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Characterising the mechanisms of cell-cell interactions sheds light on new avenues to combat infection.

A thesis submitted by

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Abstract

The spread of antimicrobial resistance (AMR) is accelerating at an alarming rate, threatening the treatment of infectious disease and other medical interventions, due to an increased risk of infection that we no longer possess effective treatments for. Long reliance on evolutionarily-static, conventional antibiotics to treat bacterial infection has fuelled resistance selection and the need for the constant discovery of new classes of antibiotics to replace those whose effectiveness is being lost.

This MRes thesis is the culmination of two rotation projects, based at the University of Birmingham and the University of Nottingham; the first investigated the underlying mechanisms behind the immune perturbation induced by vancomycin on host macrophages, ultimately resulting in an impaired control of infection, predisposing to systemic fungal infection. Future elucidation of the mechanisms underlying this predisposition to fungal infection will aid antibiotic stewardship and clinical practise regarding the implication of current and new antimicrobial therapies.

The second study investigated the role of three hypothetical proteins, predicted to be contained within a predatory vesicle whose composition and function is being actively investigated within the lab, within the predatory lifecycle of the predatory bacterium *Bdellovibrio bacteriovorus.* By providing a better understanding of the complex predatory process, this study aids the potential utilisation of *B. bacteriovorus*, or components of its predatory lifecycle, as novel antimicrobial therapeutics in the future. Future work will further confirm the roles of these proteins within the wider context of predation and infection.

To develop successful antimicrobial therapies in the future and combat the AMR crisis, the host immune system and novel or chemical antimicrobial therapies must work hand in hand. This will only be possible through the greater understanding of the interactions that occur between current and novel antimicrobial therapies and the host immune system.

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List of abbreviations

AMR	Antimicrobial resistance
ATP	Adenosine triphosphate
bp	Base pairs
CLR	C-type lectin receptor
CR3	Complement receptor 3
CXCL	Chemokine ligand
CXCR3	Chemokine receptor 3
DNA	Deoxyribonucleic acid
ESBLs	Extended spectrum β-lactamases
HGT	Horizontal gene transfer
ICU	Intensive care unit
IFN	Interferon
IL	Interleukin
IL-1β	Interleukin 1β
iNOS	Inducible nitric oxide synthase
Kb	Kilobases
LPS	Lipopolysaccharide
MBMDM	Murine bone marrow derived macrophage
mCherry	Monomeric cherry red fluorescent protein
mNeon	Monomeric neon fluorescent protein

NADH	Nicotinamide adenine dinucleotide (NAD) + hydrogen (H)
NAG	N-acetyl-glucosamine
NAM	N-acetyl-muramic acid
NFκB	Nuclear factor kappa-light-chain-enhancer-of B cells
NLR	Nod-like receptor
NLRP3	NLR Family Pyrin Domain Containing 3
NO	Nitric oxide
OMP	Outer membrane protein
OPC	Oropharyngeal candidiasis
PAMP	Pattern associated molecular pattern
PBP	Penicillin binding protein
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
TCA	Tricarboxylic acid
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
VVC	Vulvovaginal candidiasis

Chapter 1: Introduction

1. Introduction to antimicrobial resistance

1.1 Antibiotics

1.1.1 Overview

Antimicrobials have long been a vital pillar in the treatment of infectious disease, ever since the first antimicrobials were characterised and then utilised in the mid 20th century (1). Antimicrobials are compounds that inhibit the growth and replication of, or kill, microorganisms. The majority of antimicrobials are used to target pathogens that cause disease within a host, e.g. antibiotics for bacterial disease. However, antiseptics, which are used to prevent infection after injury, and disinfectants, which non-selectively eradicate a wide range of bacteria, fungi and viruses outside of the host, are also important antimicrobials for topical use and decontamination of biological and artificial surfaces.

Significant progress has been made in the treatment of other causes of mortality such as the advent of recombinant DNA technology in the development of novel biologics, vaccines and small molecule inhibitors to treat cancers and autoimmune disease, which has led to an increase in life expectancy (2). Treatment of infectious disease has also been massively advanced by antimicrobials, whereby complex surgical interventions for the treatment of cancer and iatrogenic disease caused by the immunomodulation associated with organ transplantation are commonplace and well managed, without fear of infection (2). However, little progress has been made in the discovery of novel antimicrobials for the treatment of infectious disease. The emergence of highly mobile mechanisms of resistance amongst microorganisms that commonly cause clinical disease is accelerating. The loss of some antimicrobials to resistance, combined with a lack of discovery of new antimicrobials, threatens not only the progress made in the last century in the treatment of infectious disease, but also threatens to revert

clinical practise back to a time where routine surgical interventions for the treatment of trauma, cancer and organ rejection will also not be possible.

1.1.2 Modes of action

Since the discovery and utilisation of penicillin to treat bacterial infection in the 1940s, multiple different classes of antibiotics have been discovered and utilised for the treatment of infectious disease. These classes differ in their target range, with some being broad spectrum whereby they target a wide array of bacterial species, and others being more targeted with a narrower spectrum of activity. These classes also have differing mechanisms of action whereby some inhibit bacterial growth and replication (termed bacteriostatic) through the interruption of cellular processes such as DNA replication, translation and transcription, whilst others kill bacteria (termed bactericidal) through the perturbation and detrimental alteration of cellular components such as the bacterial cell wall. A summary of the common targets of bactericidal and bacteriostatic antibiotics is shown in Figure 2A.

1.1.2.1 Bactericidal antibiotics

The majority of bactericidal antibiotics target the bacterial cell wall or membranes, both of which are vital for cell integrity and viability (3). Cell wall targeting antibiotics kill the cell by interfering with the biosynthesis of peptidoglycan, which generally results in a structural defect within the cell wall and subsequent lysis of the bacterial cell. Peptidoglycan is the primary component of the bacterial cell wall and is typically synthesised by multienzyme complexes in a highly sequential process (4). Peptidoglycan is comprised of multiple subunits, namely N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) chains, crosslinked by D-amino acid containing peptide chains (5,6). The NAG and NAM peptidoglycan subunits are originally synthesised within the cytoplasm of the bacterial cell and attached to a lipid anchor to facilitate their transport across the cytoplasmic membrane; one of these carriers is lipid-II (7). Complexes of penicillin binding proteins (PBPs) synthesise and remodel the peptidoglycan cell envelope

during bacterial growth and division, incorporating these subunits (8). PBPs belong to three designated classes; PBP_A are peptidoglycan synthases that polymerise the peptidoglycan monomers e.g. NAG and NAM into long chains. PBP_B contains the PBPs responsible for crosslinking of the bacterial cell wall during elongation and division. PBP_C PBPs are responsible for the cutting and remodelling of peptidoglycan and includes the D,D-carboxypeptidases responsible for removing the D-Alanine peptide crosslinks between the peptidoglycan subunits, and the D,D- endopeptidases that hydrolyse these crosslinks (Figure 1) (4,8). This constant turnover and remodelling of peptidoglycan allows for growth and division of bacterial cells (9); the degradation of peptidoglycan, once synthesis is blocked by the actions of cell wall targeting antibiotics, results in bacterial cell death and lysis (4).

β-lactams such as penicillins and cephalosporins typically mimic the D-Alanine-D-Alanine substrate, which is required for the formation of peptide crosslinks, irreversibly binding to the bacterial cell wall transpeptidase and carboxypeptidase enzymes, preventing transpeptidation and incorporation of peptidoglycan subunits into the existing cell wall and resulting in turgor-mediated cell lysis (Figure 1) (10,11). Glycopeptide antibiotics such as vancomycin also form blocking complexes on lipid-II precursors, preventing D-Alanine-D-Alanine bond formation and transpeptidation, once again inhibiting peptidoglycan biosynthesis and incorporation into the cell wall (12). Other antibiotics such as bacitracin and phosphonic acids (e.g. fosfomycin) interfere with cell wall biosynthesis by interfering with the lipid-II NAG/NAM carriers or UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) complex inhibition, causing cell lysis in a similar manner (13).

Instead, some bactericidal antibiotics, such as nisin, daptomycin and polymyxins B and E, target the bacterial cell membranes, either preventing lipid incorporation or incorporating themselves into the membranes, resulting in pore formation and hydrophobic disruption of the membrane (14).

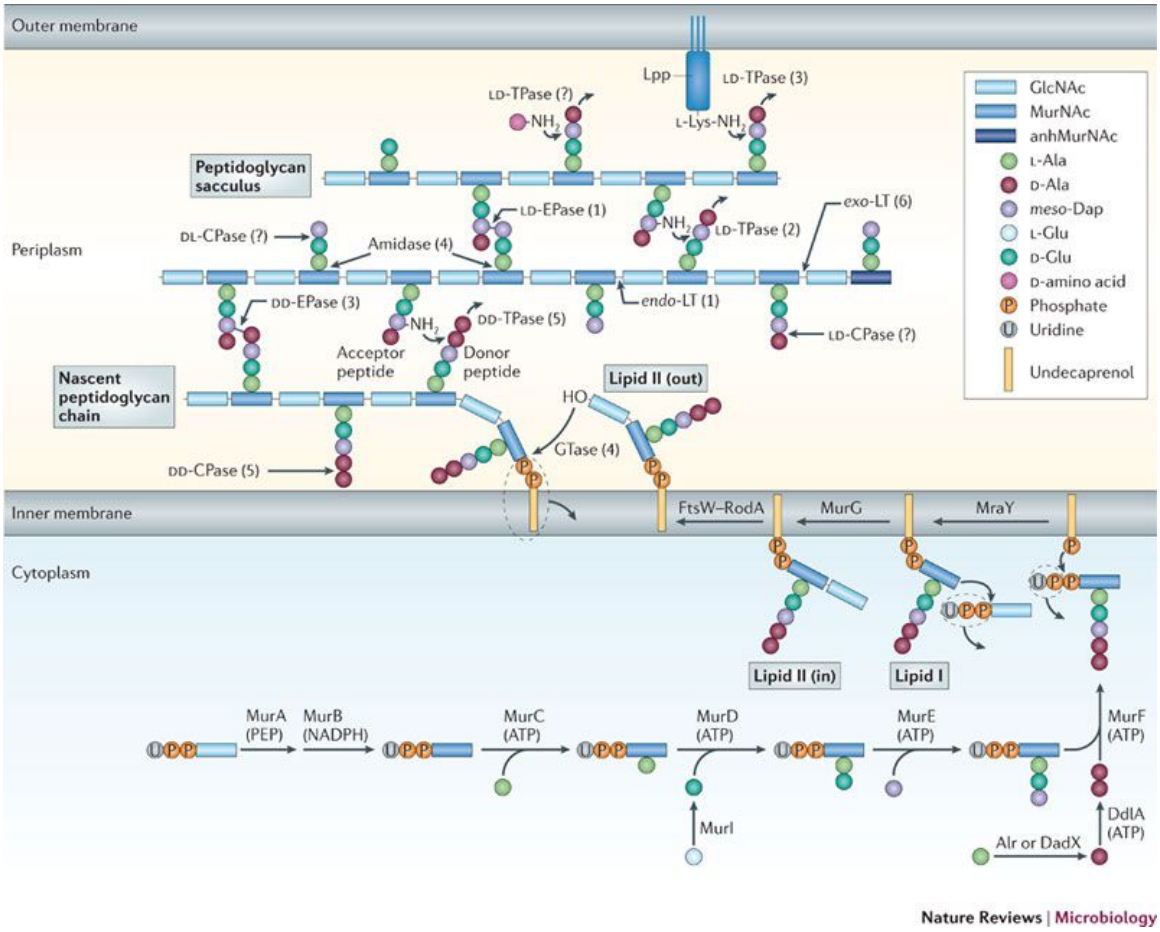


Figure 1: Bacterial synthesis of peptidoglycan
Peptidoglycan within the bacterial cell wall is constantly degraded and re-synthesised, allowing for cell growth and division. Peptidoglycan subunit precursors, predominantly N-acetyl glucosamine (NAG/GlcNac) and N-acetyl muramic acid (NAM/MurNAC), are synthesised within the bacterial cell cytoplasm and linked to a undecaprenyl phosphate lipid carrier to facilitate their transport across the cytoplasmic membrane. Peptidoglycan subunits are then incorporated into growing peptidoglycan chains by glycosyltransferases and DD-transpeptidases, linking the subunits into the existing peptidoglycan structure via their D-Ala D-Ala peptide termini. Dynamic remodelling of the peptidoglycan cell wall, resulting in the removal and de-crosslinking of peptidoglycan, also occurs via the action of DD-, LD- and DL- carboxypeptidases and DD- and LD- endopeptidases. Figure taken from Typas et al, 2012.

Alternatively, fluoroquinolones, which target the DNA gyrase and topoisomerases that control DNA coiling and winding during DNA replication, interfere with the uncoiling process and induce DNA fragmentation, SOS responses and subsequent cell lysis (15,16). The majority of bactericidal antibiotics affect growing cells, interfering with their usual biosynthetic processes and making them more vulnerable to osmotic and cellular stresses and lysis, the exceptions of which being the membrane active antibiotics that may also affect non-growing cells. Ultimately, the actions of bactericidal

antibiotics predominantly act on structural components of the bacterial cell, inducing cell death and frequently causing cell lysis (Figure 2A).

1.1.2.2 Bacteriostatic antibiotics

In contrast to the actions of bactericidal antibiotics, the bacteriostatic antibiotics target cellular process but do not induce cell lysis, instead inhibiting cell growth until the immune system neutralises the cell. The majority of bacteriostatic antibiotics interfere with DNA and RNA synthesis, transcription and translation. For example, rifampicin inhibits the DNA dependent RNA polymerase and prevents transcription and translation by interfering with RNA synthesis and elongation (17). Tetracyclines and aminoglycosides bind to the 30s subunit of the bacterial ribosome, preventing tRNA binding to the mRNA-ribosome complex and thus preventing translation initiation and peptide elongation (18,19). Erythromycin and chloramphenicol bind to the 50s subunit of the bacterial ribosome and inhibit translocation and transpeptidation of the growing peptide chain by interfering with peptidyl transferase activity, preventing the transfer of amino acids into the growing peptide chain (20,21).

In general, bacteriostatic antibiotics bind reversibly to the transcriptional and translational machinery of the bacterial cell, preventing growth and replication of the bacterial cell (Figure 2A). This controls the infection until a time when the host immune system detects and neutralises the infectious organisms. Crucially, this provides a reduced selection pressure for resistance to evolve as the antibiotic is only inhibiting growth and not killing the organism, whilst also not inducing cell lysis and causing the release of a high antigenic load into the host.

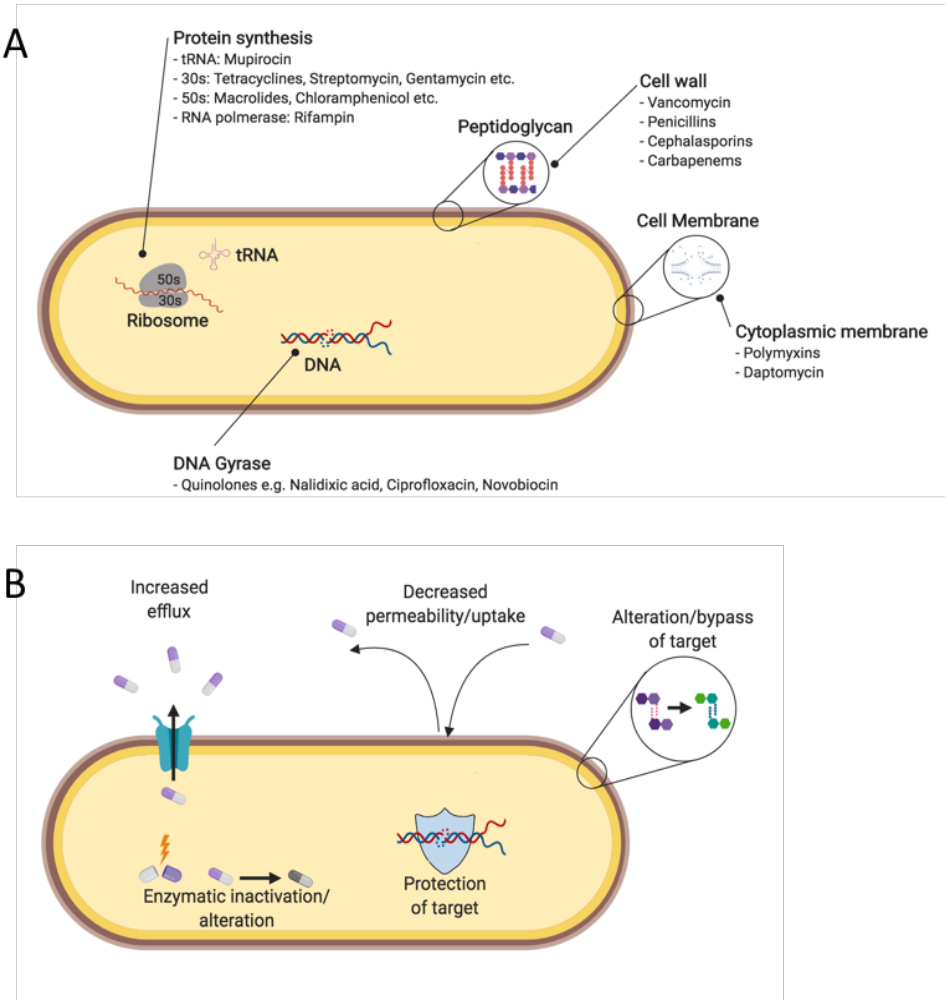


Figure 2: Common targets for antibiotic therapy and their associated resistance mechanisms
A: Antibiotics target an array of bacterial cellular components via a variety of different mechanisms, exerting either a bactericidal or a bacteriostatic effect. The most common targets of antibiotics are shown, alongside examples of the antibiotics that target them. B: Various mechanisms of resistance have evolved that negate the actions of antibiotics, depending on their individual targets and modes of action. Typical resistance mechanisms are shown above. Figure generated using Biorender software using data from Blair et al, 2015.

In summary, different antibiotic classes can determine the path for resolution of infection, either through a messy, highly immunostimulatory pathway of antibiotic-mediated bacterial lysis and immune stimulation, or through a quieter, bacteriostatic pathway that relies on the host immune system to resolve the infection fully. However, the ability of antibiotics to inhibit bacterial growth or kill bacteria entirely is under threat.

1.2 Antimicrobial resistance

1.2.1 Overview

Even before the widespread production and use of antibiotics, resistance was already being observed via a penicillinase produced by *Escherichia coli* firstly in 1940 (22) and then resistance in *Staphylococcus aureus* in 1942, mediated by an unknown non-penicillinase mediated mechanism (23), culminating in 80% of *S. aureus* isolates in the late 1960s being penicillin resistant (24). Attempts to resolve this led to the seeking of alternative antimicrobial compounds either from the environment, where most antimicrobial compounds originate and are used by microorganisms as a competitive advantage, or via the chemical modification of existing antimicrobial compounds. Shortly after the modification of penicillin to form methicillin, resistance via the modification of a penicillin binding protein within *S. aureus* was quickly observed (covered further in 1.2.2.2) (25,26). This pattern of novel antibiotic discovery and subsequent resistance development was repeated throughout the so-called “golden age” of antibiotic discovery whereby novel compounds were quickly synthesised to replace their insufficient counterparts. However, this surge of antibiotic discovery abruptly ended after the 1980s, with only 2 novel classes of antibiotics, oxazolidinones and cyclic lipopeptides, being introduced into clinical practise within the last 30 years (27).

Antimicrobial resistance is of particular concern within the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa & Enterobacter spp.*), where an increased dissemination of multidrug resistance within a clinical setting is especially prevalent. The emergence of resistance has been accelerated due to the presence of other causes of mortality in the population, increased selective pressures due to higher antibiotic and antiseptic use in clinical settings and poor infection control (28). This is amplified through the “treat now, diagnose later” culture present within clinics where broad spectrum antibiotics are used to control infection before diagnosis of the causative agent and susceptibility testing can be undergone (highlighting the need for better diagnostics). Resistance amongst the

ESKAPE pathogens may include the spread of extended spectrum β-lactamases (ESBLs) within *Enterobacter spp.*, methicillin resistance in *S. aureus,* carbapenem resistance in *A. baumannii* and vancomycin resistance amongst *Enterococcus spp.* (29).

