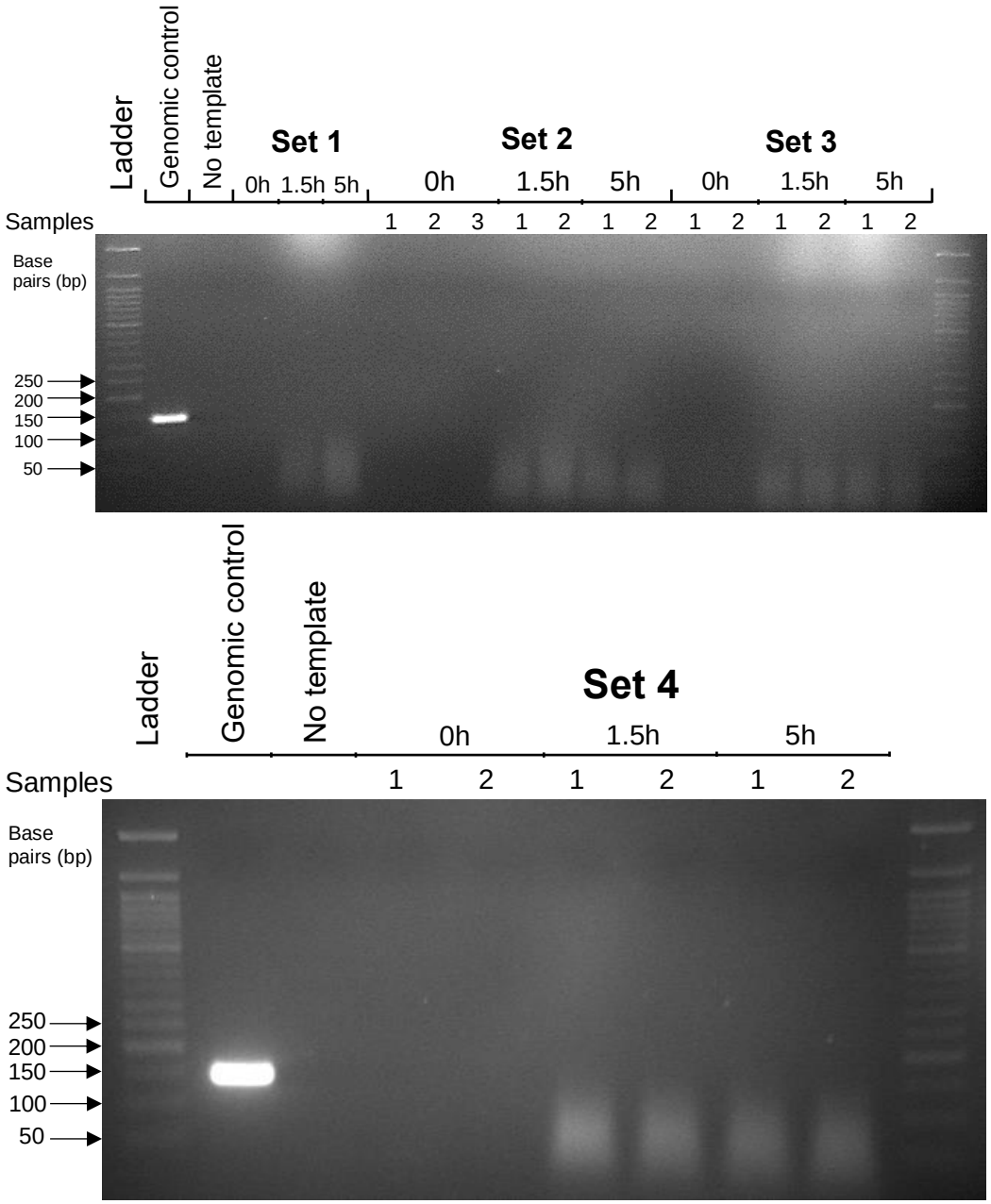
In the *A. baumannii* 5075 mutant strains, the bacterial numbers for all strains stabilised at 6 hours. Only the cfu/ml of the Evolved LOS+ strain increased from 0 to 6 hours without dropping. The cfu/ml of the other strains decreased from 0 to 2-4 hours. From 6 to 24 hours, the cfu/ml of all strains decreased steadily.