One further contributory factor to the emergence of antibiotic resistance stems from the widespread use of antiseptic disinfectants, most prominently triclosan. Triclosan, originally used as an antiseptic, is a broad spectrum antibacterial and antifungal compound that targets a multitude of components within cells, however its primary target is the enoyl-acyl carrier protein reductase, a component of fatty acid biosynthesis (30,31). Triclosan is found in a wide array of household products, including toothpaste, hand soaps and kitchen utensil handles, as well as in a healthcare setting in surgical gowns, surgical handwash and surface disinfectants (30,32). Biocides, like triclosan, play an important role in limiting sources of infection, especially within a healthcare setting where individuals are more vulnerable to infection (33).

However, prevalent use of triclosan at sub-lethal concentrations in household products (34), selects for resistance gene expression for triclosan, but more importantly for resistance to a wide array of antibiotic classes also. Triclosan use can select for antibiotic resistance via multiple mechanisms, whether it be through the induction of oxidative stress, causing mutations that upregulate β-lactamase expression, or through the selection for horizontal gene transfer of multidrug efflux pumps (35,36). This is of growing concern as the widespread use of triclosan throughout society selects for triclosan resistance and therefore may select for resistance to clinically relevant antibiotics via similar mechanisms (37).

Much like antibiotic resistance, resistance to triclosan can either be intrinsic or acquired through mutation (30), amplification e.g. hyperexpression of already-present efflux pumps (37,38) or horizontal gene transfer (covered in 1.2.2) (39). Resistance can occur through the alteration of target sites, active efflux or enzymatic degradation/inactivation, the same mechanisms that predispose to antibiotic resistance (see 1.2.2) (Figure 2B) (32). The most common form of resistance is mediated through the decreased accumulation

of triclosan within the cell, either through increased efflux (using efflux pumps highly similar to those conferring resistance to antibiotics) or alteration of the bacterial outer membrane composition to decrease permeability (37,40), either through modification of hydrophobicity (41) or outer membrane ultrastructure and composition (42–44). Triclosan has more extensively been demonstrated to be a substrate for multiple clinically relevant, antibiotic-resistance conferring efflux pumps including AdeABC, AbeM and AdeIJK in *A. baumannii* (45–47), AcrAB-TolC in *E. coli* and *S. typhimurium* (48,49) and MexAB-OprM, MexCD-OprJ and MexEF-OprN in *P. aeruginosa* (see 1.2.2.1) (48,50), furthering the suggestion that co-resistance may occur.

Initial investigations had suggested that target-based cross-resistance between antibiotics and biocides does not occur, as biocide resistance genes were no more prevalent in clinical multi-drug resistant isolates that they were in their susceptible counterparts (51,52), and triclosan was still effective against multidrug resistant bacteria such as MRSA, where it had been used to control multiple outbreaks in hospitals (53). However, mutations within a membrane-related fatty acid synthase gene (InhA) in *Mycobacterium smegmatis* was shown to confer resistance to triclosan and isoniazid, a front line therapy for tuberculosis (54) and triclosan resistance has been shown to be co-acquired with plasmid-mediated mupirocin resistance in MRSA clinical isolates (55), suggesting co-resistance may be possible.

Further details on the use of triclosan, and its contribution to the emergence of antimicrobial and antibiotic resistance is covered in (37).

Despite being a natural occurrence, antimicrobial resistance (AMR) has dramatically risen in prevalence over the last few decades, accelerated by the selective pressure of inappropriate and extreme use of antibiotics by humans in hospitals, veterinary medicine and agriculture. Antimicrobial resistance threatens the treatment of all infectious disease, not just those caused by bacteria. A lack of new antifungal development, combined with the loss of some therapies to resistance has also threatened the future of fungal disease treatment, a rising concern due to the increase in fungal disease prevalence due to the emergence of an ageing and more immune

susceptible population ((see Chapter 2, section 1.1) and covered further in (2)). Resistance to anti-viral and anti-protozoan drugs such as anti-malarial drugs is also of great concern. This thesis will focus on the development of resistance to antibiotic treatment.

One contributing factor to the emergence of antimicrobial resistance is a lack of stewardship surrounding the use of these antimicrobial compounds. Extended courses of broad spectrum antibiotics are frequently prescribed to individuals in intensive care units (ICUs), organ transplant recipients and those with compromised immunity such as individuals with AIDS, all of whom are at increased risk of infection (2). Treatment is frequently given prophylactically, even when no clinical signs of infection are present owing to the potential for severe consequences if infection did occur (29). This increased dependence on antibiotics within modern healthcare has contributed to the recent increase in AMR, highlighting the urgent need for better stewardship and control over these compounds (29). There are also emerging questions regarding the off-target effects of long-term antibiotic administration on the host immune response, something that this thesis will build on.

1.2.2 Mechanisms of AMR

Antimicrobial resistance can either be intrinsic, where it is naturally encoded within the genome of the organism that produces the antimicrobial compound, or acquired, where it develops due to random mutation or acquisition of a genetic element upon exposure to a selection pressure, where it subsequently propagates and increases in frequency within the population due to the selective advantage it provides (56). Acquired resistance can be highly transferrable (see 1.2.3) and is usually initially obtained from the producer organism via the acquisition and integration of their genetic material via horizontal gene transfer, or via mutation, negating the mechanism of action of the antibiotic on the cell or excluding the antibiotic from the cell altogether (56,57). This form of AMR poses the greatest concern as it is this form that frequently occurs and spreads in a clinical

setting and prevents the effective treatment of infectious disease. The main mechanisms of antimicrobial resistance are summarised below.

1.2.2.1 Decreased penetration and increased efflux

One group of mechanisms of antibiotic resistance are the decreased penetration or increased efflux of antibiotics from the bacterial cell. This may occur due to the decreased permeability of the bacterial cell membrane to antibiotics, either through the alteration of charge on the outer membrane, preventing lethal concentrations of antibiotic entering the bacterial cell, or through the alteration or upregulation of porin expression, which decreases the uptake of β-lactams, tetracyclines and fluoroquinolones across the outer membrane e.g. the dysregulation of *P. aeruginosa* OprD expression results in resistance to multiple antibiotic classes (Figure 2B) (57,58).

Alternatively, there may be an upregulation of efflux pumps either in Gram-positive or Gram-negative bacteria, whereby the antibiotic is effluxed from the cell before bactericidal concentrations can be reached (Figure 2B) (59). Efflux pumps are usually intrinsically coded for within the bacterial chromosome, exporting the antibiotic substrate and making them intrinsically resistant, or exporting a structurally similar substrate. Their expression is then upregulated by mutation making the bacteria increasingly antibiotic resistant, or the active site mutates making the efflux pumps capable of exporting alternative substrates (59). Efflux pumps can be categorised into 5 different families depending on their host range, mode of efflux, substrate etc.: major facilitator superfamily (MFS) (60,61), resistance nodulation division (RND) such as the AcrAB-TolC pump found in *Enterobacteriaceae* (62), small multidrug resistance family (SMS), the ATP binding cassette family (ABC) and the multidrug and toxic compound extrusion family (MATE) (59). Whilst some efflux pumps are encoded for intrinsically within the chromosome, such as the RND efflux pumps that confer resistance to a wide array of antibiotics including tetracyclines, chloramphenicol, β-lactams and fluoroquinolones (57), others are encoded by a mobile genetic element,

therefore the spread of efflux pump mediated antimicrobial resistance is also a serious threat to the treatment of infectious disease (57).

1.2.2.2 Alteration of target

As mentioned in 1.2.2, an alternative mechanism of antibiotic resistance is to alter the target of the antibiotic, thus preventing the antibiotic from binding. This can be achieved either through protection or modification of the target site (Figure 2B) (57). Genes encoding target site protecting proteins are typically encoded of mobile genetic elements, including a large number of plasmids and broad host range conjugative transposons, facilitating their spread throughout bacterial populations (63) (see 1.2.3). Protective proteins include the TetM, TetO and Qnr proteins which provide tetracycline resistance and fluoroquinolone resistance respectively (57). These proteins work in a variety of different ways, either by dislodging the antibiotic from the target site and altering the ribosomal conformation to prevent re-binding (TetM) or competing with the antibiotic to bind to the target site and altering the target binding site also (TetO) (64,65). Alternatively, the Qnr (quinolone resistance protein), which is encoded on a conjugative plasmid, competes for the DNA binding sites on the DNA gyrase and topoisomerase IV molecules (66) conferring low level resistance to fluoroquinolones. Additionally, Qnr proteins have been seen to promote single point mutations in the gyrase target site, leading to emergence of highly resistant isolates (67).

Instead, bacteria may modify their target site to prevent antibiotic binding or to decrease the binding affinity of the target site, a mechanism which is common in multidrug resistant clinical isolates (57). This may occur through the generation of point mutations within the genes encoding the target site e.g. single amino acid mutations in the *rpoB* gene encoding the binding pocket of the RNA polymerase targeted by rifampicin results in high levels of resistance (68) and multiple single amino acid changes in the DNA gyrase and topoisomerase IV molecules leads to fluoroquinolone resistance accumulation over time (69). Resistance may also occur through the enzymatic modification of the target site e.g. methylation of the erythromycin binding site on the ribosomal 50s subunit results in macrolide resistance (70). Finally, modification of the target site may be achieved through replacement

or bypass of the target, meaning the binding of the antibiotic is no longer detrimental to the bacterial cell. The primary example of this is seen in methicillin resistant *S. aureus* (MRSA) whereby the replacement of a penicillin binding protein with PBP2a (encoded by *mecA* on the mobile Staphylococcus cassette chromosome mec (SCCmec)) which has a lower binding affinity for methicillin, results in a bypass of the initial PBP target so methicillin has no effect on peptidoglycan synthesis within the cell (Figure 2B) (71).

1.2.2.3 Alteration of drug

Another mechanism of antibiotic resistance is the chemical alteration of the antibiotic itself. This mechanism is usually encoded by mobile genetic elements that are readily transferred throughout neighbouring cells via conjugation, and commonly provides resistance to aminoglycosides and chloramphenicol (57). Modification may consist of acetylation of the hydroxyl or amine groups on the antibiotic, phosphorylation or adenylation, either preventing binding via steric inhibition due to the antibiotic no longer fitting in the target binding site, or altering the nature of the antibiotic molecule making binding occur with a lower affinity (Figure 2B) (57).

Alternatively, bacteria may modify the antibiotic molecules structure and destroy the molecule (Figure 2B). The prime example of this is β-lactamase-mediated cleavage of the amide bond within the β-lactam ring within penicillins and other β-lactams, leading to a complete loss of antibacterial activity (57). Separate classes of β-lactamases have varying mechanisms of cleavage, either through the use of a serine molecule (serine β-lactamases) or via a zinc ion (metallo-β-lactamases), some of which have a broad spectrum (extended spectrum β-lactamases/ESBLs) and others with a more narrow target range (carbapenemases) (57), where all β-lactamases alter the antibiotic molecule and negate its antibacterial activity. One example of a highly mobile drug alteration mechanism is that of the ESBL CTX-M which confers cephalosporin resistance to *Enterobacteriaceae* after being first obtained from an intrinsically resistant environmental organism (57,72). The

genes encoding this form of resistance have been shown to be associated with transposon elements, conjugative plasmids and phage-like sequences, all of which would readily aid dissemination of these genes and cephalosporin resistance (57,73).

As discussed above, antimicrobial resistance may arise through a multitude of mechanisms. However, it is important to consider that resistance to one antibiotic can occur via multiple mechanisms, or one mechanism can encode resistance to multiple antibiotics. For example, resistance to fluoroquinolones can occur through a mutation in the target gyrase site, an increase in efflux pump expression or via restriction of the target site through the production of a protection protein (Qnr) (57). Conversely, the upregulation of a single RND efflux pump AdeABC in *Acinetobacter baumannii* confers resistance to tetracyclines, aminoglycosides, chloramphenicol, fluoroquinolones and an array of other antibiotics (74,75). Another important consideration is that multiple resistance-determining genes may be co-inherited together, conferring multiple drug resistance via multiple mechanisms in one single genetic transfer event e.g. New Delhi metallo-β-lactamase spread on some plasmids has been shown to also confer resistance to other β-lactams via other extended spectrum β-lactamases, macrolide resistance via methylation enzymes and fluoroquinolone resistance through Qnr protein expression (57). It is important to consider this co-acquisition and co-expression of multiple resistance mechanisms when seeking out alternative solutions to the AMR crisis.

1.2.2.4 Community mediated resistance

Bacteria may also resist the action of antibiotics through community mediated changes in susceptibility rather than single cell actions (76). A prime example of this is the formation of biofilms (communities of bacteria surrounded by extracellular DNA, extra polymeric matrices, polysaccharides etc.) whereby quorum sensing mediated communication is instrumental in their formation (77). Biofilms have been shown to resist the actions of the immune system and antimicrobials (where less susceptible cells can easily propagate) and

are regarded as sites of high genetic exchange, making them a key distribution site of mobile genetic element encoded resistance genes (77). They are also common and extremely problematic in a clinical setting, where most chronic infections occur as biofilms rather than planktonic infections. A more comprehensive review of the role of biofilms in antimicrobial resistance is covered in the following reviews: (76,77).

1.2.3 The rise and spread of AMR

As discussed in 1.2.2, antibiotic resistance has always been present in bacterial populations, long before even the first antibiotics were formally discovered, due to them being a natural product produced by microorganisms as a competitive growth advantage against others. However, the accelerated rates of antibiotic resistance and its increased mobility and spread throughout the world is of great concern. Most genes encoding antibiotic resistance are obtained via horizontal gene transfer (HGT). HGT may occur through transformation (the uptake of naked DNA from the environment), transduction (usually bacteriophage mediated injection of DNA into the bacterium), conjugation (the exchange of genetic material between two cells via pili) or the transfer of mobile genetic elements (either plasmids, transposons or integrons) between bacteria (57). The main reason behind this acceleration in AMR development is the reckless overconsumption of antibiotics within a clinical setting that has become increasingly more reliant on them for everyday medical interventions (2). The over-prescription of antibiotics for diseases that could be treated alternatively or with more appropriate interventions represents one pillar of the AMR crisis. The lack of stewardship regarding antibiotic administration, whereby they are available over the counter in some countries like the USA and Bangladesh (78) and can be used as animal growth promoters within agriculture (a practise that is banned within Europe but is still prevalent around the world) (79) when alternatives such as vaccination of livestock would provide more appropriate interventions against infection (80) are also contributory factors. Overall, the overconsumption and inappropriate use of antibiotics around the world over the last few decades has led to a scenario where, without the rapid discovery

of novel antimicrobial treatments, the treatment of infectious disease could regress significantly, representing a significant health burden. This highlights the need for development of novel antimicrobial treatments, alongside the conservation and best use of current antimicrobial therapies, to treat infectious disease in the future.

1.2.4 Overcoming the AMR crisis

As the majority of antibiotics are isolated from nature in the first instance, resistance mechanisms to these compounds will already exist within the environment even before their discovery and clinical use, either within the producing organism itself which is likely to be intrinsically resistant, or within neighbouring closely related species. This suggests the use of evolutionary static compounds as antimicrobial therapies is already a short-lived strategy owing to the tools to disarm them already being widespread. The use of alternative antimicrobial approaches has subsequently been considered, whereby the prevention of infection, the enhancement of existing antimicrobial compounds and the use of “living” antimicrobial therapies (see 1.2.4.4 and 1.2.4.6) represent potential solutions to the AMR crisis.

1.2.4.1 Adjuvants

One potential solution to AMR is the use of antibiotic adjuvants alongside conventional antibiotic therapy to extend the lifespan of conventional antibacterial therapies whilst novel antimicrobial therapies are developed. These compounds have little antimicrobial activity themselves, but when combined with conventional antibiotics, enhance their efficacy either through the targeting of resistance mechanisms e.g. clavulanic acid cleaves the TEM β-lactamase, restoring β-lactam sensitivity, and efflux pump inhibitors inhibit the export of the antibiotic from the cell, restoring drug efficacy (81). Alternatively, adjuvants may alter the bacterial cell itself making the antibiotic more potent e.g. loperamide makes the bacterial cell membrane more permeable to some antibiotics (81,82). Adjuvants provide a way to continue the use of current conventional antibiotic therapies, even when some

resistance has developed, making them a promising avenue for combating AMR. However, they face the same issues as conventional antibiotics whereby the economic support and interest is lacking, slowing the development of further adjuvant therapies, and the development of resistance is inevitable due to their evolutionarily-static nature (83).

1.2.4.2 Vaccination

Vaccination represents another potential tool in the treatment of infectious disease and the curbing of antimicrobial resistance. Much like the importance of improved hygiene practices and infection control within healthcare settings, vaccination also plays a role in the prevention of infection. Vaccination prevents disease as vaccines are administered prophylactically and so this negates the need for antibiotics to be prescribed (either correctly or inappropriately for non-bacterial disease), thus limiting the emergence of AMR and the selection pressure to evolve resistance in both commensals and pathogens (84). Viral vaccines also have a role in the prevention of AMR emergence as they indirectly limit the prevalence of secondary bacterial infections and so decrease antibiotic usage further (80). Vaccination has been pivotal in the eradication of smallpox and the near eradication of polio from the World (although lack of compliance threatens the latter) (85). One example of the role of vaccination in the fight against AMR is that of the *Haemophilus influenzae* Type B vaccine (86). Treatment of *H. influenzae* Type B infection was complicated by the presence of a high proportion of β-lactamase positive bacterial strains (16.6%) in the 1970s (86,87). However, after the introduction of a *H. influenzae* Type B vaccine, cases of *H. influenzae* drastically dropped (86), along with the prevalence of β-lactamase genes within the bacterial population (88), demonstrating that vaccination against infectious disease not only provides an alternative form of treatment, but can also actively reverse the emergence of resistance to antimicrobials. In a more direct approach, vaccines for *Clostridium difficile* and *S. aureus* infection are now being developed and are in late stage clinical trials, in the hope that their use may follow suit and slow the development of AMR further (80).

Vaccination isn't only important for the treatment of human disease; it also plays an arguably more important role in agriculture. Despite being outlawed for use as agricultural growth promoters in Europe, antibiotics are still administered in extreme quantities around the World, especially in the USA and Asia whereby the prevention of infection within livestock leads to higher yields (79). The outright banning of antibiotic use in agriculture (both as non-therapeutic growth promoters and for disease treatment) would be met with great opposition due to its potential impacts on food production and the economy. However, the development of vaccines against agricultural diseases may represent a cost-effective and equally efficacious approach to preventing disease and maximising yields, whilst minimising antibiotic use and AMR development. The development of vaccines for agricultural disease should be made a priority in the ongoing battle with AMR (79).

Potential fallbacks of vaccination include the potential for resistance to evolve, or the emergence of new strains due to selection against some strains, facilitating the emergence and increased prevalence of others (85,86). One key example of this is the *Streptococcus pneumoniae* type A vaccine whereby mutations occur which render the vaccine ineffective, and some serotypes emerge and increase in prevalence at the expense of others (85,86). This suggests that vaccines will need to be constantly modified to cover further serotypes and remain effective, increasing the economic cost and providing an ongoing solution to infectious disease rather than a permanent one.

1.2.4.3 Anti-virulence factors

Another potential alternative to the use of antibiotics is the use of anti-virulence factors. These compounds prevent the establishment of infection within a host by inhibiting the mechanisms required for colonisation, establishment or toxin release etc., without exerting a bactericidal or bacteriostatic effect on the bacterium, allowing the immune system to eradicate the pathogen without as strong a selection pressure as antibiotic use (81,89). Anti-virulence factors may target specific virulence factors

relevant to infection, such as Type 3 secretion system assembly (90) and toxin production (91), virulence regulators for quorum sensing (92) or virulence factors relevant to immune system resistance and persistence within the host such as biofilm formation (93) and capsule formation (94,95).