7.02 Appendix C – Fluorescent microscopy

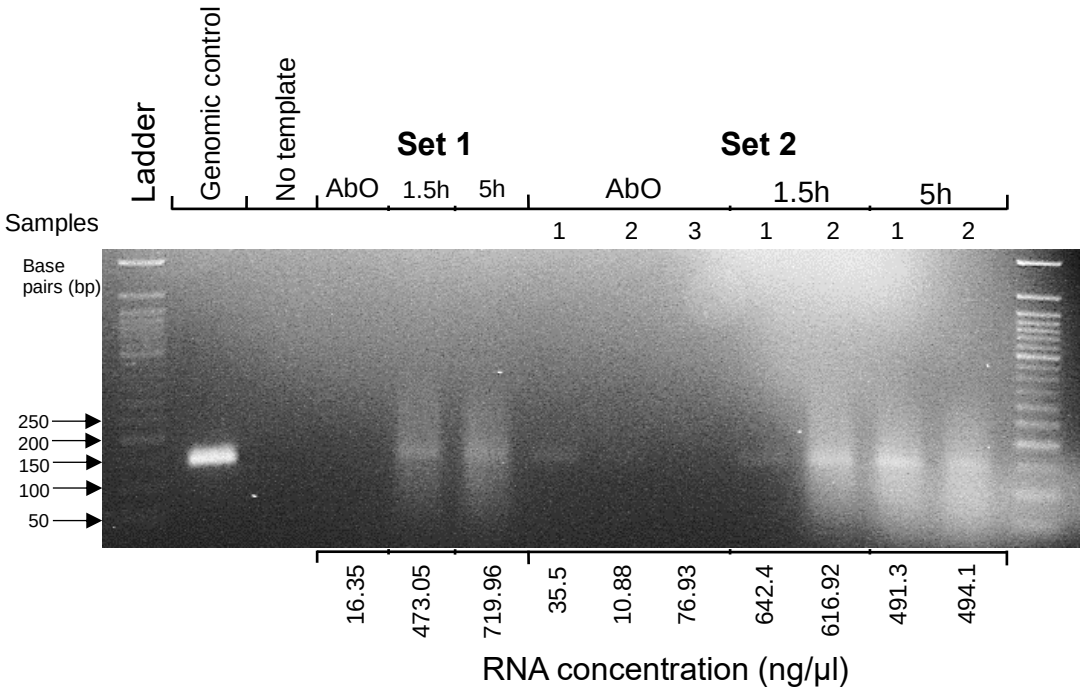


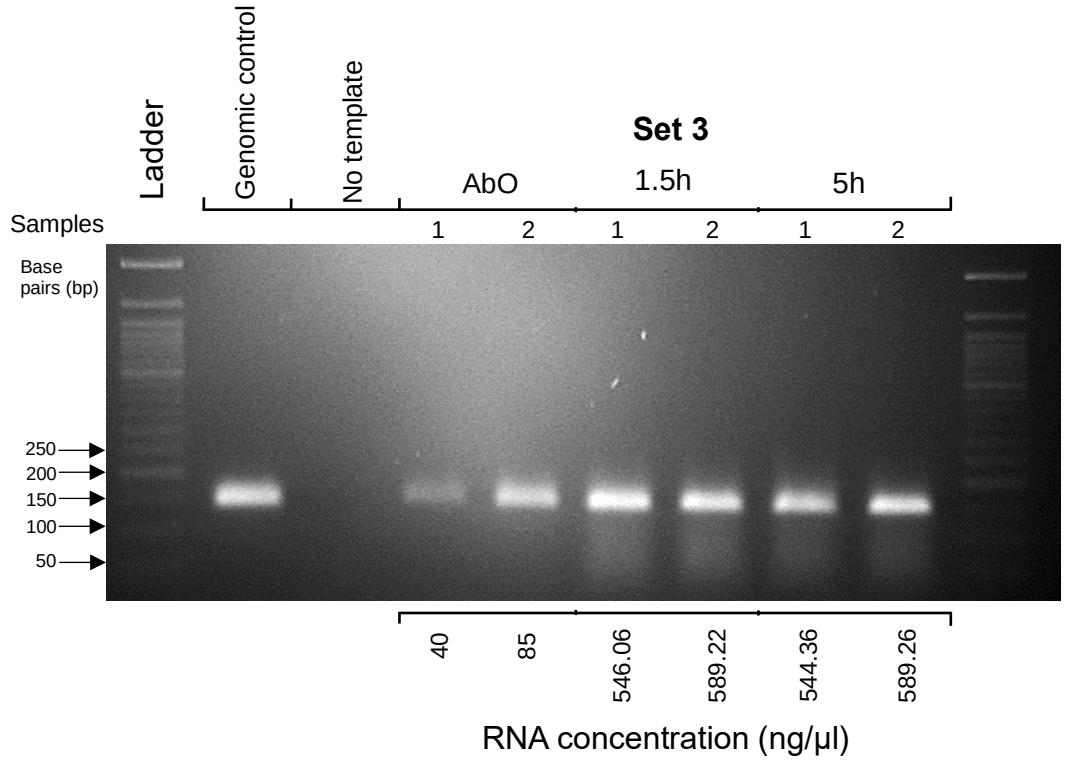
Fluorescent microscopy using LIVE-DEAD staining of *B. bacteriovorus* HD100 preying on *A. baumannii* ATCC 19606 Ancestor +/- strains. Low level (10^6 cfu/ml) and high level (10^8 cfu/ml) *B. bacteriovorus* were used to prey on 10^8 *A. baumannii* Ancestor LOS+ or LOS- strains. *B. bacteriovorus* was added to the prey at 6 hours. Images were taken at 6, 24 and 30 hours. *A. baumannii* cells are often long and can be curved. *B. bacteriovorus* cells are tiny curved cells that can be seen attached to the bigger *A. baumannii* cells. Bdelloplasts are red circular cells and can often be seen with *B. bacteriovorus* cells inside. Legend: Green = live cells; Red = dead cells; green arrow = *B. bacteriovorus* cells; red arrow = *A. baumannii* cells; blue arrow = bdelloplast; yellow arrow = debris; A+ = Ancestor LOS+; A- = Ancestor LOS-; h = hours. Scale bar = 5 µm. Magnification 1000x.

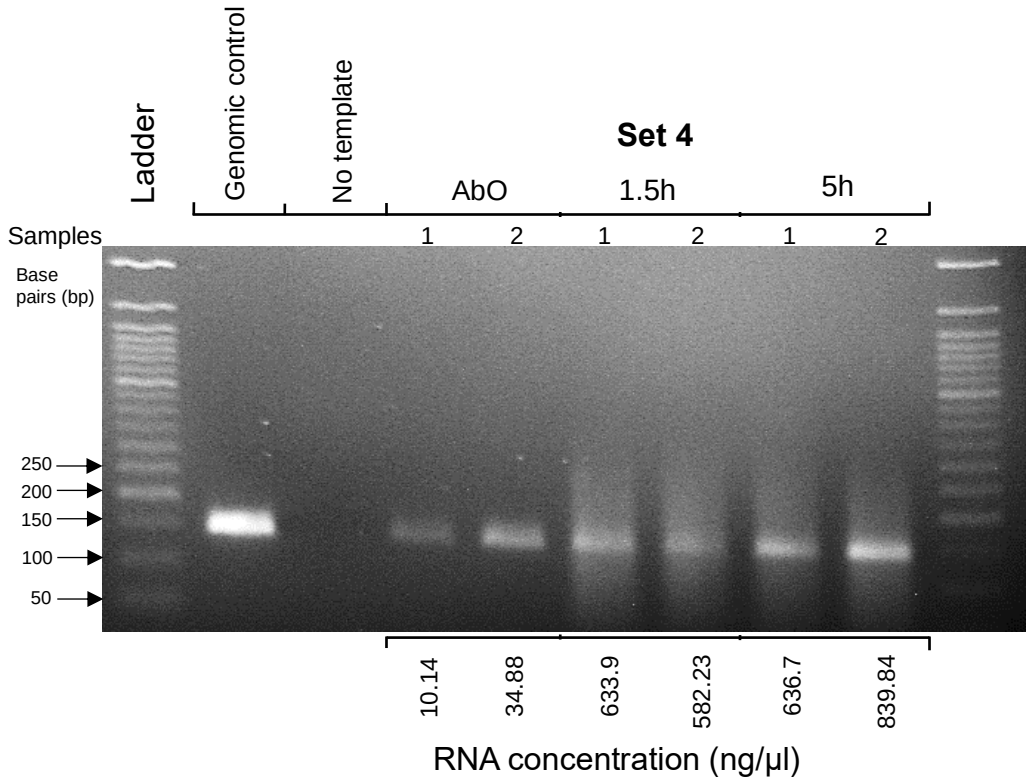
7.03 Appendix D – Quality control of RNA samples



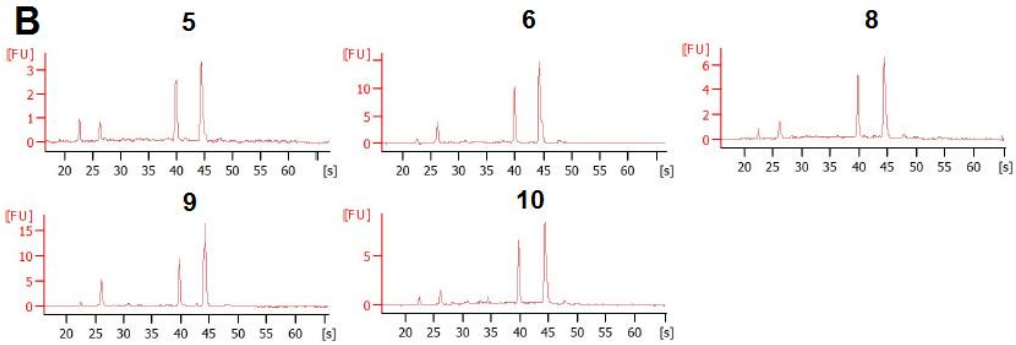
D1: Testing for presence of genomic contamination in the total RNA samples of *A. baumannii* 11W359501 extracted from zebrafish post-injection. Four sets of experimental repeats were done as follows: Set 1: 1x sample for 0h, 1.5h and 5h; Set 2: 3x samples for 0h, 2x samples for 1.5h and 5h; Set 3: 2x samples for 0h, 1.5h and 5h; Set 4: 2x samples for 0h, 1.5h and 5h. A genomic positive control with pure *A. baumannii* 11W359501 genomic DNA and a negative no template control without DNA was used for each 0.8% agarose gel run. All samples were PCR amplified for *recA* where the product in the genomic control matches the base pair length (Table 3.012). No contamination was observed in any of the four sets.