One example of a target for anti-virulence therapy is quorum-sensing, a communication system between bacteria that relies on the diffusion of small molecules to aid population density sensing and coordination of gene expression, including that of virulence factors such as adhesin expression, secretion system expression and toxin secretion (96). Successful chemical quorum sensing inhibitors have been utilised previously in clinical trials (97). However, *P. aeruginosa* isolates have already emerged that are resistant to anti-quorum sensing treatments through the upregulation of efflux pumps (much like the mechanism behind resistance to some antibiotics (covered in 1.2.2.1)), whereby the isolates cause a higher mortality in the presence of this anti-virulence therapy (98,99). As, the rate of mutation and gene acquisition within bacterial species is highly species specific, some anti-virulence factors may be highly effective in some species with lower mutation rates, such as *S. aureus*, but redundant in others, such as *P. aeruginosa* where resistance quickly emerges (100). Other anti-virulence factors currently in clinical trials include pilicides and type 3 secretion system inhibitors, some of which may have a broader host range and may target multiple species within the Enterobacteriaceae (101), and a *S. aureus* toxin-targeting anti-virulence factor that has been shown to speed up *S. aureus* infected wound healing and cause a stronger and more effective immune response to *S. aureus* in this scenario (102).

Anti-virulence therapies represent a more targeted treatment approach as they will be bacterial species specific in most cases and so should have minimal side effects on the host and gut microbiota. However, as they have a narrow host range, the advancement of clinical diagnostics will be needed before they can be fully utilised and further study will be required to find the most effective targets with the lowest likelihood of resistance formation (101).

One further potential caveat of anti-virulence factor use is that they rely on a functioning host immune system to neutralise the pathogen, something that may be lacking in individuals on ICU wards or with treatment-mediated or disease-mediated immunosuppression in individuals who are at increased risk of infection in the first instance (2). Treatment of these individuals may still be effective as disease would not be able to establish, but without being reinforced by a host-mediated clearing mechanism they may not be as effective. One solution to this would be the administration of a combination therapy of anti-virulence factors and bactericidal antibiotics. However, this still represents the use of a static therapy against a living, evolving organism, meaning that even though the pressure to develop resistance may be lower than that seen with conventional antibiotics, if pathogenesis is essential for bacterial viability then resistance is likely to develop.

Another important consideration is the differential gene expression that occurs between planktonic cells and biofilms and within biofilms themselves, whereby the bacteria at the surface of the biofilm will express different genes and virulence factors to those on the inside, who will likely express very few virulence factors in comparison. One example of this is the expression of fermentation associated genes in anoxic areas of *S. aureus* biofilms, where acidic pH tolerance genes will likely be upregulated but adhesins for host tissue cells will not be expressed (103). Comparatively, in surface biofilm cells and planktonic cells, adhesins, toxins and proteases are all upregulated so would make good targets for anti-virulence therapy, but will be effective against this subset only (103). This has potential implications for anti-virulence factor treatment as it means that treatment would only target a subset of the population, leaving the remainder unaffected and still capable of establishing infection. Once again, the solution to this would be to administer anti-virulence factors as part of a combination therapy with conventional bactericidal antibiotic treatment, but this approach isn't without drawbacks, as mentioned previously.

1.2.4.4 Phage therapy

The use of bacteriophage in the treatment of bacterial infection has long been of interest. Bacteriophage are viruses that target and infect bacteria in a highly receptor specific manner (104), making them a potential form of narrow range antimicrobial therapy for both planktonic and biofilm infections (105). Bacteriophage exhibit two lifestyles, whereby they either invade the bacterial cell and manipulate the host machinery to replicate viral DNA and capsid proteins before lysing the cell (lytic cycle), or integrate their genome into the host genome, replicating alongside the bacterial cell covertly (lysogenic cycle) (104). The latter may have a contributory role to the development of AMR as the process whereby the bacteriophage extracts itself from the host bacterial genome may also lead to the extraction of some host genomic material, facilitating the transfer of bacterial DNA and possibly antibiotic resistance genes between bacterial hosts (104). The lytic cycle, however, is of great interest in the reduction of AMR development. After bacteriophage entry and host machinery manipulation occurs to replicate the viral DNA and capsid proteins, an array of hydrolytic and degradative enzymes are released, some of which have bactericidal and bacteriostatic properties, such as holins, which create small pores in the cytoplasmic membrane of the bacterial cell and endolysins and peptidoglycan hydrolases which digest the bacterial peptidoglycan and lyse the cell, resulting in the release of viral progeny and death of the bacterium (104).

Bacteriophage have also been used as tools for genetic modification of pathogens, whereby bacteriophage are used to deliver CRISPR-Cas9 nucleases into bacteria, which subsequently can be targeted to excise antibiotic resistance encoding genes and restore susceptibility to antibiotic treatment (106,107) or to knockout virulence factor encoding genes. However, one major drawback to this approach is that the target site, i.e. the gene nucleotide sequence, must be known and so this approach would only be effective against known and well characterised resistance and virulence factor mechanisms, not those that occur as a result of single point mutations in antibiotic target sites or for virulence factors that are poorly studied.

Potential drawbacks of the use of bacteriophage in treatment of infectious disease also lie in their high specificity. Despite this high specificity limiting the off-target side effects on the host and the commensal microbiome (something that antibiotics are known for), the high specificity of bacteriophage treatments means that a whole library of bacteriophage will need to be developed to treat infectious disease (one for each strain type), making treatment development costly, and better diagnostics will be needed to diagnose the causative organism before treatment can begin (104). Other drawbacks include the potential for an immune response towards the bacteriophage, that may result in inflammation and harm to the host or may neutralise the bacteriophage before they have sufficient dwell time to prey upon the pathogenic bacteria (104). Resistance may also occur through the mutation of pathogenic surface receptors, negating bacteriophage entry, although the live nature of this treatment approach may mean that co-evolution between bacteriophage and pathogen may occur, slowing resistance formation (104).

1.2.4.5 Enzybiotics

An alternative that builds on the use of bacteriophage as antimicrobial therapies is the use of the bacteriostatic and bactericidal enzymes that bacteriophage (108,109) or other bacteria (110) produce as antimicrobial therapies. These enzymes may include peptidoglycan hydrolysers such as Gp181, a peptidoglycan hydrolase produced by the *P. aeruginosa* bacteriophage φKZ (108), phospholipases or other hydrolytic enzymes, all of which encompass a wide variety of different targets, modes and chronologies of action (111). Endolysins from bacteriophage have been shown to be effective against *Streptococcus spp.* in murine models (112,113) as well as *Enterococcus spp.* (114)*, Clostridium perfringens* (115)*, P. aeruginosa* (108,109) and *Bacillus anthracis* (116)*,* demonstrating their potential for infectious disease treatment.

Enzymes from bacteriophage typically consist of a peptidoglycan binding domain which targets the enzyme towards its substrate, and an active

catalytic domain that degrades the cell wall through the cleavage of peptidoglycan crosslinks (117). One advantage of the use of enzybiotics over bacteriophage is their versatility, whereby, due to their modular nature, enzymatic domains can be swapped between enzymes to increase their antimicrobial properties such as efficacy and their biophysical properties such as their stability and solubility (118). They can also be modified to kill Gram-negative bacteria from the outside of the cell, which does not occur naturally as the majority of bacteriophage-mediated lysis occurs from within (119).

Enzymes from the predatory bacterium *Bdellovibrio bacteriovorus* may also have potential for the treatment of Gram-negative infections (110). As part of the predatory lifecycle of *B. bacteriovorus* (detailed later in Chapter 3), an array of peptidoglycan hydrolases and other peptidoglycan-modifying enzymes are used to gain entry into Gram-negative prey, modifying the prey cell wall to promote establishment of *B. bacteriovorus* inside the periplasm where it subsequently grows and feeds upon the bacterial cell (110). One example is the *dacB* DD-endo/carboxypeptidase enzymes that are expressed upon prey entry, ultimately causing prey cell rounding and creating a niche for *B. bacteriovorus* growth (110). These enzymes could be alternatively utilised to alter bacterial cell morphology, preventing bacterial growth and establishment of infection through their bacteriostatic actions. Enzymes from *B. bacteriovorus* have not been utilised in the treatment of Gram-negative bacterial infections yet but they show great promise as treatment options for infectious disease in the future.

The use of enzybiotics for the treatment of infectious disease is easier to validate compared to living therapies, but the advantage of co-evolution between pathogen and bacteriophage/bacterium is lost, meaning resistance to these single enzymes is highly likely unless administered in combination with other antimicrobial therapies. This is an important consideration for the potential use of enzybiotics in the future.

1.2.4.6 *Bdellovibrio bacteriovorus*

As discussed in the above section, the use of static antimicrobial therapies i.e. anti-virulence factors and other conventional treatments or molecules, suffers from the fundamental flaw that resistance will inevitably evolve, rendering the treatment ineffective. The use of living antimicrobial therapies, such as bacteriophage, that can co-evolve alongside the pathogen is an alternative approach that should lead to a lower likelihood of resistance development. However, bacteriophage often only bind to a single receptor on the target bacterium, giving them a narrow spectrum of action, and are potentially immunogenic, creating barriers to their use as antimicrobial therapies. Further work is needed to develop novel antimicrobial therapies that work alongside the host immune response to eradicate infection, whilst retaining their ability to co-evolve alongside pathogens to minimise resistance formation. One example of such a living therapy is the predatory bacterium *Bdellovibrio bacteriovorus* (120,121), which will be covered in Chapter 3 of this thesis.

2. The innate immune system

2.1 Overview

Humans are exposed to a range of microorganisms on a daily basis either through inhalation, ingestion or injury, the majority of these exposures being asymptomatic. The human body is home to a diverse commensal microbiota therefore it is a fine balance between preventing and responding to infection whilst avoiding eradication of the beneficial commensal microbiome or causing inflammation (122). The use of conventional antibiotics to treat infectious disease frequently upsets this balance through the non-specific eradication of the host microbiota, perturbing the host immune response in the process. The interplay between antibiotic treatment and the host immune system has been frequently overlooked and so the consideration of the host immune system in the development of new antimicrobial approaches and the most prudent use of existing antimicrobials is of great importance.

The immune response is comprised of two systems, a dendritic cell mediated adaptive immune response consisting of a random and diverse array of T and B cells with differing pathogen recognition receptors (PRRs), that when stimulated by the binding of an appropriate pathogen associated molecular pattern (PAMP) undergo clonal selection and expansion to mount a slower, adaptive, memory-based response. This adaptive immune response is complimented by a quicker, less fine-tuned innate immune response responsible for the rapid initial control of infection (123,124). Phagocytes are the primary mediators of the innate immune system. They are selectively distributed around the body, mostly at sites where microbial exposure is high such as at the mucosal surfaces (125). Detection by phagocytes can occur via two modes; opsonised detection where Fc receptors and complement receptors recognise particles coated in antibodies or complement by the host which aids recognition, or non-opsonised detection where PAMPs are recognised by PRRs on the host phagocyte cell membrane (125).

Multiple phagocyte cell types coordinate to form the innate immune system. Neutrophils rapidly migrate to and control infection, responding to chemokine ligand 1 (CXCL1) and other chemoattractants produced by epithelial cells,

macrophages and mast cells, subsequently killing the detected pathogens. Other cell types such as natural killer (NK) and mast cells also play important roles within the innate immune response. However, arguably the most important cell type that senses and responds to infection within the innate immune system is the macrophage which will be the focus of the remainder of this section.

2.2 Macrophage sensing

Macrophages are some of the first phagocytic cells to sense and respond to infectious stimuli. They have important roles in environmental sensing, microbial killing, uptake of apoptotic and necrotic cells, tissue healing and antigen presentation to T cells (126). The origins and expression profiles, i.e. surface markers, vary between tissues facilitating the detection of various tissue types, metabolites, pathogens and antibodies (126). Tissue-resident macrophages exist in a resting state, termed M2, whereby they promote tissue repair and homeostasis. Upon the detection of infection, interleukin-12 (IL-12) and inducible nitric oxide synthase (iNOS) are released promoting differentiation into M1 type macrophages, facilitating the development and polarisation of tissue-specific macrophage phenotypes (126).

Macrophages express 6 types of pathogen recognition receptors that are key for sensing and responding to infection; Toll like receptors (TLRs), Nod (nucleotide oligomerisation domain) like receptors (NLRs), intracellular RNA receptors, intracellular DNA receptors, C-type lectin receptors and scavenger receptors (126). The detection of different PAMPs is facilitated by these different PRRs, resulting in the activation of alternative pathways.

2.2.1 Toll-like receptors

Toll-like receptors are expressed on macrophages and other host cells such as neutrophils, dendritic cells, T cells and epithelial cells (127). TLRs are capable of sensing a wide range of microbial ligands, which are usually released in higher concentrations upon the initial activation of the immune

system as it causes damage to the microbial cells. TLR-1/2/4/5 and 6 are expressed on the cell surface of the host cell whereas TLR-3/7/8 and 9 are expressed within the endosome where they induce interferon (IFN) production through the activation of interferon regulatory factor family transcription factors (126,128).

TLRs form one of the central classes of PRRs that are important in the detection of PAMPs from bacteria, fungi, viruses and protozoa, with some TLRs being more important in the detection and sensing of bacterial ligands and others being more ubiquitous (Figure 3) (126,129). For example, TLR-2 detects peptidoglycan and lipoproteins and so is instrumental in the detection of both Gram-positive and Gram-negative bacteria. TLR-4 is instrumental in the detection of LPS and lipoteichoic acids, making it important for the detection of Gram-negative bacteria (123,128). Alternatively, TLR-3 and TLR-9 detect double stranded DNA and CpG DNA respectively, and so are important for the detection of viruses and bacterial cells (when lysed) (123).

Whilst some TLRs are capable of binding to microbial ligands individually, others require additional host proteins and TLRs to bind. One example of this is the complexation of TLR-2 with TLR-1 and -6 to recognise peptidoglycan, emphasising the extensive crosstalk that occurs within the microbial detection system (123). Most TLRs signal through a multi-protein complex comprised of the MyD88 (myeloid differentiation primary response protein 88) adaptor protein and interleukin (IL)-1R associated kinase (IRAK) and TNF-receptor associated factor 6 (TRAF-6) accessory proteins, leading to NFκB transcription factor activation (123,126). Alternatively, some TLRs e.g. TLR-3 signal through the TRIF (TIR domain containing adaptor inducing interferon β) adaptor, leading to type 1 interferon production or through multiple adaptors e.g. TLR-4 signals through TIRAP (TIR-domain containing adaptor protein), TRIF and MyD88 adaptor protein complexes (Figure 4) (123,126). TLRs and IL-1Rs rely on these TIR domain-containing adaptor proteins to activate the downstream transcription factors, e.g. NFκB and MAP kinases, and induce downstream target gene expression (123).

2.2.1.1 Peptidoglycan detection

TLR-2 detects peptidoglycan within the bacterial cell wall. Usually peptidoglycan is masked in Gram-negative bacteria by the outer membrane and LPS. However, after some bacterial cells have been lysed by the immune system or upon entry into the host, exposed peptidoglycan fragments may be present. Hexokinases recognise N-acetyl glucosamine (NAG) and muramyl dipeptide (MDP) N-acetyl muramic acid (NAM) subunits and initiate a signalling cascade resulting in caspase-1 dependent NLRP3 inflammasome assembly and proinflammatory cytokine release e.g. IL-1β and activation of the innate immune response (128).

2.2.1.2 LPS detection

The detection of LPS in Gram-negative bacteria is predominantly mediated by TLR-4. Initially, LPS is detected by LPS-binding protein at the cytoplasmic membrane of the host cell (in collaboration with CD14) and delivered to a TLR-4-MD2 complex, resulting in myddosome assembly (a complex of MyD88 and other adaptor proteins). CD14 also delivers the dimerised TLR-4 to the endosome, promoting TRIF adaptor mediated signalling through a putative triffosome (a complex of TRIF and other adaptor proteins). Both of these signalling cascades converge and initiate phagocytosis, production of reactive oxygen species (ROS) and the induction of inflammation. Furthermore, upon trafficking of LPS to the cytosol, it is bound by caspase-11, initiating the formation of a non-canonical inflammasome (leading to host cell pyroptosis) and a canonical NLRP3-mediated inflammasome (resulting in IL-1β processing and release), amplifying the proinflammatory innate immune response (Figure 4) (128).

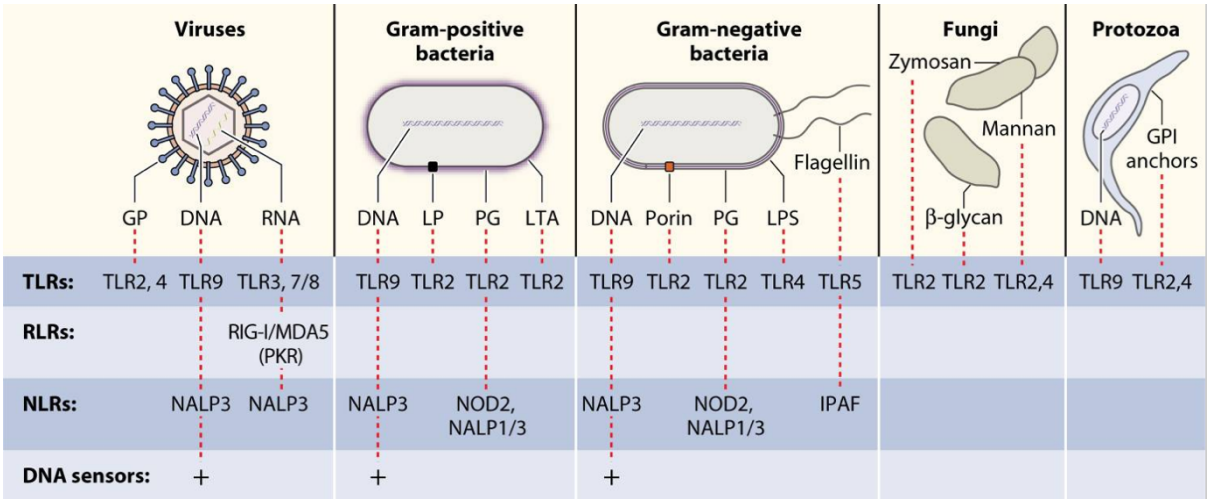


Figure 3: Recognition of pathogen associated molecular patterns by different pattern recognition receptors.
The detection of PAMPs by host PRRs is what triggers the initiation of the innate immune response, whether this be towards viruses, bacteria, fungi or protozoa. The majority of PAMPs are unique to a specific class of microbial pathogen. However, some PAMPs are ubiquitous and are represented in multiple classes of pathogen, such as DNA and RNA. The major PAMPs for each class of microorganism are shown, along with the host cell PRRs that detect their presence. GP, surface glycoproteins; LP, lipoproteins; PG, peptidoglycan; LTA, lipoteichoic acid; LPS, lipopolysaccharide; GPI, glycophosphatidylinositol; TLR, Toll-like receptors; RLR, RIG-1 like receptors; NLR, Nod-like receptors. Figure taken from Mogensen, 2009.

2.2.2 Nod-like receptors

Nod-like receptors are responsible for the regulation of a number of key inflammatory pathways and have a crucial role in inflammasome assembly. NLRs do not recognise PAMPs; instead they detect cellular stress such as reactive oxygen species and potassium efflux that are frequently associated with tissue damage and infection (Figure 3). For example, in response to NLRP3-mediated detection of cellular stress, inflammasome assembly occurs in a canonical caspase-1 dependent or non-canonical caspase-11 dependent manner, prompting the recruitment of phagocytes, proinflammatory cytokine release and phagocytosis of the cell not through the induction of transcriptional responses but through the release of already synthesised pre-existing IL-1 cytokines, amplifying the immune response further and causing localised inflammation (128). Alternatively, when sensing certain bacteria, the NLRC4 inflammasome is activated by the ASC (Apoptosis-associated speck-like protein containing CARD) adaptor protein, stimulated by the detection of flagellin, Type 3 or 4 secretion system rod and needle components or other bacterial components within the cytosol of the

cell, prompting the formation of a pyroptome that causes localised inflammation and macrophage pyroptosis (126).