Ladder
Genomic control
No template
Set 1
Set 2
AbO
1.5h
5h
AbO
1.5h
5h
Samples
1
2
3
1
2
1
2
Base pairs (bp)
250
200
150
100
50
16.35
473.05
719.96
35.5
10.88
76.93
642.4
616.92
491.3
494.1
RNA concentration (ng/µl)


Ladder
Genomic control
No template
Set 3
AbO
1.5h
5h
Samples
1
2
1
2
1
2
Base pairs (bp)
250
200
150
100
50
40
85
546.06
589.22
544.36
589.26
RNA concentration (ng/µl)



D2: Testing for RNA viability of *A. baumannii* 11W359501 samples. AbO is made from *A. baumannii* RNA only, so zebrafish RNA was not present. Zebrafish were sacrificed at 1.5h and 5h post-injection and total RNA was extracted. Four sets of experimental repeats were done as follows: Set 1: 1x sample for AbO, 1.5h and 5h; Set 2: 3x samples for AbO, 2x samples for 1.5h and 5h; Set 3: 2x samples for AbO, 1.5h and 5h; Set 4: 2x samples for AbO, 1.5h and 5h. A genomic positive control with pure *A. baumannii* 11W359501 genomic DNA and a negative no template control without DNA was used for each 0.8% agarose gel run. All samples were RTPCR amplified to *recA* where the products match the genomic control. No product was observed for Set 1 AbO and Set 2 AbO Sample 3. Products were observed for all other samples. RNA concentrations were measured immediately after RNA was extracted and displayed below each lane. Abbreviations: AbO = *Acinetobacter baumannii* only. It is unknown why RNA concentration does not increase with growth, but it is assumed this is due to RNA degradation.



D3: Electropherograms of RNA samples show clear peaks of 16S and 23S ribosomal RNA in the correct position at 40 and 45 seconds. These samples would be sent for RNA-seq.

7.03 Appendix E

Sequence comparisons of type VI secretion system components between *S. marcescens* Db10 and *A. baumannii* 11W359501.

TssA

```
# Length: 391
# Identity:      96/391 (24.6%)
# Similarity:   160/391 (40.9%)
# Gaps:          80/391 (20.5%)

Sm_TssA            1 MAIDTDTLLAPVDADNPCGDNLEYDADFLAMEQAATGK---AEQQFGSTI     47
                     |:||...||.|::....||::..:..:|..:::|.|..   .:|  |..:
Ab_TssA            1 MSIDISELLKPINDSLLCGEDYSFSNEFHEIKKARTQDDLLLDQ--GDWV     48

Sm_TssA           48 IPEEAPDWVQVER-LATSLLTRTKDLRIMLYLTRAWTQLRGLPGYADGLT     96
                     ...:..||..|.: ::|.|:.:|||:|::.::..|||.|.|..|...|:|
Ab_TssA           49 AERKQADWDFVAKSVSTLLIEKTKDIRLLTWVIEAWTHLNGFEGMVKGIT     98

Sm_TssA           97 LVHQSMTRYWNTLQPPLEFDGEADPLF-----RINALADLGDKAALAASV    141
                     |.|..:.:||..:.|.:| |.:.|...     .||.|..|..|..|..:.
Ab_TssA           99 LTHTMLNQYWQDIHPIIE-DDDLDQRIGLLQGLINQLPMLLKKVPLINTA    147

Sm_TssA          142 RAAPLL---------------------KSAAGEISLRDAGALLDGSKQ-E    169
                     ....||                      :|...|:...|. |:.:.||. :
Ab_TssA          148 PYYNLLDYDNFLYHENIRRKQTEEYESQSGPSELEQFDQ-AIFNTSKTFQ    196

Sm_TssA          170 CPNFPGGRARLQ---------DELAQQDRPEGALVTRIANTLSAIRSEVT    210
                     ..|:....:.|.         |.|...|.|..|.:.....|:.
Ab_TssA          197 YSNYQEFNSVLTEWNVLKQTLDHLMGLDSPSFAAIDSAFETIH-------    239

Sm_TssA          211 RHLGESALPEMSALTKVFSLVALAGQSDAPAADEPDATPEAAAMQQPAAV    260
                           |.|.|::...|. |...|| :.|..|.....:|:....|
Ab_TssA          240 -----------STLRKIYKAEAF-GTGLAP-SQEQAAVITTPSMENQVPV    276

Sm_TssA          261 QSAT-APLNWRSAQ--IQSRDDAQLMLDKVKNYFRLHEPSHPAPLMIDRV    307
                     |..: .|:....||  :.:|:.|..:|.::.:||:.:||..|...|:
Ab_TssA          277 QIVSDQPMFQPQAQTHLANREQAMKVLQEIADYFQANEPHSPVSYML---    323

Sm_TssA          308 QRLITLDFM-------QIVRDLAPDGLNQLETILG-RPDNE    340
                     |:.|....|       |:::|..|  |..::.:|| :|.||
Ab_TssA          324 QKTIKWSQMPLHEWLAQVIKDEHP--LQMVQEMLGVQPKNE    362
```

TssB

```
# Length: 160
# Identity:      70/160 (43.8%)
# Similarity:   108/160 (67.5%)
# Gaps:          1/160 ( 0.6%)

Sm_TssB            4 SKSRSGQKFIARNRAPRVQIEYDVEIYGAERKIQLPFVMGVMADLVGKAV     53
                     :|..|.||.:.|.|.||||:.|||||..|:...:||||:|||.|....:.
Ab_TssB            2 AKRESVQKKLQRIRPPRVQLTYDVEIGDAKEAKELPFVVGVMGDFSAASE     51

Sm_TssB           54 DPQASVEERKFMEIDVDNFDERMKSLKPRVAYQVDNTLTGE-GKLNIDLT    102
                     ..:..::::||:.:|:||.||.|:||.||.|:|||||||.| ||:.:|||
Ab_TssB           52 LERTKLKDKKFINVDLDNIDEVMESLSPRAAFQVDNTLTEEGGKMAVDLT    101

Sm_TssB          103 FESMEDFSPAAIARKVDALDNLLDARTQLSNLLSYMDGKNGAEELISKIL    152
                     |:|||||.|..:.::||.|..|::||.:|::|.:.:.....|:|:.::|
Ab_TssB          102 FKSMEDFRPENVVQQVDPLKKLVEARERLTDLRNKISNSERLEDLLDEVL    151

Sm_TssB          153 QNPALLQSLT    162
                     :|...::.|:
Ab_TssB          152 KNTDQIRKLS    161
```