Both TLRs and NLRs are crucial classes of PRRs in the innate immune response, directed towards the detection of a range of bacterial, fungal and viral PAMPs (Figure 3). Other receptors present on/in the macrophage have a more focused pathogen detection range. The intracellular RNA and DNA PRRs have an important role in the detection of viral genome components and lysed bacterial cells. RIG1, an intracellular RNA receptor, signals through a MAVS (mitochondrial antiviral) adaptor protein to activate IRF-3 and -7 transcription factors prompting the release of IFN-α and β (Figure 3). The intracellular DNA receptors TLR-9 and STING (stimulator of interferon genes) subsequently also signal through the same pathway, aiding the detection and immune response towards viral and bacterial DNA.

2.2.3 C-type lectin receptors

C-type lectin receptors are capable of recognising carbohydrate ligands on the epithelial and endothelial cell surface as well as on the surface of pathogens and on the surface of apoptotic and necrotic cells, facilitating uptake. They can be classed as collectins, selectins, lymphocyte lectins and proteoglycans amongst others depending on their target cell type. CLRs typically interact with ITIM (immunoreceptor tyrosine-based inhibition motif)-domain containing adaptor proteins where they have an inhibitory and suppressive effect on immune cell activation or with ITAM (immunoreceptor tyrosine-based activation motif)-domain containing adaptor proteins with a stimulatory effect, although other ITAM/ITIM independent adaptor proteins are also used. CLRs have been shown to have a role in the detection of bacterial pathogens (130,131) and some viruses (132), but their most definitive role is in the detection of fungal ligands during infection e.g. Dectin-1 and mannose receptor have important roles in the detection of β(1,3)-glucan, mannose and other fungal ligands (126,133).

Upon CLR activation, intracellular signalling pathways are initiated, triggering phagocytosis, proinflammatory cytokine release, activation of the respiratory

burst etc. via the activation of ITAM and other signalling motifs within the CLRs or by association with other signal transducing adaptor molecules (133) For example, CLR-mediated activation of Syk (spleen tyrosine kinase) leads to the formation of a CARD9:BCL10:MALT1 (Caspase recruitment domain-containing protein 9: B-cell lymphoma 10: Mucosa-associated lymphoid tissue translocation protein 1) multiprotein complex that subsequently activates NFκB, driving proinflammatory cytokine release and the release of TNF-α, whilst also shaping Th_1 and Th_{17} immune responses (Figure 4) (133). TNF receptors then bind soluble and surface expressed TNF molecules, resulting in caspase activation, inflammasome formation and apoptosis, or further activation of the NFκB and MAP kinase transcription factors, resulting in further cytokine release and amplification of the proinflammatory response.

In summary, macrophages possess a diverse array of membrane bound pattern recognition receptors that, when bound to by microbial ligands, initiate signalling cascades throughout the cell. These signalling cascades involve the transduction of a signal through Syk or other ITAM-containing adaptor proteins within multiprotein complexes, which subsequently induce the activation of transcription factors such as NFκB or activator protein 1 (AP1), ultimately leading to amplification of the innate immune response through proinflammatory cytokine release and phagocyte recruitment, whilst also prompting inflammasome assembly and apoptosis, ultimately resulting in increased microbial killing.

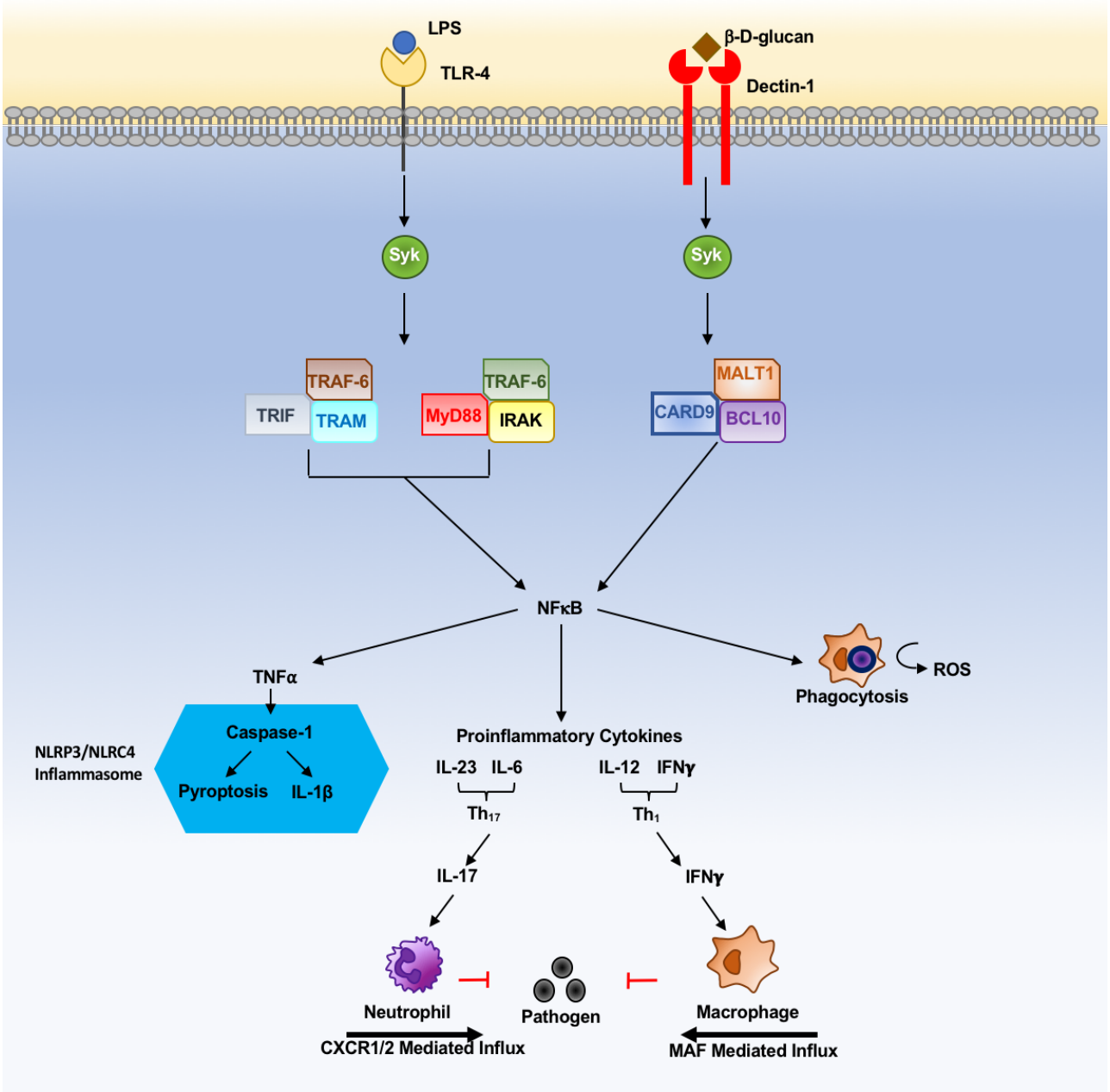


Figure 4: Detection of and responding to microbial molecules by the host immune response. Pathogen associated molecular patterns (PAMPs) in the microbial cell are detected by pattern recognition receptors (PRRs) on the plasma membrane of the host phagocyte cell. This initiates a signalling cascade through an ITAM- containing adaptor protein e.g. Syk into a multiprotein complex of other adaptor and accessory proteins. Signal transduction leads to the activation of transcription factors such as NFκB, resulting in inflammasome assembly and proinflammatory cytokine release, prompting further phagocyte recruitment and microbial killing. LPS, lipopolysaccharide; TLR, Toll-like receptor; Syk, spleen tyrosine kinase; ITAM, immunoreceptor tyrosine-based activation motif; TRIF, TIR domain containing adaptor inducing interferon β; TRAF, TNF receptor associated factor; TRAM, TRIF-related adaptor molecule; MyD88, myeloid differentiation factor 88; IRAK, interleukin-1 receptor associated kinase; CARD9, caspase recruitment domain containing protein 9; MALT1, mucosa-associated lymphoid tissue translocation protein 1; BCL10, B-cell lymphoma 10; NFκB, nuclear factor kappa B; ROS, reactive oxygen species; TNFα, tumour necrosis factor alpha; IL, interleukin; NLR, Nod-like receptor; CXCR, chemokine ligand receptor; IFN, interferon; MAF, macrophage activating factor; Th, T helper. Adapted from Clark & Drummond, 2019.

2.3 Consideration of the innate immune response within infectious disease treatment

Recently, there has been an increased focus on the importance of a collaborative effort of treatment approaches and the immune system in treating infectious disease. The use of treatments that control infection without complete eradication, thus allowing the immune system to neutralise the infection, is thought to be an alternative approach that may curb the formation of resistance to treatments such as antibiotic therapy. The interplay between and effects of the immune system on antibiotic therapy and the effects of antibiotics on the functioning of the immune system is only recently being considered. Whilst there has long been a known association between immunosuppression, either through iatrogenic interventions such as organ transplantation, or through disease such as AIDS, and opportunistic bacterial and fungal infection, the link between antibiotic use and predisposition to infection is only just being realised (2). This is of great concern due to large numbers of individuals in ICU wards, undergoing organ transplantation or immunomodulatory treatments, being on long-term courses of antibiotics, which may, in turn, be predisposing them to fungal or multidrug resistant bacterial infection, resulting in mortality.

The consideration of the participation of the immune system in novel treatment approaches, and how treatment may affect the sensing of pathogens by macrophages and initiation of the innate immune response is of great importance. One such novel treatment approach that was recently found to use this collaboration between treatment and the immune system is the use of the predatory bacterium *Bdellovibrio bacteriovorus.* Studies by Willis et al. (120), demonstrated that not only did *B. bacteriovorus* persist within a zebrafish larvae host non-pathogenically, and not perturb the functioning of the immune response, *B. bacteriovorus* was also able to prey upon an antibiotic resistant strain of *Shigella flexneri* within zebrafish larvae and drastically improve survival (120). One further, revolutionary finding of this study was achieved through the depletion of zebrafish leukocyte numbers, impairing the innate immune response (120). Leukocyte (predominantly macrophage) depletion demonstrated that, in addition to *in-*

vivo predation depleting *S. flexneri* numbers significantly, the most beneficial outcome and greatest resolution of infection arose from the collaboration of the host immune response with *B. bacteriovorus* predation (120). This demonstrates how the interplay between *B. bacteriovorus* treatment, and the host immune response is beneficial for resolution of infection, and how the synergy between the immune response and treatment is an important consideration, both for conventional and novel antimicrobial therapies (134).

2.3.1 Use of *Bdellovibrio bacteriovorus* in infectious disease treatment

2.3.1.1 Characterisation of the immune response to *B. bacteriovorus*

One important consideration when advocating the use of the living, predatory bacterium *B. bacteriovorus* as an antibacterial therapy is that it involves the introduction of a second bacterium, with many potentially immunostimulatory components such as LPS and peptidoglycan, to a scenario that already contains an infectious organism with immunostimulatory LPS and peptidoglycan, potentially triggering a stronger immune response that may harm the host or eradicate *B. bacteriovorus* before it has sufficient dwell time to prey on the pathogens.

As previously mentioned (above), initial studies characterising the interplay between the host immune system and *B. bacteriovorus* were undertaken by studying predation of an antibiotic resistant strain of *Shigella flexneri* by *B. bacteriovorus* within a zebrafish larvae model (120). This study demonstrated that *B. bacteriovorus* becomes localised within macrophages and is slowly degraded within the phagosome. Prior to degradation, *B. bacteriovorus* was shown to assist the immune system in promoting larvae survival and resolution of infection, whereby the best survival and clearance of infection was seen in immune-competent larvae (120), therefore it was concluded that a collaboration between the host immune response and *B. bacteriovorus* was important in combatting infection. However, this study was unable to characterise the interactions between *B. bacteriovorus* and the host immune cells on a molecular level, and to discern the immune response that occurs towards *B. bacteriovorus*.

A further study (121), sought to characterise the immune response towards *B. bacteriovorus* to determine how it is taken up and trafficked throughout the immune system and whether it has any impact on macrophage viability or phagosome maturation. It was shown that *B. bacteriovorus* persists within the macrophage with a sufficient dwell time that predation upon pathogenic organisms would be possible, without perturbing the immune response or phagocyte viability and without causing high proinflammatory cytokine release (121). This builds on previous studies (135–141) that have found a lack of cytotoxicity within *in-vitro* cell culture and *in-vivo* animal models by further characterising the interactions of *B. bacteriovorus* with the host immune system.

Further research into the immunostimulatory properties of *B. bacteriovorus* have shown it to be surprisingly minimally immunogenic within the host (121,142). Despite containing LPS, peptidoglycan and flagellins, all of which have been shown to be highly immunostimulatory when expressed by other bacteria, studies in our lab showed that *B. bacteriovorus* induces a cytokine response that is significantly lower than other bacteria such as *Klebsiella pneumoniae* and *Salmonella typhimurium,* with TNF-α induction being shown to be 100 x less in *B. bacteriovorus* stimulated human macrophages compared to human macrophages stimulated by *E. coli* K12 (142). *B. bacteriovorus* stimulation of human macrophages was also shown to cause less IL-6 induction compared to *E. coli* K12 stimulation, and less TNFα, IL-6 and IL-1β induction (2 fold to 8 fold less) compared to stimulation by *K. pneumonia*e and *S. typhimurium* LT2 (121,142).

One potential explanation for this lower immunostimulatory state is the expression of an alternative lipid A polar head group within *B. bacteriovorus* LPS, whereby an α-d-mannose group is present instead of a phosphate group, negating the LPS's usual negative charge and giving it a lower binding affinity to host TLR-4 and other PRRs (142). Another contributory factor to the low immunogenicity of *B. bacteriovorus* is that the flagellins that comprise the single polar flagellum of *B. bacteriovorus* are sheathed by a lipid membrane, a feature that is unusual in many bacteria (143). This membrane

sheath masks the flagellins from detection by host PRRs such as NLRC4, making *B. bacteriovorus* less immunostimulatory still (Figure 5: top, left) (143).

However, *B. bacteriovorus* stimulation of human macrophages is not completely immunologically silent, whereby some proinflammatory cytokine induction is seen e.g. at 4-8hrs of stimulation, TNF-α levels peak in *B. bacteriovorus* stimulated human macrophages (121). This is likely due to the random lysis of *B. bacteriovorus* upon exposure to macrophages whereby some predatory bacteria will be non-specifically targeted by the host macrophage for degradation or will randomly lyse. This lysis of the predator cell would result in a higher concentration of immunogenic fragments being presented to the macrophages, whereby the usually masked flagellins and peptidoglycan would be exposed and detected by the host phagocyte PRRs, resulting in activation and proinflammatory cytokine release (121). However, even at the peak of TNF-α induction, the cytokine levels still do not exceed those induced by the pathogens *K. pneumoniae* and *S. typhimurium* and so *B. bacteriovorus* can still be considered to be minimally immunogenic when exposed to human macrophages (121), suggesting it may be suitable for the treatment of bacterial infection within human hosts.

2.3.1.2 Immune sensing of infection

2.3.1.2.1 Overview

The host immune system typically processes and detects whole antigens upon the surface of living cells. Some antigenic material may be withheld from detection by the immune system due to it being masked by other components e.g. the peptidoglycan of Gram-negative organisms is masked by the outer membrane and LPS and so is not detected by host phagocyte PRRs. Additionally, bacterial DNA is withheld within the cell and so remains undetected by host phagocytes. There is also a slowing of the immune response due to a lower frequency of antigenic material being present i.e. one cell compared to multiple fragments. Other immunostimulatory antigens e.g. flagellin are not usually masked in bacteria, or they have a typical LPS

structure that is immunogenic, and so some proinflammatory cytokine induction occurs (Figure 5: top, right) (123). However, upon bacterial entry into the host, random lysis of the infectious organism may occur, leading to PAMP detection by host PRRs e.g. peptidoglycan detection by TLR-2 and bacterial DNA detection by TLR-9. Cell lysis and PAMP detection accelerates the innate immune response in response to antigenic stimulation, leading to phagocytosis and digestion of pathogens and antigen presentation, amplifying the proinflammatory cytokine and innate immune response. This targeted antigen presentation amplifies the immune system, whilst the presence of a higher concentration of immunogenic fragments, due to cell degradation and lysis, results in the further acceleration and tailoring of the innate immune response.

It therefore follows that alternative means of cell lysis and degradation would also amplify the immune response due to the increased availability and presence of immunogenic material. One means of degradation and subsequent stimulation has been seen through the use of bactericidal antibiotics, whereby antibiotics kill the bacterial cell but frequently also trigger cell lysis and so simultaneously stimulate the immune response towards the pathogen, leading to a higher concentration of antigenic material being exposed to the immune system (compared to a living, whole cell), and a heightening of the immune response. Cell lysis and immune system acceleration is not seen to such an extent with bacteriostatic antibiotic treatment, where they inhibit the growth of the pathogen without lysing the cell and so bacteriostatic antibiotic treatment does not stimulate the immune system further than the presence of the infectious organism alone. Predation of Gram-negative pathogens by *B. bacteriovorus* enhances the lytic, immunogenic response seen with bactericidal antibiotic treatment and as mentioned previously (section 2.3), work from our lab has demonstrated that the combination of predation by *B. bacteriovorus* and the actions of host immune leukocytes combines to give maximal pathogen clearance (120).

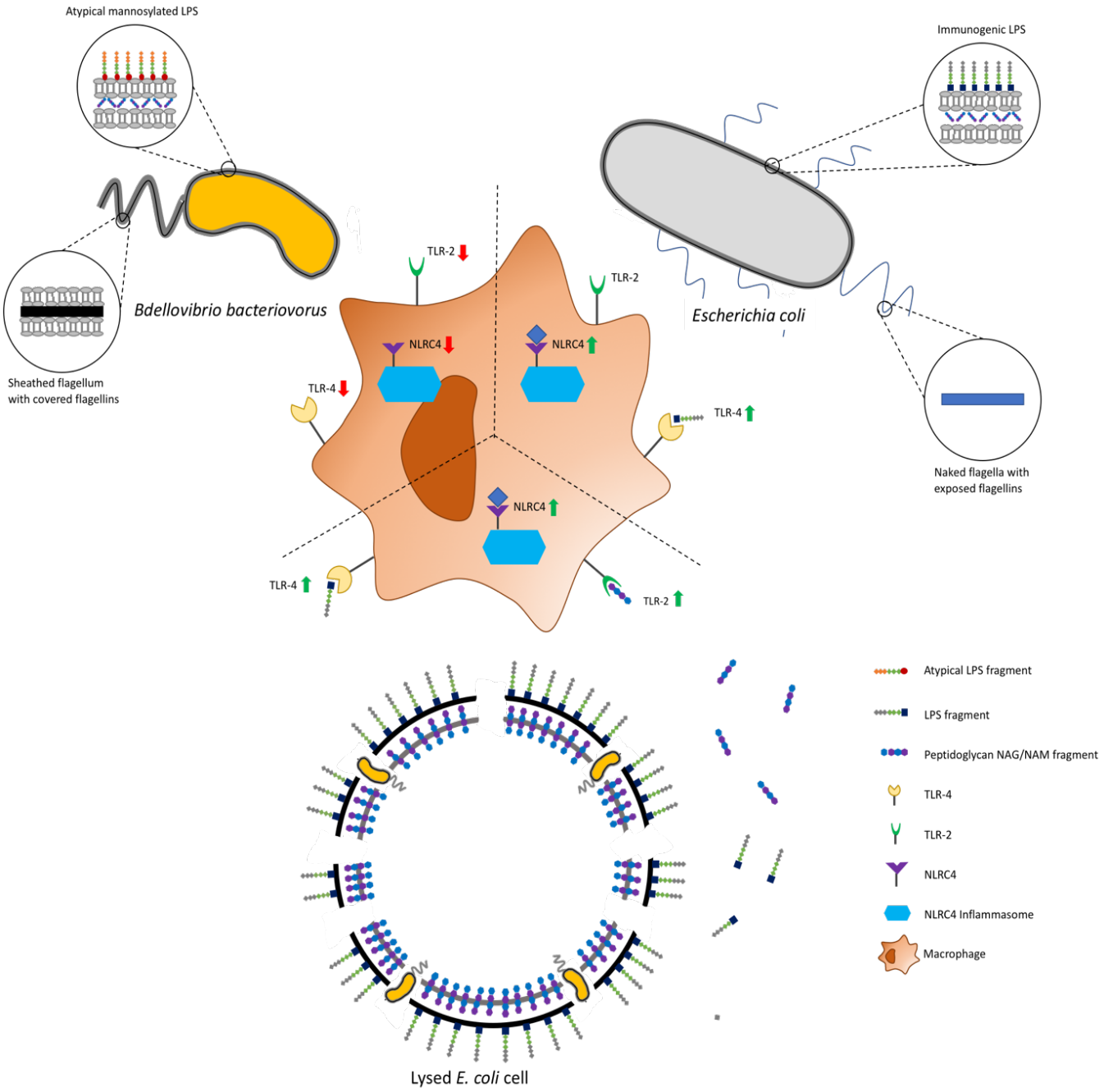


Figure 5: Summary of predicted immunogenicity of *Bdellovibrio bacteriovorus* and a typical pathogenic bacterium *Escherichia coli* pre- and post-predation.
Top left: *B. bacteriovorus* is minimally immunogenic to host macrophages due to an atypical mannosylated LPS structure, masked peptidoglycan and a membrane sheathed flagellum, minimising detection by the host macrophage pathogen recognition receptors TLR-2, TLR-4 and NLRC4. Top, right: *E. coli* is mildly immunogenic due to LPS and flagellin being immunostimulatory to host macrophages, resulting in activation of host PRRs TLR-2, TLR-4 and NLRC4. Lower, middle: Upon *E. coli* cell lysis post- *B. bacteriovorus* predation, antigenic microbial ligands such as LPS, peptidoglycan and flagellin are present in higher concentrations causing greater levels of activation of host PRRs, resulting in further activation of the host innate immune system. LPS, lipopolysaccharide; TLR, Toll-like receptor; NLR, Nod-like receptor; NAG, N-acetyl glucosamine; NAM, N-acetyl muramic acid.

2.3.1.2.2 Immune response to *B. bacteriovorus* predation

With predation of pathogenic bacteria by *B. bacteriovorus*, cell lysis and increased presentation of antigenic material to the immune system occurs. The lysis of prey cells leads to the revealing and presentation of broken cellular fragments from prey, including those containing peptidoglycan which is usually masked from host immune detection by the infectious bacterium. Broken fragments of LPS are also present, resulting in the increased presentation of these antigens in a higher than normal concentration to the host immune system (Figure 5: lower, middle). Cell lysis and fragmentation of the carbohydrates on the surface of the pathogen cell results in the formation of small vesicles studded with a diverse array of O-antigens, surface antigens and peptidoglycan-derived carbohydrates from the infectious bacterium, which are then detected by the host resulting in a higher degree of immune stimulation.

However, *B. bacteriovorus* also limits immune stimulation through its use of some of the prey cell components in its own growth. The DNA of the pathogenic prey cell is consumed by *B. bacteriovorus* during predatory growth, whilst RNA and proteins are degraded and the monomers repolymerised into components that are required for predator growth, whereby they are incorporated into the growing *B. bacteriovorus* cell (144,145).

This contrasts to the actions of bacteriophage on bacterial cells (as mentioned in 1.2.4.4), whereby they hijack host cell machinery during their lytic growth cycle to synthesise their genome and capsid proteins (146). After attachment, bacteriophage inject their DNA into the prey bacterium and redirect the host cell machinery to stimulate genome replication of the phage DNA, whilst blocking prey RNA translation and slowly degrading the prey DNA (146,147). Capsid proteins and further bacteriophage DNA are then synthesised, where the individual components then assemble into phage progeny (146,147). Upon the induction of lysis of the bacterial cell by the bacteriophage, bacteriophage progeny are released completing the replication cycle (146). However, prey bacterium DNA and components thereof are also released, resulting in a wasteful and immunogenic process

(compared with *B. bacteriovorus* predation where the majority of host DNA and components are used and incorporated into *B. bacteriovorus* progeny making it comparably more immunologically silent). This also heavily contrasts to the actions of bactericidal antibiotics, which lyse bacterial cells and expel immunogenic material such as bacterial DNA, RNA, LPS, peptidoglycan and other bacterial components, all of which trigger various host pattern recognition receptors and induce a strong proinflammatory immune response.

Predation of pathogenic bacterium may be considered as an awakening stimulus to the host immune response (120,134). Previously, the whole, living, pathogenic bacterium may have evaded detection by the host immune response or resisted engulfment and antigen presentation. *B. bacteriovorus* kills and degrades the pathogenic bacteria cells, resulting in a higher concentration of immunogenic antigens being shown to the host immune response where they can be detected, activating the host PRRs and inducing the signalling cascades that result in increased phagocyte recruitment, phagocytosis, proinflammatory cytokine production and pathogen killing, without overloading the host immune response and causing extensive inflammation and harm (120,134).

2.4 Research aims

To develop successful antimicrobial therapies, host immunity and novel and chemical antimicrobial approaches must combine for a successful anti-infective outcome. For this to happen it is imperative that we understand the interactions that occur between current and novel antimicrobial therapies and the host immune system.

In this thesis, I explore the results obtained from two rotation projects undertaken as a joint endeavour between the University of Birmingham and the University of Nottingham.

In my first rotation project, undertaken at the University of Birmingham, I used a combination of tissue culture and molecular biology to probe the

interactions that occur between fungi and the host immune system, and how vancomycin treatment may alter this interaction and perturb the antifungal immune response.

In my second rotation project, undertaken at the University of Nottingham, I focused on one potential novel antimicrobial therapy of the future, the use of *Bdellovibrio bacteriovorus* as a “living antibiotic”. It is vital that we understand the intricate mechanisms that underly the predation process before we can consider using *B. bacteriovorus*, or any components of its predatory lifecycle, as antimicrobial therapies. Through the use of molecular cloning techniques, I investigated the role of three hypothetical proteins predicted to be contained within a vesicle associated with predation, to elucidate on the complex biological killing of one bacterium by another.

Taken together, these projects provide insight into how cell-cell interactions can be studied to inform the development of new avenues to combat infection, highlighting the need for increased consideration and a higher depth of understanding when administering current antibiotic therapies and when considering novel antimicrobial therapies in the future. This research is relevant to the future development of *B. bacteriovorus* as a novel antimicrobial treatment and also the best use of current antimicrobial therapies to achieve the most successful infection outcome.

I will now introduce each project in turn and will explain my findings further in chapters 2 and 3.



UNIVERSITY OF BIRMINGHAM

CHAPTER TWO: Characterisation of the immunomodulatory effects of vancomycin in *Candida albicans* infection

University of Birmingham rotation project

Supervised by Dr Rebecca Drummond

Abstract

Fungal infections are associated with a high mortality, making the increase in fungal disease prevalence a significant challenge for infectious disease treatment and a major threat to human health. Long-term courses of antibiotics are regularly administered to immunocompromised individuals. However, certain antibiotics have been demonstrated to be a collaterally-damaging risk factor for the development of fungal infections, whereby they have been shown to perturb mucosal and systemic antifungal immunity, through their localised effects on host phagocytes, and wider effects on global immune regulation. The underlying mechanisms behind such antibiotic-mediated predisposition to fungal infection are yet to be found.

In this project, a combination of tissue culture and molecular techniques were used to investigate the mechanisms of vancomycin-mediated susceptibility to *C. albicans* infection. Macrophages were found to have a decreased ability to kill *C. albicans* when treated with vancomycin. This was attributed to a decrease in mitochondrial membrane potential, which forms a key component of the TCA cycle and immune response, alongside decreased expression of the pattern recognition receptors Dectin-1 and Complement receptor 3 and decreased proinflammatory IL-1β cytokine secretion. These data suggest that a localised immune defect is being induced in macrophages by vancomycin, which would result in an inability to control fungal infection in an *in-vivo* scenario resulting in systemic fungal infection and increased mortality.

Understanding the mechanisms underlying the vancomycin-induced susceptibility to candidemia will lessen the significant health burden this disease confers and will aid the wider consideration of fungal infections with regards to shaping antibiotic stewardship and clinical practise. This study helps to elucidate the wide-ranging implications of antibiotics in human disease. Further work will aim to elucidate the mechanism behind vancomycin-induced predisposition to fungal infection in the hope that these findings may then be implicated in the shaping of antibiotic stewardship and clinical practise.

1. Introduction

1.1 Overview of antibiotic treatment

1.1.1 The commensal microbiome

The human body plays host to a wide array of bacteria, fungi and other microorganisms on the skin, within the gut and on other mucosal surfaces, all of which comprises the commensal microbiome of the host. The commensals within the body persist due to a level of tolerance present within the host immune system, whereby they exist in a state where overgrowth, chronic infection and disease is prevented but persistence is ensured (Figure 6A). Upon perturbation of the microbiota, through antibiotic treatment, disease or immune suppression, a subset of this commensal microbiome may become pathogenic and establish as opportunistic infections. One such example of this is the fungal commensal *Candida albicans,* a dimorphic fungus that persists within the gut, skin and vulvovaginal tract but can cause invasive mucosal and systemic infection (Figure 6B). The commensal microbiota plays an array of unknown functions in host metabolism and physiology, whilst also protecting the host against invasive fungal infection. Further understanding of this balance between control and tolerance, and how the immune responses are elicited and quenched within healthy individuals has important implications for the treatment of fungal disease in individuals without a functioning immune system.

1.1.2 Long-term antibiotic administration

Broad-spectrum antibiotics are prophylactically administered to several patient groups who are at risk of bacterial infection, including those in the ICU, recipients of organ transplants and patients with acquired immunodeficiency (e.g. HIV) (29). Modern healthcare has become increasingly dependent on antibiotics to allow the continued execution of surgical procedures and due to iatrogenic interventions, such as immunomodulatory treatment post-organ transplantation, increasing the proportion of immunocompromised individuals within the population. This increased dependency is threatened by the emergence of antibacterial

resistance, making the control of even minor infections increasingly difficult. It is therefore crucial that antibiotic stewardship is improved, and antibiotics are deployed in hospitals more effectively (29). Another emerging, crucial issue with the increased use of antibiotics is the amplified predisposition to fungal infection. Antibiotics have been cited as an independent risk factor for the development of both mucosal and invasive *Candida* infections (148), and so the determination of the mechanisms that underlie the predisposition to infection require immediate research.

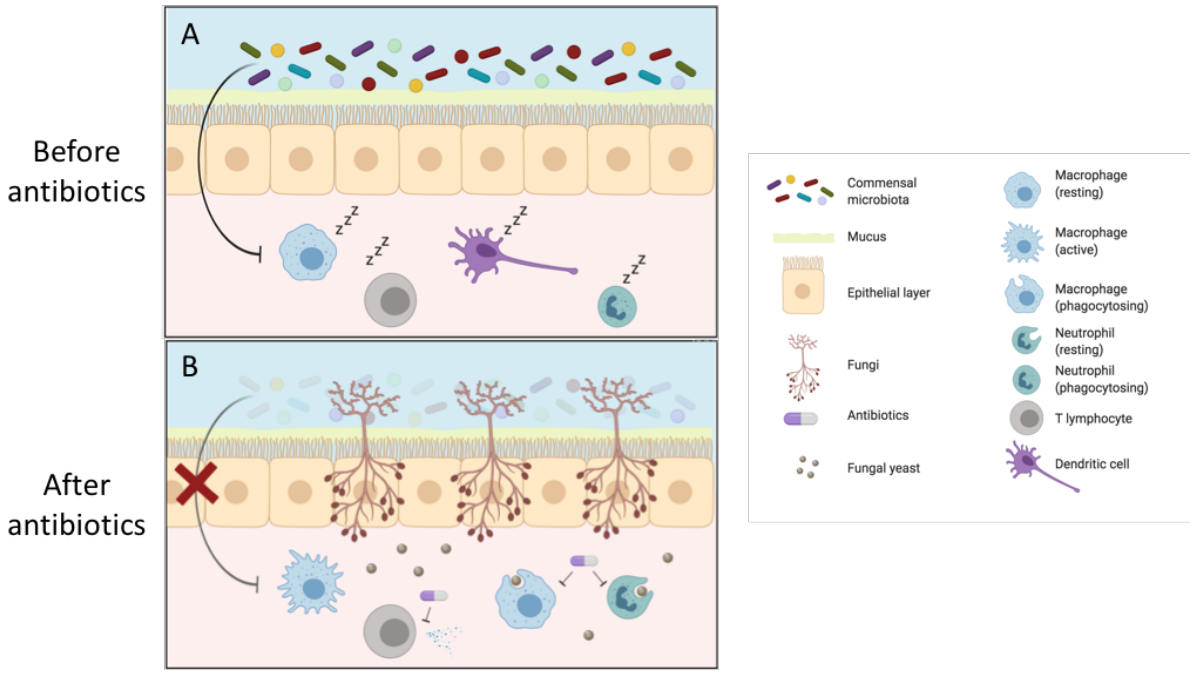


Figure 6: Antibiotic-mediated predisposition to superficial and systemic fungal infection
A: In a healthy host, the commensal microbiome exists in a state of symbiosis with the host, whereby overgrowth is prevented by the actions of the host immune system and by physical crowding and spatial limitation by the other members of the microbiota, whilst eradication is prevented by the induction of a hyporesponsive immune state, maintained by the microbiota. B: Upon antibiotic treatment, selected components of the commensal microbiome are eradicated, reversing this hyporesponsive immune state and allowing for the overgrowth of commensal fungi e.g. *C. albicans*. This results in superficial and mucosal infection on body surfaces, promoted further by inflammation caused by the overstimulated immune system in response to the remaining microbiota. Fungi may also penetrate into tissues of the gastrointestinal tract and enter the bloodstream, whereby disseminated systemic infection may occur. This is amplified by the potential effects of antibiotics on host phagocytes, where they may affect cytokine release, phagocytosis and fungal killing, hindering the control of fungal infection. Figure generated using Biorender software.

1.1.2.1 Vancomycin

One antibiotic that is regularly administered to at-risk patient groups as part of a cocktail of antimicrobial drugs is vancomycin. Vancomycin is a large, tricyclic glycopeptide molecule with bactericidal properties (Figure 7) (149,150). Its mode of action consists of it covalently and irreversibly binding to the D-Ala D-Ala peptidoglycan substrate preventing its incorporation into the bacterial cell wall and predisposing to bacterial cell lysis (Figure 1; see 1.1.2.1) (149). Due to its poor tissue penetration and its tendency to cause ototoxicity and nephrotoxicity when administered in large doses, it is used as an antibiotic of last resort against Gram-positive nosocomial infections (as it is too large to enter the Gram-negative bacterial cell) where penicillin resistance is suspected such as in MRSA infection, where penicillin intolerance within the patient is present, or when infection is life threatening such as in sepsis (149). Vancomycin also perturbs the membrane permeability of the bacterial cell, which may also contribute to its bactericidal effect (149).

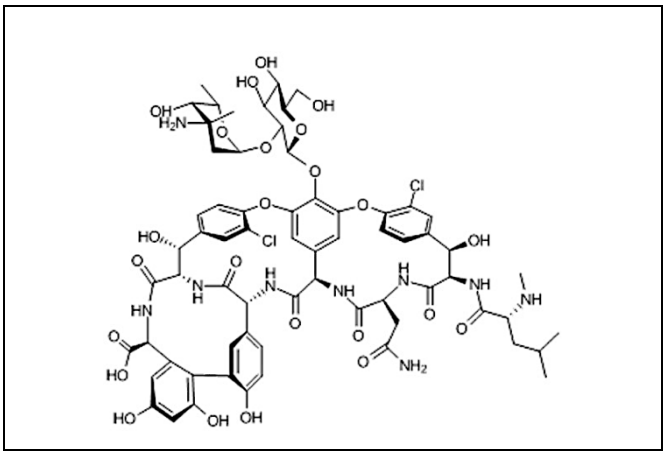


Figure 7: The chemical structure of vancomycin
Image taken from Nitanai et al, 2009

1.2 Overview of fungal infection

Fungal infections are responsible for 1.5 million life threatening infections every year, as well as causing over 1 billion superficial infections annually (151), so the need for effective antifungal treatments and vaccines and for

rapid diagnostics to allow for prompt diagnosis to combat fungal infection is essential (152). Fungal infections are typically opportunistic, whereby a localised perturbation of the immune response is required for infection to establish. They may occur due to the overgrowth of fungi within the commensal microbiome or establishment of infection by the environmental fungi that the human body is exposed to on a daily basis, if an insufficient immune response is mounted (152). Due to an increase in the proportion of the population that are immunocompromised, either through disease such as AIDS, iatrogenic interventions such as immunomodulatory therapies post-organ transplantation, or individuals within ICU wards, the burden and prevalence of fungal disease is increasing (2,152). Immunocompromised individuals are at risk of bacterial infection, which has the potential to be life-threatening, so they are treated with long term antibiotic treatment, negating the risk of most bacterial infections but still leaving the individual at risk of developing fungal infection. Emerging evidence suggests that long term antibiotic treatment may be affecting the host immune response, further predisposing to fungal infection. The mechanisms behind this alleged predisposition are currently unknown and will form the focus of this study.

1.2.1 *Candida albicans*

1.2.1.1 Overview

Candida albicans is a white, asporogenous, ascomycete yeast that is a commensal of the skin, gut and mucosal surfaces of the human body. It can undergo a dimorphic switch into a hyphal form when prompted by environmental conditions such as nutrient levels and temperature, and become pathogenic, causing opportunistic infection (Figure 8) (153–155). *C. albicans* is one of seven medically important *Candida* species but accounts for the majority of *Candida* fungal infections (153). Fungi have a thick cell wall that is the first point of contact for the host immune response. The cell wall is a highly crosslinked structure composed of an outer layer of O- linked and N-linked glycoproteins, predominantly linked to mannose sugars, and an inner layer of chitin, β (1, 3) glucan and β (1, 6) glucan which play key

structural roles in the cell (156,157). The cell wall composition varies between the different morphologies of *C. albicans*, whereby, upon the switch to hyphal growth, highly immunogenic β-glucan residues are no longer masked by the mannoprotein outer layer, accounting for the difference in immunogenicity between yeast and hyphal forms and the immune tolerance towards commensal yeast (158). In a host with a functioning immune system, the immune response would react to the hyphal *C. albicans,* controlling infection and restoring symbiosis. However, in immunocompromised individual this morphological switch signals the start of a chronic *C. albicans* infection.

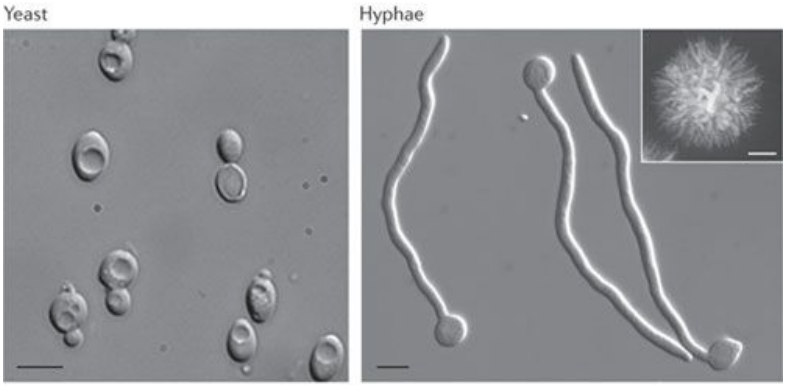


Figure 8: The opportunistic fungal pathogen *C. albicans*

Microscopy images of the two most common morphologies of the opportunistic fungal pathogen *C. albicans.* Figure is taken from Gladfelter & Sudbery, 2008.

1.2.1.2 Pathology of *C. albicans* infection

C. albicans infection can take two forms: superficial, mucosal infection that whilst not life-threatening, may have a significant health burden on individuals, or systemic, disseminated disease which is life-threatening and carries a high mortality (157).

Mucosal infection frequently occurs in individuals with localised immune system dysregulation, the two most common forms of which being

oropharyngeal candidiasis (OPC), which is common in individuals with HIV that are not on anti-retroviral treatment, and vulvovaginal candidiasis (VVC), which is common in women taking antibiotics (157). Mucosal infection usually results from localised dysbiosis of the commensal microbiota and an excessive immune response and inflammation, making it treatable with a combination of topical antifungals and immune modulatory drugs such as interferon-1 receptor antagonists for VVC which aims to mediate the excessive inflammation occurring with the vaginal tract, allowing for the re-establishment of the vaginal microbiome and the calming of dysbiosis (159,160).