TssC

```
# Length: 498
# Identity:     223/498 (44.8%)
# Similarity:   329/498 (66.1%)

Sm_TssC           1 MSNSPQQQNALQTTETFSSDEFSALLNKEFRPKTDQAKEAVENAVKTLAQ     50
Ab_TssC           1 MNNT--QSAAMPLVENEEVNLLDSIVEQSRIARNEEEHSRAKSLIGELAK     48

Sm_TssC          51 QALENTVTVSSDAYRTIQALIAEIDEKLSQQINQIIHHDDFQKLEGAWHG    100
Ab_TssC          49 EVMAGTITVSENMTLSIDKRIAEIDALISKQLSQIMHNEQFQKIESTWRG     98

Sm_TssC         101 LHYLVNNSETDEMLKIRFMSISKQELGRTLKRYKG-VGWDQSPIFKKVYE    149
Ab_TssC          99 LYYFCQETPSNPLIKIRMLNTTKKEL---VKDFQGATDFDQSTLFKKIYE    145

Sm_TssC         150 EEYGQFGGEPFGCLVGDYYFDHSPQDVELLGEMAKIGAASHCPFIAGTAP    199
Ab_TssC         146 EEYGSFGGAPYSALIGDFEFDRTPSDMYLLEQISHVAAAAHAPFISAASP    195

Sm_TssC         200 SVMQMESWQELSNPRDLTKIFQNTEYAAWRSLRESEDARYLGLVMPRFLA    249
Ab_TssC         196 SILGLESFTDIDRPRDVSKIFETAEYVQWRSFRDSEDSRYVALTLPHVLG    245

Sm_TssC         250 RLPYGIRTNPVDE-FDFEEDTDGATHGNYTWTNAAYAMAANINRSFKEFG    298
Ab_TssC         246 RLPYHPKEGTATEGFNFIEDVSGENHNEYLWMNAAYAFGTRLTNAFDMHG    295

Sm_TssC         299 WCTAIRGVESGGAVENLPCHTFPSDDGGVDMKCPTEIAISDRREAELAKN    348
Ab_TssC         296 WCAAIRGVEGGGLVEGLPVHTFKTQDGEVVFKCPTEIAITDRREKELSDL    345

Sm_TssC         349 GFMPLVHRKNSDFAAFIGAQSLQKPAEYYDADASANAQLSARLPYLFACC    398
Ab_TssC         346 GFIPLVHCKNTDYAAFFGAQSTQKPKKYDNDTANANSALSSQIQYIMAVS    395

Sm_TssC         399 RFAHYLKCIVRDKIGSFRERDDMERWLNDWIMNYV---DGDPANSSQETK    445
Ab_TssC         396 RIAHYLKAMMRDKVGSFASAGNVEAFLNEWLSQYVLLDDG----ASQEAK    441

Sm_TssC         446 SRKPLAAAEVQVEEIEDNPGYYSAKFFLRPHYQLEGLTVSLRLVSKLP    493
Ab_TssC         442 AQYPLREASVKVVEDPAQPGHYKSVVFLRPHFQLDELSVSLRLVTELP    489
```

TssE

```
# Length: 138
# Identity:     38/138 (27.5%)
# Similarity:   63/138 (45.7%)
# Gaps:         25/138 (18.1%)

Sm_TssE          32 DKMQP-----VLLDMLTDDEPQKKQEEQVRNLVSHSELRRRVLRDLQWLF     76
Ab_TssE           4 DRLYPYGFRSTLFDRLIPE-----SEDYAKGL-SIQQLRESVAKDLEDLL     47

Sm_TssE          77 NCVNSESNLDLGDFPQVRRSTLNYGIASLAGKRMSDIEWLD---IQRALT    123
Ab_TssE          48 NSRVAKLDHVIDDYPLVKKSILQFGIIDFVG--LSTANPLDRDKICQSIE     95

Sm_TssE         124 DSILHFEPRI-------LPEG--LQVRCISDTGSLELH    152
Ab_TssE          96 QSIAAHEPRLRQIRVEMLLDGHNMGALCLSIQAYLNIH    133
```

TssF

```
# Length: 648
# Identity:     174/648 (26.9%)
# Similarity:   318/648 (49.1%)
# Gaps:          71/648 (11.0%)

Sm_TssF            4 KLLDYYNRELAYLREMGAEFAEQYPKVAGRLGMRGIDVADPYIERLMEGF     53
                     :||.:|.::|....:...|||::|||:|.||.:....:.||:||||::.|
Ab_TssF            4 ELLPFYEKQLQEFGQQSREFAQKYPKIAQRLSLNQEQIDDPHIERLIQAF     53

Sm_TssF           54 AFLTSRVQLKMDAEFPRFSQRLLEIIYPNYLSPTPSMAIAELQPDSSKGD    103
                     :.:.:|:..|:...:..|::.|.|:::|.||...|:.::...:..:....
Ab_TssF           54 SLIAARIDKKLTDSYDVFTRSLFEVMFPQYLRHFPACSVVSFEDINKIKQ    103

Sm_TssF          104 ISNGFVVPRGTMMDSQTLKKSGITCSYTTAHDVTLQPVRIAGVELGGIPA    153
                     ::...:||..|::.|::.|  |:.|.:.|..||.|.|:.::|::....|:
Ab_TssF          104 LTEPHLVPHHTVLKSRSFK--GVQCEFNTTQDVKLLPIHLSGLDFKTTPS    151

Sm_TssF          154 DIPLASLGLQHSGCVSALRIRLECYESVTLNN----LQLDQLMFYLAGPD    199
                         |.:.|..:   :.|.::.|.:     ||    |..::|..||....
Ab_TssF          152 ----AHMHLNQN---ATLSLKFEIF-----NNAHACLTDEKLPIYLDAIS    189

Sm_TssF          200 MQAQQLLELLMQHSVGLVC---QTVEAQPQRRALALDGLRQEGFAAEQAL    246
                     ....|:|:.:.:.....   ||:.      .|:.:.....|||.:::|
Ab_TssF          190 NFPLQVLDSIFKKGTSFAVKYGQTIV------ELSKNPFELIGFAEQESL    233

Sm_TssF          247 LPNDLRNFEGYRLLQEYFAFPARFQFFSVNGLRPLLQSVREGKKALRQFE    296
                     ||.|.....||||.|||.||.:|.:.::     |..::...:.:.:.|
Ab_TssF          234 LPLDQHTHHAYRLLMEYFCFPEKFSYLKLD-----LDFLKRIPQHVSELE    278

Sm_TssF          297 IVV--LLDRHDAALER---VVDVAHLALHCTPVINLFPKIAERIAINEKN    341
                     :::  .|:.:|.|:.|   .:::|:..|..|||||||.|.||...|..|.
Ab_TssF          279 LLIHFKLNLNDQAVVRNYSELNIANFKLFTTPVINLFEKYAEPQKIVHKQ    328

Sm_TssF          342 HEYHLVVDNIRPLDYEVFSVQRLGGSASEKRYEQEF---RPFYSTLSADD    388
                     .||.||.|...|..|:|:|:..:.....:...|:.:   .||:: :|...
Ab_TssF          329 LEYPLVTDAHHPEFYQVYSILEMNMVREKTNQEESYVPVLPFFA-MSHYH    377

Sm_TssF          389 GNYGAYFSLRREQRTLSEHARRYGTRTGYAGSEVFVSLVDERQSPWHSDL    438
                     .:...:|.....|:..|..     ...||       |::.:..:|:.:..
Ab_TssF          378 NDTARFFYSVNYQKLQSNF-----VEMGY-------SIISKNLNPFSTRS    415

Sm_TssF          439 KYLTADVLCTSRDLPLMLLQQDQGNFVMPDSIPIKQVSLRKGPTPPRPAL    488
                     .:::..:||::||||...|.|......:.||...:|..:.|.||.|....
Ab_TssF          416 DFISTKLLCSNRDLPHEALGQSNNVLNLNDSSLARRAIVLKRPTKPYKFE    465

Sm_TssF          489 AEGMITWRLISQLQLNYLSMMDGDPEQGAASLRQLLGLYGNLSEPAIAKQ    538
                     ......||:||.|.||.|::|.||   ..:.:::||.|| ||.:   :|:
Ab_TssF          466 QGQSEQWRVISHLSLNSLALMKGD---AVSHVKELLALY-NLPQ---SKE    508

Sm_TssF          539 ----IQGVRHCNLRPVYRRVP-EPGPIVFARGIAIDLTVDEQAFSGNSPY    583
                         |:.::.......::.|. :|.| :|.||:..:|.:|...|.|:|.|
Ab_TssF          509 NYLIIESIKKLEFSLTHKLVDGKPFP-MFVRGVKAELQIDSSVFRGHSLY    557

Sm_TssF          584 LLGSVLERLFSRLVAMNTFTEMTL---SSQQRGEIAYWQARMGKRTLI    628
                     :...:|.|:|:..|.:|:|.::.:   ||||  |:......:|.:||:
Ab_TssF          558 IFSQLLSRVFNLKVQINSFVDLVVKDYSSQQ--ELYQCSQNVGGKTLL    603
```