Systemic candidemia frequently occur in individuals with more widespread immune defects, whereby dysbiosis leads to hyphal growth and escape of *C. albicans* into the surrounding tissues and dissemination (153). Bloodstream infections represent one of the most frequent, life-threatening and difficult to treat forms of nosocomial mycosis, affecting over 250,000 people annually resulting in the deaths of 50,000 people a year (161). 80% of candidemia infections are caused by *C. albicans* (153). There has been an increase in both the prevalence and mortality of candidemia in recent decades, due to emerging antifungal drug resistant isolates, increased levels of immunomodulation within the population and a lack of novel and rapid diagnostic tools (153). Systemic candidemia is frequently observed in individuals after surgery or chemotherapy, whereby the barrier function within the gastrointestinal tract is perturbed, allowing for the escape of *C. albicans* into the bloodstream (162). This association can be reversed through the better understanding and consideration of risk factors and the use of azole prophylaxis after surgery or treatment to minimise the risk of fungal infection, although azole resistance is on the rise within *C. albicans* and other multidrug resistant species, such as *C. glabrata,* are now increasingly causing disease (162).

An alternative form of systemic infection frequently occurs in a hospital setting, where long term use of catheters is common such as on ICU wards (125). Catheter insertion disrupts mucosal barriers within the host, disrupting mucosal immunity (16). Biofilm formation occurs on these artificial surfaces,

after contamination by *C. albicans* from the environment or from the skin, leading to direct access to the bloodstream and the establishment and dissemination of life-threatening systemic *Candida* infection. These biofilms are commonly less susceptible to antifungal drugs and immune responses, making them harder to treat (153,163). These infections are common in individuals who are immunocompromised or on long term antibiotic treatment because, upon entry into the bloodstream, *C. albicans* is not neutralised by the host immune system, suggesting that a localised or systemic immune defect may be present in individuals who contract these infections. This potential immune defect requires a great deal of further investigation, something that this study aims to address.

1.2.2 Antifungal immune response

As previously mentioned (Chapter 1, section 2.1), the immune response is comprised of the innate immune response, mediated predominantly by myeloid cells, which controls infection, and the adaptive immune response which is initiated and co-ordinated by dendritic cells and monocytes and provides further protection against infection (124). Phagocytes are the first line of response to fungal infections, whereby they kill pathogenic organisms through oxidative mechanisms such as the generation of reactive oxygen species and reactive nitrogen species, and non-oxidative mechanisms such as antimicrobial peptide secretion and neutrophil extracellular trap formation (152). Phagocytes are highly abundant on mucosal surfaces where most encounters with fungal pathogens first occur (124).

Phagocytes, and the microbial sensing and signalling mechanisms that they initiate, are known to be crucial to the antifungal immune response due to the increase in predisposition to fungal disease that occurs when deficiencies in these molecules and pathways are seen. Phagocytes, especially macrophages and neutrophils play an important role in the control and prevention of fungal infection (161). Defects in neutrophil killing and recruitment are common in immunocompromised individuals, leaving them highly susceptible to fungal infection (161). Deficiencies or polymorphisms in

pattern recognition receptors such as Dectin-1 and TLR-3 result in decreased NFκB activation and proinflammatory cytokine IFN-γ release giving an increased susceptibility to recurrent episodes of VVC and other cutaneous and mucosal infections, such as chronic skin and nail infections, but no predisposition to systemic fungal infection (164–166), probably due to the functioning of the other PRRs within the antifungal immune response.

Mutations in the IKK gene, which encodes a NFκB transcription factor activator protein, results in susceptibility to chronic mucocutaneous candidiasis due to the impairment of IFN-γ, IL-17A and IL-22 release, all of which are pivotal in the maintenance of the mucosal barrier (167).

Alternatively, deficiencies or the absence of functional signalling and adaptor proteins such as CARD9 and Syk, and in cytokines and chemokine receptors such as TNFα and CXCR1 respectively, results in predisposition to systemic fungal infection but not does affect mucosal immunity (168,169). CARD9, like other adaptor proteins, mediates the downstream signalling from multiple CLRs within the antifungal immune response (170). Deficiency leads to a predisposition to invasive candidiasis infection and spontaneous systemic fungal infection (171–173), and a defect in cytokine and CXCR1/2 ligand production, leading to decreased neutrophil recruitment and highly invasive fungal infection development within the brain (174,175). CXCR1 is highly induced in kidneys during *C. albicans* infection. Deficiencies in the chemokine receptors CXCR1 and CCR2 in humans gives increased susceptibility to disseminated candidiasis due to a defect in phagocyte recruitment, neutrophil degranulation and killing (176–178), whilst $Cxcr1^{-/-}$ mice are unable to control *C. albicans* infection due to impaired neutrophil killing (178), demonstrating the importance of cytokine mediated signalling in the regulation and sustained action of the antifungal immune response.

1.2.2.1 *Candida* specific immune response

Similar to the wider antifungal immune response, the immune response against *C. albicans* heavily relies on C-type lectin receptors to detect the fungal cell and initiate the signalling cascade to trigger an immune response (179). *C. albicans* is predominantly detected by TLR-2, TLR-4 and Dectin-1

within the host (180). β- glucans present in the inner layer of the fungal cell wall are some of the more immunogenic residues involved in *C. albicans* sensing (179). They are shielded within the yeast form, minimising immune stimulation and resulting in tolerance to *C. albicans* within the gut and other mucosal sites. Upon the switch to hyphal growth, β-glucans become unmasked due to them non longer being shielded by mannoproteins present in the outer cell wall, leading to detection by Dectin-1 and other CLRs on phagocyte cells (179,181). Dectin-1 activation promotes proinflammatory cytokine release and internalisation of the fungal cell, frequently also triggering macrophage pyroptosis (182,183), whilst amplifying the binding of TLR-2 and TLR-4 to the fungal cell (184). Binding to TLR-2 and TLR-4 then promotes further proinflammatory cytokine release (179). Other TLRs such as the intracellular TLRs TLR-3 and TLR-9 also play a role in the anti-*Candida* immune response through the detection of nucleic acids within the cell, which would signal either the invasion of a cell by the fungi, or the phagocytosis and digestion of a fungal cell, either way alerting the immune system to the presence of the microorganism (179). Chitin within the fungal cell wall is recognised by TLR-9, whilst also modulating the immune response through the activation of a NOD2 dependent mechanism (185). A multitude of other PRRs such as DC-SIGN (Dendritic cell specific ICAM3 grabbing non integrin) and galectin-3 have roles in the recognition of mannan proteins within the cell wall, inducing T helper cell responses via further signalling cascades (179). Unmasking of β-glucan during hyphal growth also leads to Dectin-1 dependent NLRP3 inflammasome activation (186), triggering caspase-1 and caspase-8 induced cleavage of pro-IL-1β and pro-IL-18 into their biologically active forms (187–190), amplifying the proinflammatory response. Deficiency in NLRP3 results in decreased caspase-1 activation and IL-1β secretion within murine macrophages, giving increased susceptibility to infection (188,189,191). Activation of the NLRC4 inflammasome also plays a role in mucosal defence through the release of proinflammatory cytokines and antimicrobial proteins (192). Detection of *C. albicans* relies on an array of different pathways. Perturbation of these pathways, or their components, may lead to predisposition to fungal infection.

1.2.3 Predisposition to fungal infection

Antibiotics have been cited as independent risk factors for the development of both mucosal and invasive candida infections (148) and have been shown to perturb mucosal and systemic antifungal immunity, two key components of the anti-*Candida* response. Antibiotics have been shown to predispose to fungal infection through the significant disruption of the microbiota, negating colonisation resistance and enhancing the likelihood of invasive infection through system wide defects in the immune response (167). *C. albicans* infection has also been associated with the administration of antibiotics to patients at risk of infection that are fitted with intravenous catheters, such as those in ICU wards. The underlying mechanisms behind this association are largely unknown, suggesting some form of antibiotic-induced defect in antifungal immunity may be present. This combines with recent studies that show that antibiotics have been shown to perturb mucosal and systemic immunity, independently of their effects on the microbiome, where they are believed to impair the action of macrophages and lymphocytes through detection impairment and mitochondrial dysfunction (193).

1.2.3.1 Disturbance of the commensal microbiome

As mentioned in 1.1.1, disturbance of the commensal microbiota by long term antibiotic treatment represents one mechanism of predisposition to fungal infection within the host, due to the negation of spatial growth inhibition. Fungal infection also occurs due to the loss of a microbiota-mediated modulation of the immune response.

1.2.3.1.1 Innate immunity

Antibiotics have also been shown to perturb innate immunity through the elimination of certain subsets of the gut microbiota causes the loss of specific metabolites, such as adenosine monophosphate (AMP) within the gut, causing immune defects via the impairment of phagocytic killing and respiratory activity within macrophages through a decreased AMP kinase

activation (194,195). This antibiotic-mediated alteration of both host-derived and microbiota-derived metabolites perturbs the correct functioning of the immune response (194), resulting in an increased predisposition to fungal infection.

1.2.3.1.2 Adaptive immunity

Bactericidal antibiotics have also been shown to shape the adaptive immune response through their effects on the host commensal microbiome. Normally, the gut exists in a hyporesponsive state whereby PRRs do not get activated by the presence of the commensal microbiome, or its ligands, avoiding overstimulation, inflammation and microbiome eradication (196). This hyporesponsive state is mediated by an increase in the proportion of regulatory T cells (T_{regs}) within the gut, and the presence of short chain fatty acid molecules (metabolites of a subset of the commensal microbiome) that increase oxidative phosphorylation within intestinal macrophages, alternatively activating them into a hyporesponsive state (196). Antibiotics cause a reversion in this hyporesponsive state by eliminating the contingent of the microbiota that produce these short chain fatty acids, resulting in hyperactivation of gut macrophages and causing them to react to the presence of any remaining commensals that have not been eradicated by antibiotic treatment, releasing large amounts of proinflammatory cytokines, shaping the Th_1 response and causing dysbiosis within the gut (196). This also leads to the suppression of the Th_2 and Th_{17} responses, both of which are important for mucosal and antifungal immunity, whereby Th_{17} cells promote the recruitment of neutrophils to the site of infection through chemokine release, namely CXCL1 and CXCL5, and stimulate antimicrobial peptide release (197), making the suppression of these responses a crucial factor in the predisposition to fungal infection (196). The importance of the Th_{17} immune response in combatting fungal infection at mucosal surfaces is demonstrated by patients with IL-17 receptor antagonist mutations, as they are more susceptible to mucosal candidiasis (198).

Contrastingly, increased T_{reg} numbers may also predispose to infection so the microbiota also plays a key role in the balancing of the immune response, allowing it to still respond to infection when needed. A subset of the microbiota has been shown to mediate Th_{17} response activation in the intestine, modulating inflammation whilst simultaneously protecting against mucosal infection (199). Vancomycin-mediated elimination of segmented filamentous bacteria (SFB) has been shown to result in a decrease in IL-17 secretion by $CD4^+$ T cells and the Th_{17} response, increasing the number of T_{reg} cells and predisposing to *Citrobacter* infection due to intestinal macrophages being in a hyporesponsive state and not responding to infection (199,200). The balance between the Th_{17} and T_{reg} responses dictates intestinal immunity, tolerance and infection susceptibility (199). The further study of the commensals and metabolites responsible for this balance will lead to an increased understanding of the underpinning principles of intestinal immunity and will better inform clinical practise and antibiotic use.

1.2.3.2 Predisposition to *Candida* infection

1.2.3.2.1 Predisposition to mucosal candidiasis

Recurrent mucosal *C. albicans* infections have been linked to antibiotic use, especially the use of β-lactams. One key example of this is VVC. *C. albicans* forms part of the commensal microbiome within the female reproductive tract, whereby its growth is limited by the other commensals present and by the actions of the host immune system (201). Commensal lactobacilli secrete exopolysaccharides that prevent *C. albicans* overgrowth by modulating growth, adhesion and morphogenesis, all of which form vital pillars of infection establishment (201). Lactobacilli also modulate the immune response to prevent dysbiosis, through the release of an aryl hydrocarbon receptor (AhR) ligand that induces IL-22 transcription, promoting barrier function and mucosal immunity regulation, and preventing inappropriate neutrophil recruitment (202). IL-22 also induces NLRC4-mediated production of the IL-1 receptor antagonist, inhibiting NLRP3 inflammasome activation and production of the proinflammatory cytokines IL-1β, IL-1α and IL-18, all of which would cause excessive inflammation (160). The eradication of

lactobacilli from the vagina by antibiotic treatment negates this, allowing for excessive and unregulated *C. albicans* growth and a decrease in IL-22 mediated barrier function, resulting in a lack of immune response modulation, NLRP3 inflammasome activation and subsequent proinflammatory cytokine release and inflammation, whilst also promoting the establishment of mucosal *C. albicans* infection (159). This is further demonstrated by exogenous IL-1 receptor antagonist treatment being protective against VVC infection, whereby neutrophil recruitment is modulated highlighting the importance of IL-22 in the prevention of dysbiosis and disease, and by IL-22 polymorphisms which subsequently predisposes to VVC in individuals (159,160).

1.2.3.2.2 Predisposition to invasive candidiasis

Antibiotics have been seen to predispose to invasive candidiasis also, especially the use of β-lactams, aminoglycosides and vancomycin (148,203). Antibiotic-mediated elimination of *Bifidobacterium* species, Clostridial Firmicutes and Bacteroidetes allows for the expansion of Proteobacteria within the gastrointestinal tract (204), whilst also negating the secretion of antimicrobial peptides and HIF1α activation by the gut microbiota (205), allowing for overgrowth of *C. albicans* and establishment of systemic, invasive infection. Isolates from patients with invasive candidemia bloodstream infections have been shown to match those present within the individuals gastrointestinal tract, supporting the idea that antibiotic-mediated intestinal dysbiosis triggers a fungal bloom resulting in overgrowth, dissemination and invasive disease (206). This is compounded by the disruption of barrier function by chemotherapy and surgery. Whilst surveillance and azole prophylaxis can prevent gastrointestinal infection. Systemic *C. albicans* infection associated with catheter use and long-term antibiotic treatment is less well understood.

1.2.3.3 Localised effects on immune cells

Bactericidal antibiotics, such as the quinolones, aminoglycosides, vancomycin and β-lactams have been shown to cause mitochondrial dysfunction and overproduction of reactive oxygen species in phagocytes within murine models via the interruption of the TCA cycle and electron transport chain, which leads to dysregulated ROS production and oxidative damage to host DNA, lipids and proteins as demonstrated through the presence of elevated oxidative stress markers, oxidative tissue damage and an upregulation of anti-oxidative defences. The exact mechanisms by which bactericidal antibiotics cause this are yet to be fully determined (149,193), but will be investigated further in this study.

1.3 Research aims

Individuals that are at a higher risk of infection, either due to disease mediated immunosuppression or long-term ICU admission, are given long term courses of antibiotics to minimise infection risk. This has been shown to give an increased predisposition to fungal infection, leading to increased mortality amongst at risk individuals. This predisposition is thought to be due to an antibiotic-mediated immune defect.

Preliminary data (Drummond et al, unpublished) shows that vancomycin, one of the antibiotics commonly administered to at risk individuals, increases mortality when administered to mice that are subsequently challenged intravenously with *C. albicans,* mimicking catheter insertion and infection. This increase in mortality is accompanied by an increase in dissemination to organs that are atypical for *C. albicans* infection and dissemination, namely the small intestine and colon, but no difference in typical dissemination sites such as the kidneys.

It was further demonstrated that a fungal killing defect was present within murine macrophages, and that mitochondrial respiration within murine macrophages was perturbed suggesting some form of immune defect was present due to vancomycin treatment. This is suggestive of a vancomycin-mediated immune defect preventing control of *C. albicans* infection within a

murine model, resulting in colonisation of the gastrointestinal tract and disruption of the mucosal barriers. This culminates in the escape of Gram-negative commensals into the bloodstream, triggering polymicrobial sepsis and mortality. This project aims to build on these preliminary findings by assessing the ability of vancomycin-treated bone marrow derived murine macrophages to kill the fungus *C. albicans and* determining the underlying mechanisms behind this apparent defect in fungal killing, namely does vancomycin perturb fungal detection, phagocytic uptake, phagosomal maturation or fungal killing.

Understanding the mechanisms of vancomycin-mediated susceptibility to *C. albicans* infection, a life-threatening fungal infection, will lessen the significant health burden this disease confers and will aid the wider consideration of fungal infections with regards to antibiotic stewardship and clinical practise. This study will elucidate the wide-ranging implications of antibiotics in human disease.

2. Material & Methods

2.1 Fungal strains

A full list of the strains used in this project can be found in Appendix: Supplementary Table 1.

2.2 Fungal strain maintenance and culture

Candida albicans SC5314 was cultured on Yeast Peptone Dextrose Agar plates (2% Peptone, 2% Glucose, 1% Yeast extract, 2% Agar (Sigma)) and grown at 25°C for 48 hours. Single colonies were then inoculated into YPD broth (same composition, minus 2% agar) and grown at 30°C for 18 hours at 200 rpm to give the liquid fungal culture used in subsequent experiments. Glycerol stocks of *C. albicans* were stored at - 80°C. *C. albicans* fungal plates were re-streaked onto YPD agar on a weekly basis.

2.3 Culture of bone marrow derived macrophages

Healthy adult mice (C57BL/6 WT) were maintained in the University of Birmingham's Biomedical Services Unit. Mice were euthanised and legs removed. Legs were then sterilised in ethanol and any excess tissue and fat removed. Tibias and femurs were then flushed with PBS (Gibco) + 0.2% EDTA (Sigma) to remove bone marrow until bones appeared white. The bone marrow solution was filtered (0.7 nm) (Greiner Bio-one) and the cells pelleted (1500 rpm, 4°C for 4 minutes) and resuspended in RPMI (Sigma) supplemented with 20% Foetal Bovine Serum (FBS) (Gibco), 40 ng/ml macrophage colony stimulating factor (mCSF) (Invitrogen) ± 20 µg/ml vancomycin (Gibco). Cells were split into T75 flasks (Corning) and grown at 37°C, 5% CO_2 for 48 hours. Media was then replenished (as before) and cells were grown for a further 3 days before use.

2.4 Harvesting of murine bone marrow derived macrophages (MBMDM's)

Flasks containing 5-day old MBMDMs were taken and adherent cells were dislodged using a cell scraper (CoStar). Cell suspensions were then centrifuged (1500 rpm, 4°C for 4 minutes) and resuspended in RPMI. Viable cells were counted using trypan blue (Gibco) and resuspended at the desired concentration (varied per experiment) in RPMI + 10% FBS ± 20 µg/ml vancomycin. MBMDM's were then considered ready for plating and experimental use.

2.5 Fungal killing

5-day old MBMDMs were plated into 96 well plates (Corning) at $1x10^4$ cells per well to give 20 technical replicates per condition, for each of 4 conditions (± vancomycin treatment, ± opsonisation) and left to adhere for 24 hours (37°C, 5% CO_2). The plate was then centrifuged (1500 rpm, 4 minutes) and the supernatant discarded. Cells were then resuspended in 50 µl PBS ± 10% FBS (non-opsonised) or 10% mouse serum (opsonised) (BioWest). A liquid culture of *C. albicans* (prepared as detailed previously) was washed and resuspended in phosphate buffered saline (PBS). Fungal cells were then counted via a haemocytometer and diluted at $2x10^6$ cells/ml. Standards beginning at $2x10^6$ cells/ml were serially diluted (1:2) in PBS + 10% FBS to give 7 concentrations and a blank (containing no fungal cells) and plated in 100 µl volume, in duplicate. *C. albicans* was then added to each infection well at $1x10^5$ cells/well to give an MOI of 10 (macrophage: fungi) in a total volume of 100 µl. Plate were then incubated for 2 hours (37°C, 5% CO_2) and subsequently washed with sterile distilled water (dH_2O) + 0.02% Triton-X (Fisher Scientific) and PBS. Alamar blue dye (Invitrogen), a dye that measures metabolic activity and can be used as a proxy for cell viability and quantification, was then added and the plate was incubated for a further 18 hours (37°C, 5% CO_2). Fluorescence was then measured at 570 nm absorption, 590 nm emission using a Flexstation plate reader. Readings were

then blank corrected and the fluorescence of each well compared to that of the $1x10^5$ cells/well standard to give the percentage fungal killing.