TssG

```
# Length: 336
# Identity:      77/336 (22.9%)
# Similarity:   135/336 (40.2%)
# Gaps:          48/336 (14.3%)

Sm_TssG          37 DLFQ------------LLRRIDAQGGQPYLLGRAPLPRHEMLRLGQEPSL     74
                    :|||            |||.:.|..        |.|...:..:.....:|
Ab_TssG          15 ELFQRPKSFEFIQATRLLRHMPAND--------AALSWSDHFKFETSFNL     56

Sm_TssG          75 SFAPSTLAQVTPRANSPLHDVSIFSFGLFGPNGPLPLHLTEYVRERVYHH    124
                    :| |:|..:.....:..:|..::. .||.|..|.||...|..::: ....
Ab_TssG          57 NF-PATEIESLELVDERVHLTNLI-VGLTGIQGALPYTYTNKIKQ-APRQ    103

Sm_TssG         125 QDTTLLAFTNLFHHRLTLLFYRAWADAQPTVSLDRP-----DNRRFDEYL    169
                    |......|.:||:|:||..:      .:.:::...|     :|:  ::||
Ab_TssG         104 QRAETKEFLSLFNHKLTSQY------VESSITYHLPVRYEIENK--NDYL    145

Sm_TssG         170 SSLIGL-----GQPAQQERDSINAHAKHFMAGHLIRHSRDPEGLSKILQQ    214
                    ..|..|     .|..||:.|...|.....|.|    .:.....|..:|..
Ab_TssG         146 DILHALNGYVRSQHQQQDLDEYFAEFSGLMQG----QNNTVHALKTMLSC    191

Sm_TssG         215 YFKVPVRIVENVPHWLRVEPREQARLKAGRGAPRLGESAFLGIAVRDIQH    264
                    .||..:.|.|.|....::...:...| .|.....||.:.|.|..::.|..
Ab_TssG         192 IFKHEITIKEFVQESFKLAGGQLTTL-GGSQPSLLGINTFCGETIQQIDG    240

Sm_TssG         265 KFRIELGPMPQRDYDRFLPGADLCRQLRDWVRQYLGIEFVWEVRLILAKE    314
                    |..|::||:.::.|.:|||..:|..:|:..|..:.....::|||||.:.
Ab_TssG         241 KIEIQIGPLKRQQYLKFLPHQELSLKLKKIVETWCSPTLSIDLRLILDES    290

Sm_TssG         315 QAHGIQL--GGAQRLGLSSWLADHRRQRDLDDLIFS    348
                    :...::|  |....||..::|...:.....|:..:|
Ab_TssG         291 EIQPVRLTQGQESGLGQGAFLMSRKPNTHNDETCYS    326
```

TssM

```
# Length: 1317
# Identity:     354/1317 (26.9%)
# Similarity:   571/1317 (43.4%)
# Gaps:         289/1317 (21.9%)

Sm_TssM          14 LWGFV----GITALAF--------------IIWTIGPLVAIGDYRPLEPE     45
                    ||.::    .|.||:.              |.|.:....|:|
Ab_TssM           8 LWQYITNPKAIIALSLFVALMSSYTSIPRHIFWALASAYALG--------     49

Sm_TssM          46 LNRQIAIGVIYLIWFLCRLIPRLYSAWFNSRLLSNLRAAEAV----PAED     91
                       .||.|:   .|.:.|          ....:....|:|:    .||.
Ab_TssM          50 ---LIAYGI---YWLIQR----------KKHAVQGEELAQAIEKDTQAEY     83

Sm_TssM          92 GKPEVKQDDQL-AQRFDEAAQLLKKARFAPGQGDGKHRWMTRFSRQYLYQ    140
                    ||.:.|::.|| :|:..|:.||::|::....:|:..           ||:
Ab_TssM          84 GKNKDKEELQLISQQMKESIQLIRKSKLGDKKGNAA----------LYE    122

Sm_TssM         141 LPWYVIIGAPGAGKTTALVNSGLHFPLADRFGKAALRGVGGTRNCDWWFT    190
                    ||||::||.|.|||::|:.||||.||..:...|....|:.||||||:|:
Ab_TssM         123 LPWYMVIGNPAAGKSSAIYNSGLKFPFEETHQKMVSAGLSGTRNCDWFFS    172

Sm_TssM         191 NDAVLLDTAGRYTTQESQREEDAGEWQSFVALLKKYRTRQPINGVMVTIS    240
                    .:.:||||||||    |...||..||..|:.:|||.|::.|:||:::.:|
Ab_TssM         173 TEGILLDTAGRY----SVYSEDHSEWLGFLNILKKNRSKAPVNGLILIVS    218

Sm_TssM         241 VADLLSDSAEARAAQASALRKRLIELHEQLGIHFPVYVLVTKTDLLNGFM    290
                    :|:|:|.|.|.....|..||.|:.:|.|:|.:..|||::.:|.||:.||.
Ab_TssM         219 IAELVSQSPEHSLKLAKNLRARIQDLTERLEVVVPVYLVFSKMDLIAGFT    268

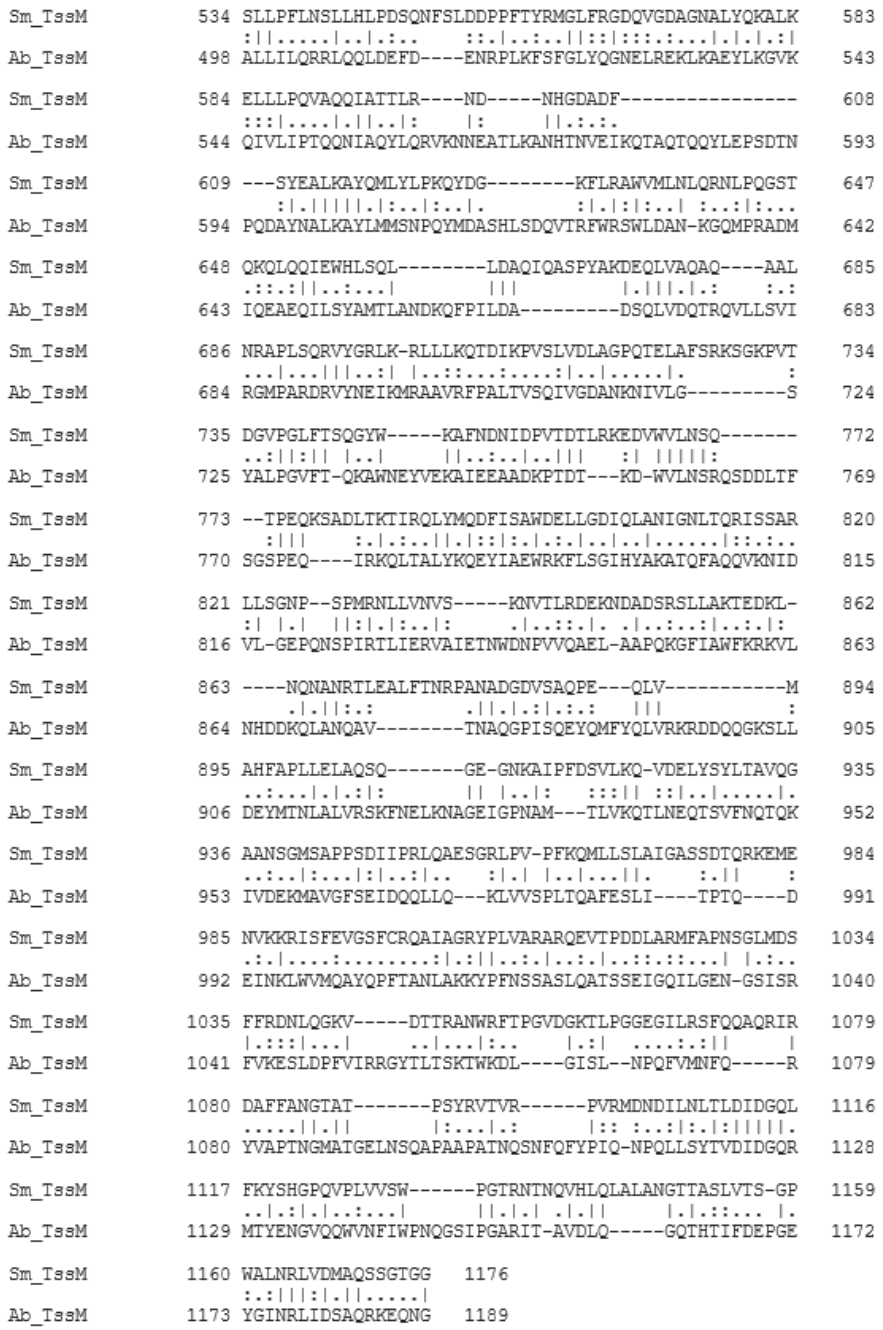
Sm_TssM         291 AYFGSFDKAQRDQIWGFTFPYERSRQPDFNLNAAFEQQYALLQQRLDAAL    340
                    .:|..::..:.:|.||.|.|||:  ...|....||:.|.:|...|....
Ab_TssM         269 EFFEFYEAQEYNQAWGATLPYEQN--SSHNAVDLFEKHYNILYDGLKGVS    316

Sm_TssM         341 PDTLLMEHDARQRAESYLFPQEFAALRPLLGQYLEQVFATSSFETRFTPR    390
                    ...|...|..........||.||..|:|.|..::..:|..:.::.:...|
Ab_TssM         317 STHLSRRHSQNISPSVMTFPLEFKTLKPALKSFIATLFEDNPYQFQPVFR    366

Sm_TssM         391 GIYFTSGTQEGLPFDRVMGELNRYLQLPTANSGVQANAAWDSVGQETPIP    440
                    |.||||..|||:....:..::.:..||    |.:..:...|.: :.||
Ab_TssM         367 GFYFTSALQEGVIESPMTEQIAQEFQL----SQIAQSEVKDQL-KTTP--    409

Sm_TssM         441 AAKGQSFFLKETLESVVFQESGLAGSN-RWWEYRNRALHWAGYIA-LAVI    488
                       ...|||.....|:.::..|...: ...:.|.|.:.:.|.:| :::|
Ab_TssM         410 ---NHGYFLKGLFSDVILKDKDLVKQHVNTSKKRQRYIAFIGALAGVSII    456

Sm_TssM         489 LAVLAMLWFTSYGNNKAYLAEVAA---KVPGVER-QGQSL-TQLGNGDMF    533
                    |.|    |..||.||:..:|||.|   ||..:|: .||.| |||.
Ab_TssM         457 LGV----WVWSYRNNQQLIAEVQADLNKVVQLEKSSGQQLSTQLE-----    497
```


Sm_TssM 534 SLLPFLNSLLHLPDSQNFSLDDPPFTYRMGLFRGDQVGDAGNALYQKALK 583
Ab_TssM 498 ALLILQRRLQQLDEFD----ENRPLKFSFGLYQGNELREKLKAEYLKGVK 543
Sm_TssM 584 ELLLPQVAQQIATTLR----ND-----NHGDADF---------- 608
Ab_TssM 544 QIVLIPTQQNIAQYLQRVKNNEATLKANHTNVEIKQTAQTQQYLEPSDTN 593
Sm_TssM 609 ---SYEALKAYQMLYLPKQYDG--------KFLRAWVMLNLQRNLPQGST 647
Ab_TssM 594 PQDAYNALKAYLMMSNPQYMDASHLSDQVTRFWRSWLDAN-KGQMPRADM 642
Sm_TssM 648 QKQLQQIEWHLSQL--------LDAQIQASPYAKDEQLVAQAQ----AAL 685
Ab_TssM 643 IQEAEQILSYAMTLANDKQFPILDA---------DSQLVDQTRQVLLSVI 683
Sm_TssM 686 NRAPLSQRVYGRLK-RLLLKQTDIKPVSLVDLAGPQTELAFSRKSGKPVT 734
Ab_TssM 684 RGMPARDRVYNEIKMRAAVRFPALTVSQIVGDANKNIVLG---------S 724
Sm_TssM 735 DGVPGLFTSQGYW-----KAFNDNIDPVTDTLRKEDVWVLNSQ------- 772
Ab_TssM 725 YALPGVFT-QKAWNEYVEKAIEEAADKPTDT---KD-WVLNSRQSDDLTF 769
Sm_TssM 773 --TPEQKSADLTKTIRQLYMQDFISAWDELLGDIQLANIGNLTQRISSAR 820
Ab_TssM 770 SGSPEQ----IRKQLTALYKQEYIAEWRKFLSGIHYAKATQFAQQVKNID 815
Sm_TssM 821 LLSGNP--SPMRNLLVNVS-----KNVTLRDEKNDADSRSLLAKTEDKL- 862
Ab_TssM 816 VL-GEPQNSPIRTLIERVAIETNWDNPVVQAEL-AAPQKGFIAWFKRKVL 863
Sm_TssM 863 ----NQNANRTLEALFTNRPANADGDVSAQPE---QLV----------M 894
Ab_TssM 864 NHDDKQLANQAV--------TNAQGPISQEYQMFYQLVRKRDDQQGKSLL 905
Sm_TssM 895 AHFAPLLELAQSQ-------GE-GNKAIPFDSVLKQ-VDELYSYLTAVQG 935
Ab_TssM 906 DEYMTNLALVRSKFNELKNAGEIGPNAM---TLVKQTLNEQTSVFNQTQK 952
Sm_TssM 936 AANSGMSAPPSDIIPRLQAESGRLPV-PFKQMLLSLAIGASSDTQRKEME 984
Ab_TssM 953 IVDEKMAVGFSEIDQQLLQ---KLVVSPLTQAFESLI----TPTQ----D 991
Sm_TssM 985 NVKKRISFEVGSFCRQAIAGRYPLVARARQEVTPDDLARMFAPNSGLMDS 1034
Ab_TssM 992 EINKLWVMQAYQPFTANLAKKYPFNSSASLQATSSEIGQILGEN-GSISR 1040
Sm_TssM 1035 FFRDNLQGKV-----DTTRANWRFTPGVDGKTLPGGEGILRSFQQAQRIR 1079
Ab_TssM 1041 FVKESLDPFVIRRGYTLTSKTWKDL----GISL--NPQFVMNFQ-----R 1079
Sm_TssM 1080 DAFFANGTAT-------PSYRVTVR------PVRMDNDILNLTLDIDGQL 1116
Ab_TssM 1080 YVAPTNGMATGELNSQAPAAPATNQSNFQFYPIQ-NPQLLSYTVDIDGQR 1128
Sm_TssM 1117 FKYSHGPQVPLVVSW------PGTRNTNQVHLQLALANGTTASLVTS-GP 1159
Ab_TssM 1129 MTYENGVQQWVNFIWPNQGSIPGARIT-AVDLQ-----GQTHTIFDEPGE 1172
Sm_TssM 1160 WALNRLVDMAQSSGTGG 1176
Ab_TssM 1173 YGINRLIDSAQRKEQNG 1189