2.6 Macrophage viability

5-day old MBMDMs were dislodged from flasks using cell scrapers and stained with trypan blue to ascertain viability. Viability was quantified via light microscopy (10 x magnification) and viable cell counting by eye.

2.7 Macrophage differentiation

5 day old MBMDMs were dislodged from flasks and stained with Cd11b-APC Cy7 (1 µl/well)(Miltenyi Biotech), anti-CD45 BV510 (1 µl/well)(Miltenyi Biotech), F480-APC (1 µl/well)(Miltenyi Biotech) and MHC-II PE-Cy7 (1 µl/well) (Miltenyi Biotech) on ice for 10 minutes. Samples were then washed with FACS buffer (PBS + 2% FBS), to prevent non-specific binding by Fc receptors, and acquired using a BD LSRFortessa 4 machine. Cells were gated by forward scatter; forward scatter width; $Cd11b^+$; $CD45^+$ $F480^+$; MHC-II count to determine macrophage differentiation profiles, using FACSDiva acquisition software and FlowJo v10 analysis software.

2.8 Macrophage gross morphology

5-day old MBMDMs were plated in duplicate in 24-well plates (Corning) at a concentration of $1x10^5$ cells/well ± vancomycin treatment into wells containing sterile cover slips and left to adhere for 24 hours (37°C, 5% CO_2). Cover slips were then removed from each well, and placed upside down onto glass microscope slides. Macrophages were then imaged at 20 x magnification under brightfield with 3 fields of view taken per condition, and gross morphology compared by eye.

2.9 Phagocytic uptake

5-day old MBMDMs were plated in triplicate in 24-well plates at a concentration of $4x10^5$ cells/well for each of six conditions (± vancomycin treatment ± *C. albicans* infection (30 minute and 60-minute incubation)) and left to adhere for 24 hours (37°C, 5% CO_2). dTomato *C. albicans* was grown overnight (same method as previously used for WT), washed and added into each infection well at a MOI of 10 for 30 minutes or 60 minutes. Plates were put on ice for 30 minutes to aid detachment of macrophages. Macrophages were detached via gentle pipetting and moved to FACS tubes. Cells were centrifuged (1500 rpm, 4 minutes) and resuspended in PBS. Samples were stained with ZombieViolet live-dead stain (1 µl/well) (Biolegend), anti-*Candida* GFP antibody (0.5 µl/well) (Invitrogen) and anti-CD45 BV510 antibody (1 µl/well) on ice for 10 minutes. Samples were washed with FACS buffer and acquired using a BD LSRFortessa 4 machine. Cells were gated by forward scatter; forward scatter width; live ($PacBlue^+$); $CD45^+$ (BV510); GFP and dTomato status to determine whether fungi were associated attached (GFP^+ $dTomato^+$) or internalised (GFP^- $dTomato^+$), using FACSDiva acquisition software and FlowJo v10 analysis software.

2.10 RNA isolation

5-day old MBMDMs were plated in triplicate in 24-well plates at a concentration of $5x10^5$ cells/well for each of four conditions (± vancomycin ± infection) and left to adhere for 24 hours (37°C, 5% CO_2). WT *C. albicans* was grown as before and added into each infection well at a MOI of 10. Cells were incubated for 6 hours (37°C, 5% CO_2). Plates were then centrifuged (1500 rpm, 4 minutes) and the supernatants harvested and flash frozen (subsequently stored at -80°C until use). 1 ml of Trizol (Ambion) was then added to each well to lyse the cells, upon which the cell suspension was flash frozen and subsequently stored at -80°C until use.

For the isolation of RNA from lysates, lysate samples were defrosted at room temperature and mixed thoroughly. 200 µl chloroform (Alfa Aesar) was added to each sample, mixed thoroughly and then incubated at room temperature

for 5 minutes. Samples were then centrifuged (12000 x g, 15 minutes, 4°C) and the top layer (containing RNA) removed. Sample preparation was subsequently carried out as detailed in the RNeasy mini handbook (Qiagen) (step 4 onwards) using a Qiagen RNeasy RNA extraction kit and stored at -20°C until use.

2.11 cDNA generation

cDNA was generated from RNA samples using PowerUp SYBR Green Master mix (Thermofisher). Briefly, 10 µl of 2x Master Mix was added to 1 µl reverse transcriptase (20x) and 9 µl RNA and cycled using the programme suggested by Thermofisher (incubation at 37°C for 1 hour followed by inactivation at 95°C for 5 minutes) to yield 20 µl of cDNA. cDNA was stored at -20°C until used.

2.12 qPCR

The genes studied, and primers used are detailed in Appendix: Supplementary Table 2. The qPCR procedure was as follows: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of annealing at 95°C for 15 seconds and extension at 60°C for 1 minute. ΔCt values were then calculated ($Ct_{target} - Ct_{GapdH}$, and relative expression calculated using the 2^{-Ct} method.

2.13 Mitochondrial staining assay

5-day old MBMDMs were plated in duplicate in 12-well plates (Corning) at a concentration of $1x10^5$ cells/well for each of four conditions (± vancomycin ± infection) into wells containing sterile cover slips and left to adhere for 24 hours (37°C, 5% CO_2). WT *C. albicans* was grown overnight as before. *C. albicans* was added into each infection well at a MOI of 2 (macrophage: fungi) and incubated for 2 hours (37°C, 5% CO_2). Wells were then stained with Mitospy Orange (0.05 µl/well) (BioLegend) for 30 minutes at 37°C.

Cover slips were then removed from each well, and placed upside down onto glass microscope slides. Macrophages were then imaged at 40 x magnification under brightfield and PE channels (exposure: 2 s; excitation: 496 nm; emission: 578 nm) with 3 fields of view taken per condition using a Leica DM6000 Fluorescent microscope. ImageJ software was then used to quantify MFI per μm^2 for 5 macrophages per field of view.

2.14 IL-1β ELISA

IL-1β sandwich ELISA was carried out via the protocol detailed in the DuoSet kit (R+D Systems) using the supernatants harvested previously.

2.15 Statistical analysis

A D'Agostino-Pearson test for normality was carried out on data sets from each experiment which indicated that the data did not fit the assumptions made for parametric testing i.e. the data did not conform to a Gaussian distribution. Due to this, the data was analysed using non-parametric tests. Statistical analysis was carried out using GraphPad Prism 7.03 analysis tool package. Significance was obtained via Mann-Whitney tests. A confidence level of 95% was applied to all statistical tests. N.S. indicates non-significant findings, * indicates $P<0.05$, ** indicates $P<0.01$, *** indicates $P<0.001$, **** indicates $P<0.0001$. n indicates the number of biological replicates performed for each experiment.

2.16 Ethics statement

All experiments conformed to ethical approval obtained by Dr Rebecca Drummond, University of Birmingham, under home office personal project license number PBE275C33.

3. Results

Fitting in with my interests in immune-antimicrobial interplay, I began my first rotation by studying the relationship between *Candida albicans* infected macrophages and antibiotic treatment.

3.1 Vancomycin negatively affected fungal killing by macrophages

Preliminary data (Appendix: Supplementary Figure 1) showed that vancomycin treated MBMDMs had a reduced capacity to kill *C. albicans* fungi, therefore we first wanted to re-confirm whether vancomycin affected macrophage fungal killing capacity. To determine whether vancomycin affects the ability of macrophages to kill *C. albicans,* MBMDMs (± vancomycin treatment ± opsonisation) were incubated with *C. albicans* and the percentage of fungi killed assessed by comparison to standards of known fungal cell number. Treatment of macrophages with 20 μg/ml of vancomycin decreased the killing of *C. albicans* in both opsonised and un-opsonised conditions (n=2, $P<0.0001$ & $P<0.001$ respectively) (Figure 9). Taken together, our data shows that vancomycin negatively affects fungal killing by macrophages. This may be due to an alteration of macrophage viability and differentiation, a defect in fungal cell recognition, a defect in phagocytic uptake or maturation, or a multitude of other mechanisms and thus required further investigation.

3.2 Vancomycin did not affect macrophage viability, differentiation and gross morphology

One explanation for the reduced fungal killing seen with vancomycin treatment may be that vancomycin is detrimentally affecting naïve macrophage development. We therefore wanted to see whether vancomycin affected macrophage viability, differentiation and gross morphology. To determine whether vancomycin treatment affected macrophage viability, the number of viable macrophages for each of 8 rounds of differentiation was measured via staining with the cell viability stain trypan blue, whereby viable cells extrude the dye and are not stained, and counted via light microscopy.

Macrophage viability was not affected by vancomycin treatment (n=8, P< 0.05) (Figure 10A). To determine whether vancomycin affected the gross

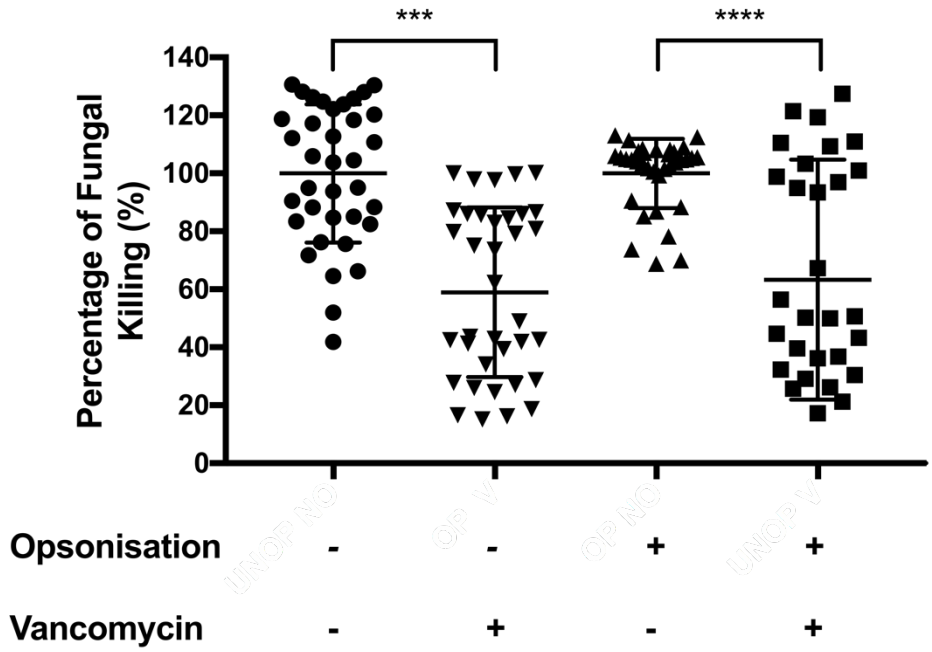


Figure 9: Vancomycin treatment decreased *C. albicans* killing by murine macrophages Treatment of murine bone marrow derived macrophages with vancomycin decreased their ability to kill *C. albicans,* independently of opsonisation, compared to untreated macrophages (n=2, P<0.0001 & P<0.001 respectively). *C. albicans* was incubated with bone marrow derived murine macrophages treated with 20 µg/ml of vancomycin (± opsonisation) and the percentage of fungal killing assessed after 2 hours using the metabolism dye alamar blue, by comparison with standards of known fungal cell count. n=2 where n represents the number of independent biological replicates obtained for this experiment. Data was analysed via Mann-Whitney U-tests due to its non-parametric nature. *** represents significance at P<0.001, **** represents significance at P<0.0001. Error bars represent the standard deviation of the data set. Data has been normalised to non-opsonised and opsonised untreated values (set at 100%) to account for variation in killing between biological repeats.

morphology of the macrophages, as the presence of blebs or other structural anomalies may indicate cell stress or apoptotic responses, macrophages were imaged via brightfield microscopy (20 x objective) and compared by eye. Vancomycin treatment did not affect the gross morphology of the macrophages (n=1) (Figure 10B). Finally, to determine whether vancomycin affected the differentiation of bone marrow progenitor cells into macrophages, the differentiation profiles of the macrophages were probed via flow cytometry, characterising them by their expression of Cd11b, a macrophage and microglial marker, CD45, a leukocyte marker, F480, a

murine macrophage marker, and MHC-II count, a marker of macrophage activation. Cells were gated by forward scatter; forward scatter width; $Cd11b^{+}$; $CD45^{+}F480^{+}$; MHC-II count. Macrophage differentiation was not affected by vancomycin treatment ($n=1$, $P<0.05$) (Figure 10C). Taken together, our data shows that vancomycin does not affect the viability, differentiation profile or gross morphology of macrophages, and thus the defect in fungal killing seen is not due to developmental differences.

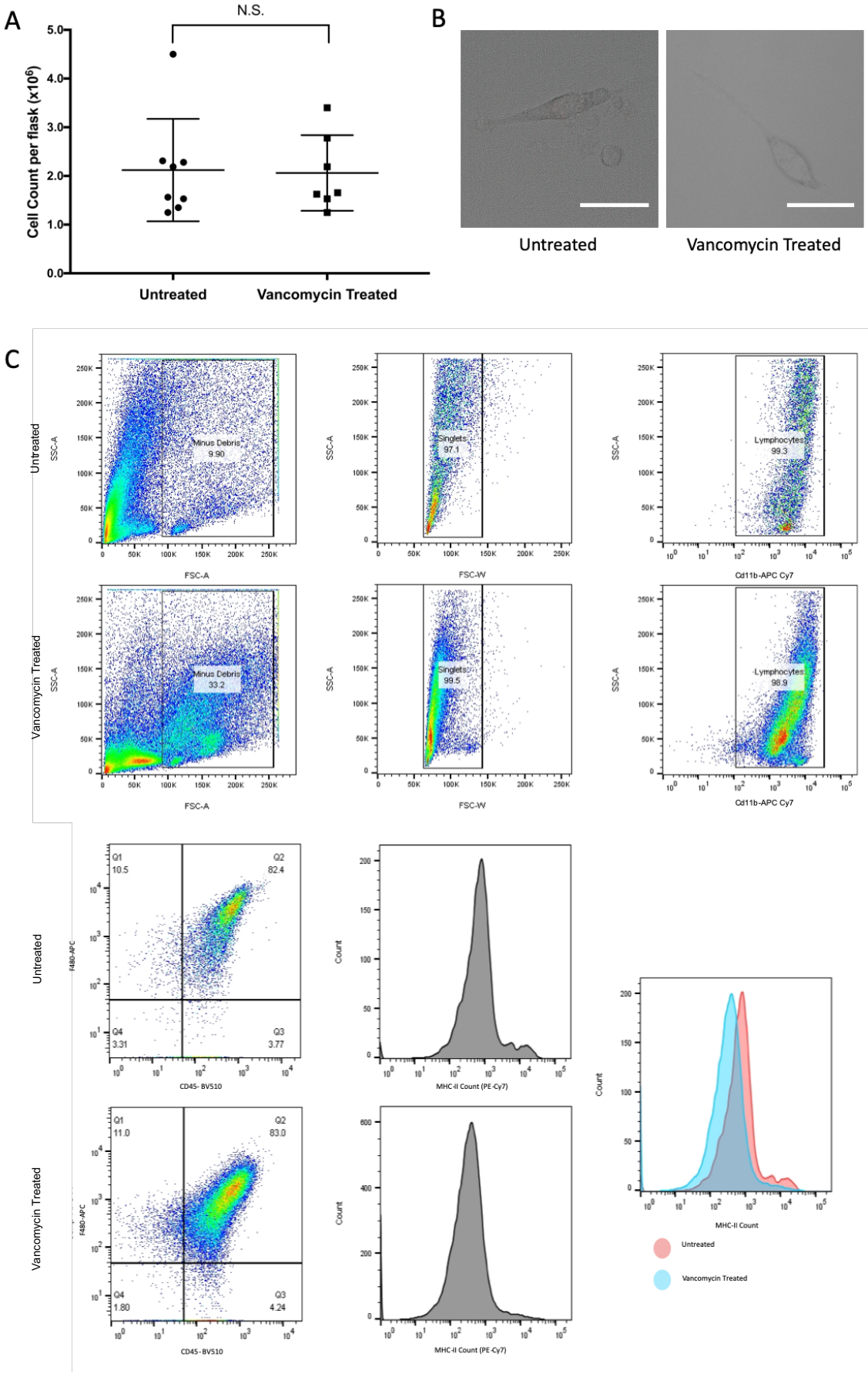

A
N.S.
Cell Count per flask (x10^6)
5.0
4.0
3.0
2.0
1.0
0.0
Untreated
Vancomycin Treated
B
Untreated
Vancomycin Treated
C
Untreated
Vancomycin Treated
SSC-A
FSC-A
Minus Debris
9.90
Minus Debris
33.2
FSC-W
Singlets
97.1
Singlets
99.5
Lymphocytes
99.3
Lymphocytes
98.9
Cd11b-APC Cy7
Q1
10.5
Q2
82.4
Q4
3.31
Q3
3.77
Q1
11.0
Q2
83.0
Q4
1.80
Q3
4.24
F480-APC
CD45- BV510
Count
MHC-II Count (PE-Cy7)
MHC-II Count
Untreated
Vancomycin Treated

Figure 10: Vancomycin did not affect macrophage viability, differentiation or gross morphology Treatment of bone marrow derived progenitor cells with vancomycin throughout differentiation into macrophages did not affect the viability, differentiation rate, maturation or gross morphology of macrophages. Bone marrow progenitor cells were treated with 20 µg/ml of vancomycin after harvesting and throughout their differentiation into macrophages. A: The number of viable macrophages per treatment group was quantified via trypan blue viability staining and manual counting. B: The gross morphology of macrophages from both treatment groups was compared via microscopy (brightfield, 20x objective) for 3 fields of view. Representative images shown. C: The differentiation and maturation of macrophages from both groups was determined via flow cytometry using CD11b, CD45, F480 and MHC-II as markers of macrophage differentiation and activation. Cells were gated on forward scatter; forward scatter width; $Cd11b^{+}$; $CD45^{+}F480^{+}$; MHC-II Count. A: n=8 where n represents the number of independent rounds of differentiation carried out. Data was analysed using Mann-Whitney U-tests due to the non-parametric nature of the data. N.S represents non significance at $P<0.05$. Error bars represent the standard deviation of the data set. B/C: n=1 where n represents the number of biological replicates obtained for the experiment. B: White scale bars represent 25 µM.

3.3 Vancomycin did not affect association and uptake by macrophages

Phagocytosis forms a key part of the antifungal immune response; we next wanted to test if vancomycin affected phagocytic uptake by macrophages. Defects in association and binding may account for the reduced fungal killing seen with vancomycin treatment. A defect in internalisation would indicate that vancomycin was not affecting binding but may be having an effect on the macrophage cytoskeleton or intracellular trafficking capabilities, in turn perturbing phagocytic uptake, maturation and fungal killing. To assess macrophage-fungi association and phagocytic uptake, macrophages were infected with a dTomato strain of *C. albicans,* for either 30 minutes or 60 minutes to account for both binding and internalisation and stained with an anti-*Candida* antibody. Phagocytic ability was then assessed via flow cytometry by determining whether vancomycin affected the proportion of fungal cells A: associated, B: bound and C: internalised by the macrophage. Cells were gated on forward scatter; forward scatter width; $CD45^{+}$ $dTomato^{+}$ (associated); $dTomato^{+}GFP^{+}$ (bound) or $dTomato^{+}GFP^{-}$ (internalised). Percentage of macrophages associated with fungi represents the number of macrophages ($CD45^{+}$) co-associated with *C. albicans* ($dTomato^{+}$) out of the whole macrophage population (all $CD45^{+}$ events). Percentage of macrophages bound with fungi represents the number of $dTomato^{+}GFP^{+}$ events out of the whole $CD45^{+}dTomato^{+}$ (associated) population. Percentage of macrophages with fungi internalised represents the number of

dTomato^{+}GFP^{-} events out of the whole CD45^{+}dTomato^{+} (associated) population (N.B. GFP antibody cannot enter macrophage thus GFP^{-} fungi must be internalised). Vancomycin did not affect association, binding or internalisation of *C. albicans* by macrophages (n=2, P<0.05) (Figure 11). Taken together, our data showed that vancomycin does not affect the ability of macrophages to phagocytose *C. albicans* and so a defect in phagocytic uptake is not responsible for reduced fungal killing.