VgrG

```
# Length: 795
# Identity:     190/795 (23.9%)
# Similarity:   295/795 (37.1%)
# Gaps:         212/795 (26.7%)

Sm_VgrG          15 QLLFRSLDGIEALSTPFDFSIELLSTDARLDRKALLGQPLTLEIPTQGFL     64
                    |:..:.:||:.||:......:..|||:|.:..|:.:|....::|.|:
Ab_VgrG          33 QVFLQRIDGVHALNDGLKAELLCLSTNATIQLKSFIGVQAAVDIVTE---     79

Sm_VgrG          65 SAPRYLNGKITAI------AVSSEEIGGTRYAVYNLHVQPDLWPMTKDRN    108
                          .|::|.:      |...:..|........|.....||...  ||
Ab_VgrG          80 ------RGELTRVTGIITHAQQGQSDGSLTLYKLTLEDPTALWKYR--RN    121

Sm_VgrG         109 FRIFQEQTVPQIVKTLLAE---------HNVQLE-DQLTGDYRLWGYCVQ    148
                    .|:|..::|.:|.:.|..|         .::.|: ..||..|.:..:.:|
Ab_VgrG         122 SRVFMNKSVVEIWEILFKEWQTKNPLFAASLSLDLSGLTQTYDVRPFVMQ    171

Sm_VgrG         149 YNESSFNFISRLMELEGIYYYFKHEMGKH------------TLVLGDAPH    186
                    :|||.:||::||:..|.|.:..  :..:|            .|.|.||..
Ab_VgrG         172 HNESDWNFLTRLLRSENISWLI--DEAQHIVPSTETPIQAQKLRLIDANS    219

Sm_VgrG         187 HHQPYPGYEMIPYHLTPSGGSTSEEGISQWTLSDRVTPGIYSLDDYDFRK    236
                    .:||. ..:.|.||        ....:.|:....|:| ...||      :
Ab_VgrG         220 QYQPL-DRKTIRYH--------RSSAVEQYDSMTRLT-AERSL------Q    253

Sm_VgrG         237 PNAWLFQARQNPVSPTPGQIDVYDWPGR-YTEHQQGEFYARVR---QEAW    282
                    ||....|..|..:      :|..:..|. .::||..|.|....   ::||
Ab_VgrG         254 PNVMHIQRWQAEI------LDQEEGIGSVQSKHQHSEHYDNATLGLEQAW    297

Sm_VgrG         283 ------------------------------------QAEHQQIRGTATALG    297
                                                       :|:.::.....|...
Ab_VgrG         298 NYSPAWIGDLKGEDGVTKSGNQQVERLNQNLNNYYEAQAKRFIAQTTVRD    347

Sm_VgrG         298 IAPGSTFTLYNAP----HADDNREYLTLQANYH----------------L    327
                    ...|..|.|...|    |...:|.:|.:..::.                |
Ab_VgrG         348 AYVGYYFELNEHPEIDQHESTDRSFLIISKSFFNQNNLPKDLNDRINGLL    397

Sm_VgrG         328 KENRYA-SGDDQSSEHRIDFIVLP----ADVPWHPPQQATWPKTHGPQTA    372
                    .::.:| ...:.|.|.:.:.::|.    ...|.:.||..: |.|| ||.|
Ab_VgrG         398 AQSNWAIQNPENSDERQANQLILQRRHIPTTPAYSPQIHS-PVTH-PQRA    445

Sm_VgrG         373 RVVGPAGESIWTDKYGRIKVKFHWDRFGPKDDGS-------------SCW    409
                    :||||.||.|:.|::|||||:|.:.|   .||.|             |.|
Ab_VgrG         446 KVVGPEGEEIYVDEWGRIKVRFLFTR---SDDHSHDGGAGTNNNDTDSAW    492

Sm_VgrG         410 VRVSSAWAGQGYGGVQIPRVNDEVVVDFINGDPDRPIVTGRVYNEASMPP    459
                    :.|.:.|||:|||...:||:.:.||:||.|||.|||.|.||:: ||...|
Ab_VgrG         493 IDVLTPWAGEGYGVRFLPRIGEIVVIDFFNGDIDRPFVMGRIH-EAQRQP    541

Sm_VgrG         460 ------WALPAAATQMGFMSRTKDGTADNANALRFEDKAGAEQVWIQ---    500
                          ..||......|.  |:|:.:......|||:|..|.....:|
Ab_VgrG         542 TKFDNKGKLPDTKKLSGI--RSKEVSGGGFGQLRFDDTPGQISTQLQSSH    589

Sm_VgrG         501 --AERNM-------DTQVKND--ESHTIANDHTHLVGGNQIKRVVLNQAT    539
                      ::.||       |.....|  |...:..|....:...|...|..::..
Ab_VgrG         590 GASQLNMGKLSHPKDKAESEDRGEGFELRTDQWGALRAGQGLLVSTHKQD    639

Sm_VgrG         540 GVKG------------ESSALTGKTRSDAVVNAFT---------------    562
                    ..||            |.|....|..||...|..|
Ab_VgrG         640 NAKGEHLDAEVAKKQLEGSQTNSKALSDIAKNQKTDEIESIEQLKDFASQ    689

Sm_VgrG         563 --------------LGSGESLRLECGESVIELLANGQIN-ITGTSFNITV    597
                              |.|.:.:.|...|. |.:.|:.||| |.|.|.||:.
Ab_VgrG         690 IQQQIAKFEKALLLLSSPDGIALSSSED-IHISADAQINQIAGDSINIST    738

Sm_VgrG         598 KEDGEINTGGQLDL-NQPGGAARTAAPGGGHQAAIQSAVDQLFPK    641
                    :::...:...:|.| ....|....||.|.....|...|:|.|..|
Ab_VgrG         739 QKNVIAHAQNRLSLFAAQSGLKAVAAQGKVEIQAQSDALDVLANK    783
```

7.04 Appendix F

Compilation of interesting differentially regulated genes from RNA-seq data. Fold change is ordered in descending fashion.

Group	Gene Name	Product	Fold change
T6SS			
	tssC	Type VI secretion system sheath subunit TssC	**22.00**
	tssE	type VI secretion system baseplate subunit TssE	**5.50**
	tssA	type VI secretion system protein TssA	**5.33**
	tssM	type VI secretion system membrane subunit TssM	**4.50**
	vgrG	type VI secretion system tip protein VgrG	**4.11**
	tssB	type VI secretion system contractile sheath small subunit TssB	**3.67**
	tssG	type VI secretion system baseplate subunit TssG	**3.00**
	vgrG	type VI secretion system tip protein VgrG	**2.67**
	vgrG	type VI secretion system tip protein VgrG	**2.41**
	clpV1	Protein ClpV1	**2.17**
	tssF	type VI secretion system TssF	**2.00**
	tagF	type VI secretion system-associated protein TagF	**1.40**
	tssK	type VI secretion system baseplate subunit TssK	**1.09**
	-	PAAR domain-containing protein	**0.69**
Sidero-phores			
	entS	Enterobactin exporter EntS	**3.33**
	entB	Enterobactin synthase component B	**0.35**
	entE	Enterobactin synthase component E	**0.27**
	pfeA	Ferric enterobactin receptor	**0.16**
	bfr_2	Bacterioferritin	**1.93**
	bfr_1	Bacterioferritin	**1.55**
	bfd	Bacterioferritin-associated ferredoxin	**0.35**
	iutA_1	Ferric aerobactin receptor	**1.20**
	iucC	Aerobactin synthase	**0.39**
	iutA_2	Ferric aerobactin receptor	**0.33**
	-	IucA/IucC family siderophore biosynthesis protein/Aerobactin synthase	**0.22**
	fatA_2	Ferric-anguibactin receptor FatA	**1.17**
	angR	Anguibactin system regulator	**0.45**
	fatB	Ferric-anguibactin-binding protein FatB	**0.12**
	fatA_1	Ferric-anguibactin receptor FatA	**0.09**
	fatD	Ferric-anguibactin transport system permease protein FatD	**0.09**
	fatC	Ferric-anguibactin transport system permease protein FatC	**0.06**

	fhuA_3	Ferrichrome outer membrane transporter/phage receptor	**0.54**
	fhuA_1	Ferrichrome outer membrane transporter/phage receptor	**0.08**
	besA	Ferri-bacillibactin esterase BesA	**0.43**
	feoB	Fe(2+) transporter FeoB	**0.43**
	-	Fur family transcriptional regulator	**0.39**
	-	ferrous iron transport protein A	**0.35**
	hemP	hemin uptake protein HemP	**0.28**
	-	(2Fe-2S)-binding protein	**0.28**
	fhuE_2	FhuE receptor	**0.15**
	vibB	Vibriobactin-specific isochorismatase	**0.11**
	yusV	putative siderophore transport system ATP-binding protein YusV	**0.07**
Porins			
	dcaP	DcaP porin	**27.00**
	nicP_1	Porin-like protein NicP	**2.00**
	oprB_1	Porin B	**1.26**
	oprD	Porin D	**0.81**
	nicP_2	Porin-like protein NicP	**0.60**
	oprB_2	Porin B	**0.50**
	tsx	ion channel protein Tsx	**0.40**
Mla genes			
	-	antisense: mlaC	**13.75**
	-	antisense: mlaC	**4.92**
	-	antisense: mlaF	**1.42**
	mlaD	putative phospholipid ABC transporter-binding protein MlaD	**1.32**
	mlaF	putative phospholipid import ATP-binding protein MlaF	**1.23**
	mlaC	putative phospholipid-binding protein MlaC	**1.10**
	mlaE	putative phospholipid ABC transporter permease protein MlaE	**0.82**
Toxins			
	cvaA	Colicin V secretion protein CvaA	**2.44**
	prtN	pyocin activator protein PrtN	**2.33**
	apxIB	Toxin RTX-I translocation ATP-binding protein	**1.83**
	ltxB	Leukotoxin export ATP-binding protein LtxB	**1.23**
	tdeA	Toxin and drug export protein A	**1.20**
	-	zonula occludens toxin	**0.27**
T2SS			
	gspJ	type II secretion system protein GspJ	**2.40**
	xcpQ	Type II secretion system protein D	**1.39**
	epsE_1	Type II secretion system protein E	**1.33**
	epsF_1	Type II secretion system protein F	**1.15**
	epsF_2	Type II secretion system protein F	**0.50**
	espE_2	type II secretion system protein E	**0.35**

Lipo-proteins				
	lspA_3		Lipoprotein signal peptidase	**1.03**
	lspA_1		Lipoprotein signal peptidase	**0.18**
	lspA_2		Lipoprotein signal peptidase	**0.18**
	blc_1		Outer membrane lipoprotein Blc	**1.78**
	blc_2		Outer membrane lipoprotein Blc	**0.58**
	lolB		Outer-membrane lipoprotein LolB	**1.01**
	lolD		Lipoprotein-releasing system ATP-binding protein LolD	**0.77**
	-		copper resistance lipoprotein NlpE	**2.88**
	-		entericidin A/B family lipoprotein	**2.47**
	pal		Peptidoglycan-associated lipoprotein	**2.34**
	lnt		Apolipoprotein N-acyltransferase	**0.94**
Pili				
	-		antisense: pilT_2	**1.09**
	-		antisense: pilQ	**0.42**
	-		antisense: pilQ	**0.14**
	-		antisense: pilT_2	**0.09**
	-		antisense: pilT_1	**0.00**
	pilT_1		Twitching mobility protein	**0.46**
	pilT_2		Twitching mobility protein	**0.26**
	-		CsuAB/ spore coat protein U domain containing protein	**11.00**
	-		CsuC/P pilus assembly protein, chaperone PapD	**0.33**
	filC		putative pilus assembly protein FilC	**2.14**
	pilV		type IV pilus modification protein PilV	**0.29**
	pilQ		Type IV pilus biogenesis and competence protein PilQ	**0.27**
	-		PilN domain-containing protein	**0.21**
	pilP		pilus assembly protein PilP	**0.16**
	fimA		Fimbrial protein	**0.16**
	outQ		Type IV prepilin-like proteins leader peptide-processing enzyme	**0.55**
	-		pilus assembly protein/type IV fimbrial biogenesis protein	**0.28**
Misc.				
	-		PEGA domain-containing protein	**0.48**
	-		chlorhexidine efflux PACE transporter AceI	**0.42**

7.05 Appendix G

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Mechanisms of Resistance

Colistin Resistance in *Acinetobacter baumannii* Is Mediated by Complete Loss of Lipopolysaccharide Production

Jennifer H. Moffatt, Marina Harper, Paul Harrison, John D. F. Hale, Evgeny Vinogradov, Torsten Seemann, Rebekah Henry, Bethany Crane, Frank St. Michael, Andrew D. Cox, Ben Adler, Roger L. Nation, Jian Li, John D. Boyce

DOI: 10.1128/AAC.00834-10

Genome sequencing. The genome sequences of strains ATCC 19606 and 19606R were determined using 36-bp paired-end sequencing chemistry on an Illumina Genome Analyzer II apparatus (Illumina) at the Micromon Sequencing Facility (Monash University). Raw sequence data from both strains were independently aligned to the published strain ACICU genome sequence (12) using the SHRiMP program (27) (average read depth, ~110), as described previously (31). The raw read data were also assembled *de novo* using Velvet software (39) and a CLC genomics workbench (CLCbio, Denmark) to confirm the mutation.