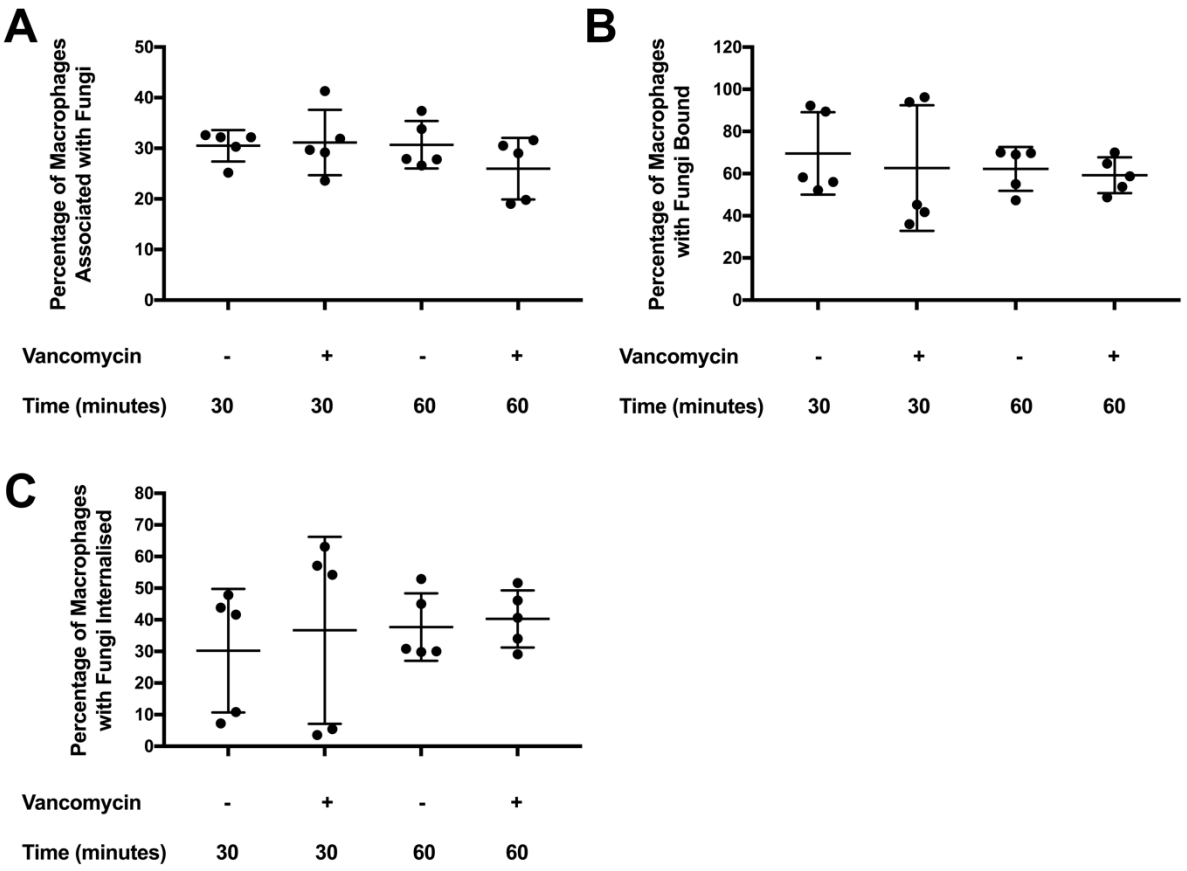


Figure 11: Vancomycin treatment did not affect macrophage-*C. albicans* association or phagocytic uptake
Vancomycin treatment did not affect the ability of macrophages to phagocytose and uptake *C. albicans* (n=2, P<0.05). dTomato *C. albicans* (fluorescent in the PE channel) was incubated with macrophages at a MOI of 10 for either 30 minutes or 60 minutes and the proportion of events (phagocytes) positive for dTomato, CD45 and GFP markers indicating that fungi were either A: associated (CD45^{+}dTomato^{+}), B: bound (dTomato^{+}GFP^{+}) or C: internalised (dTomato^{+}GFP^{-}) by the macrophages was determined by flow cytometry. An example gating strategy can be found in Appendix: Supplementary Figure 2. Data points represent technical replicates for two independent experiments. n=2 where n represents the number of independent biological replicates obtained for this experiment. Data was analysed via Mann-Whitney U-tests due to its non-parametric nature. N.S. represents non-significance at P<0.05, Error bars represent the standard deviation of the data set.

3.4 Vancomycin affected pattern recognition receptor expression on macrophages

Vancomycin did not affect phagocytic uptake, but its effect on other aspects of phagocytosis were unknown, namely: Does vancomycin affect pattern recognition receptor expression and pathogen detection, phagosome maturation and cytokine expression post-phagocytosis? To determine whether vancomycin affected fungal detection by the macrophage, RNA was isolated from vancomycin treated macrophages, both in the presence and absence of *C. albicans* infection, from which cDNA was generated and probed for the expression of a series of pattern recognition receptors and detection markers via qPCR (standardised to the housekeeper gene GapdH). The markers investigated were as follows: ClqA (complement component chain A) which forms part of the serum complement mediated system of pathogenic detection, CR3 (complement receptor 3) which detects non-opsonised β-glucan and can trigger phagocytosis and oxidative killing, Dectin-1 (Clec7a) which recognises β-glucan and initiates a signalling cascade, MINCLE (Clec4e/macrophage inducible calcium dependent lectin receptor) which recognises α-mannan and Mrc1 (mannose receptor) which detects mannose within the fungal cell wall. Perturbation of these PRRs would result in the absence of detection and signalling response to fungal cell presence, giving a dysregulated signalling response and inadequate immune activation. Our data showed that vancomycin did not affect the expression of ClqA, MINCLE and Mrc1, in the presence and absence of infection ($n=2$, $P<0.05$), but did result in significant downregulation of CR3 in an infection scenario ($n=2$, $P<0.05$) and downregulation of Dectin-1 during infection, although not significantly ($n=2$, $P=0.0513$) (Figure 12A-E).

Previous experiments (Figure 11) suggested that vancomycin does not cause a defect in fungal binding and phagocytic uptake. The expression of Msr1 (macrophage scavenger receptor) which plays a role in the endocytosis of microorganisms and their pathogenic ligands was also assessed to confirm that no defect in fungal uptake was present. A defect in fungal uptake would result in reduced fungal killing and control of infection. Our data shows that

vancomycin did not affect the expression of Msr1, independent of infection (n=2, P<0.05) (Figure 12F), in line with our previous findings.

Next, we wanted to test whether vancomycin affected the amplification of the immune responses, post-phagocytosis, through the modulation of cytokine release. To determine whether cytokine expression was altered by vancomycin, the expression of IL-10, an immunomodulatory cytokine, and CXCR3, a receptor for CXCL9/10/11 chemokines which are involved in leukocyte recruitment and amplification of the immune response, were also assessed. Our data showed that vancomycin did not affect the expression of IL-10 in the presence and absence of *C. albicans* infection (n=2, P<0.05), but did significantly upregulate CXCR3 expression in the absence of infection (n=2, P<0.05) (Figure 12G & H).

One final potential explanation for the vancomycin-mediated fungal killing defect is that vancomycin may be influencing the activation state of the macrophage cells, causing a hyperresponsive state which may result in an inflammatory phenotype and premature cell apoptosis, or a hyporesponsive state resulting in an insufficient immune response towards infection and decreased fungal killing. To determine whether vancomycin alters the activation state of macrophages, either into a more inflammatory M1 phenotype or the more tolerant, immunomodulatory M2 phenotype, the expression of NOS2 (nitric oxide synthase 2), responsible for the production of cytotoxic nitric oxide reactive oxygen species, and Arg1 (arginase 1), which suppresses CD4+ T cell responses and modulates the immune response, were assessed. Our data showed that vancomycin did not affect the expression of NOS2 by macrophages, independent of infection, and thus did not alter the activation state of the cell (n=2, P<0.05) (Figure 12I & J).

Taken together, our data shows that vancomycin downregulated the expression of the PRRs Dectin-1 and CR3 by macrophages during our experimental *C. albicans* infection and upregulates the expression of the chemokine receptor CXCR3 in the absence of infection, potentially perturbing the initiation and co-ordination of the antifungal immune response. We next sought to determine the downstream effects of the reduced PRR expression

and detection capabilities of the macrophages on the antifungal immune response.

3.5 Vancomycin perturbed macrophage mitochondrial function in the absence of infection

A large proportion of macrophage function and killing ability is reliant upon the TCA cycle. The ratio between oxidative phosphorylation and glycolysis during infection is indicative of the immune status of the cell and thus by studying the membrane potential of mitochondria we can make judgements about the immune response capabilities of the cell. Preliminary data suggests that vancomycin affects macrophage respiratory activity (Drummond et al., unpublished), whilst bactericidal antibiotics have been demonstrated to affect oxidative phosphorylation and glycolysis within macrophages (149). Firstly, I sought to determine whether vancomycin affects glycolysis, through determination of the expression of three enzymes (Hexokinase 2, Enolase 1 and Pyruvate Kinase) which act at various stages of the glycolysis pathway (determined by qPCR relative to the housekeeper gene GapdH). HK2 (Hexokinase 2) catalyses the first step of glycolysis where glucose is converted to glucose-6-phosphate, whereby expression would indicate initiation of the glycolysis pathway. ENO1 (Enolase 1) and PK (Pyruvate Kinase) catalyse the penultimate and last stages of the glycolysis pathway, whereby expression would indicate the completion of glycolysis within the cell. Vancomycin did not affect the expression of HK2, ENO1 and PK within macrophages, independent of infection, suggesting that vancomycin does not affect glycolysis ($n=2$, $P<0.05$) (Figure 13).

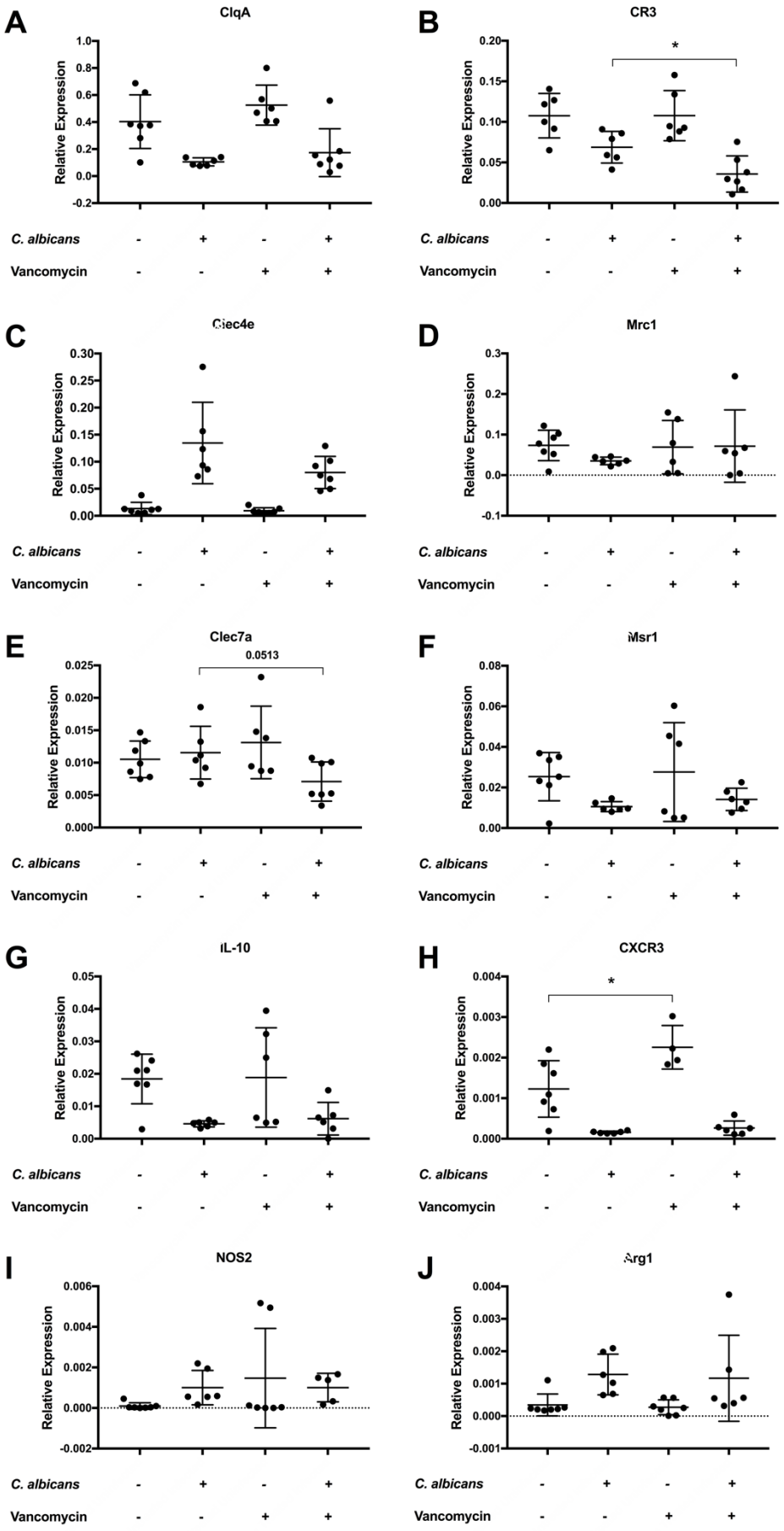

A
ClqA
Relative Expression
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
C. albicans - + - +
Vancomycin - - + +
B
CR3
*
Relative Expression
0.20
0.15
0.10
0.05
0.00
C. albicans - + - +
Vancomycin - - + +
C
Clec4e
Relative Expression
0.30
0.25
0.20
0.15
0.10
0.05
0.00
C. albicans - + - +
Vancomycin - - + +
D
Mrc1
Relative Expression
0.3
0.2
0.1
0.0
-0.1
C. albicans - + - +
Vancomycin - - + +
E
Clec7a
0.0513
Relative Expression
0.025
0.020
0.015
0.010
0.005
0.000
C. albicans - + - +
Vancomycin - - + +
F
Msr1
Relative Expression
0.08
0.06
0.04
0.02
0.00
C. albicans - + - +
Vancomycin - - + +
G
IL-10
Relative Expression
0.05
0.04
0.03
0.02
0.01
0.00
C. albicans - + - +
Vancomycin - - + +
H
CXCR3
*
Relative Expression
0.004
0.003
0.002
0.001
0.000
C. albicans - + - +
Vancomycin - - + +
I
NOS2
Relative Expression
0.006
0.004
0.002
0.000
-0.002
C. albicans - + - +
Vancomycin - - + +
J
Arg1
Relative Expression
0.004
0.003
0.002
0.001
0.000
-0.001
C. albicans - + - +
Vancomycin - - + +

Figure 12: Vancomycin altered pattern recognition receptor-mediated recognition of *C. albicans*
Vancomycin decreased macrophage expression of Dectin-1 (Clec7a) and Complement receptor 3 (CR3) in infection and increased the expression of chemokine receptor 3 (CXCR3) in the absence of infection. RNA was isolated from macrophages ± vancomycin treatment ± *C. albicans* infection, from which cDNA was generated and probed for A-E: detection markers and pattern recognition receptors, F: a phagocytic uptake receptor, G & H: cytokine/chemokine ligands and receptors and I & J: activation markers. Each data point represents a technical replicate for each of 2 independent experiments. n=2 where n represents the number of independent biological replicates obtained for this experiment. Data was analysed via Mann-Whitney U-tests due the non-parametric nature of the data. Changes in expression between treatment groups were non-significant (n=2, $P<0.05$) unless indicated otherwise. * indicates significance at $P<0.05$. Error bars represent the standard deviation of the data set.

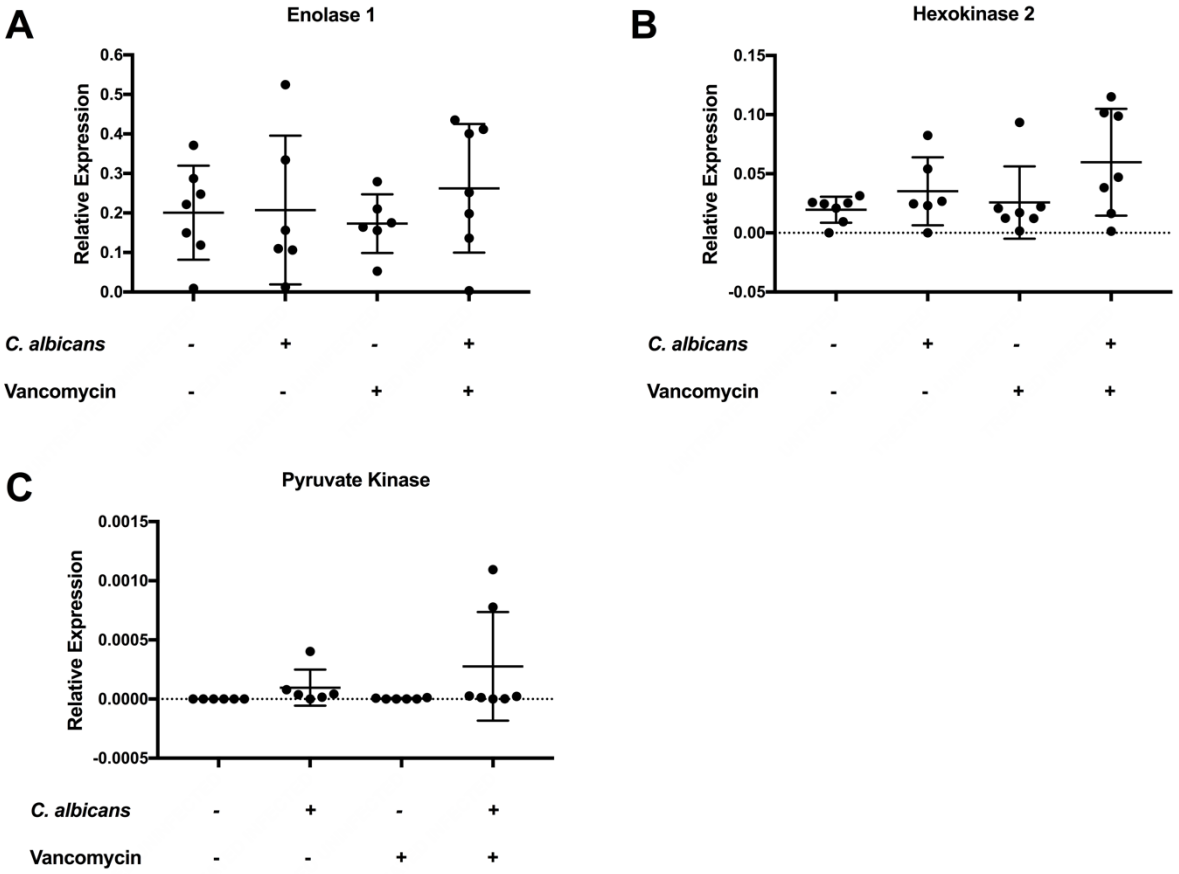


Figure 13: Vancomycin did not affect glycolysis within macrophages
The treatment of macrophages with vancomycin did not affect the expression of ENO1, HK2 or PK, three glycolytic enzymes situated at various stages in the glycolytic pathway, in both the presence and absence of infection. RNA was isolated from 10^6 murine bone marrow derived macrophages ± vancomycin ± *C. albicans* infection (MOI of 10), from which cDNA was generated. cDNA was then probed for the glycolytic enzyme genes ENO1, HK2 and PK. Each data point represents a technical replicate for each of 2 independent experiments. n=2 where n represents the number of independent biological replicates obtained for this experiment. Data was analysed via Mann-Whitney U-tests due to its non-parametric nature. Non significance (n=2, $P<0.05$) was concluded throughout unless stated otherwise. Error bars represent the standard deviation of the data set.

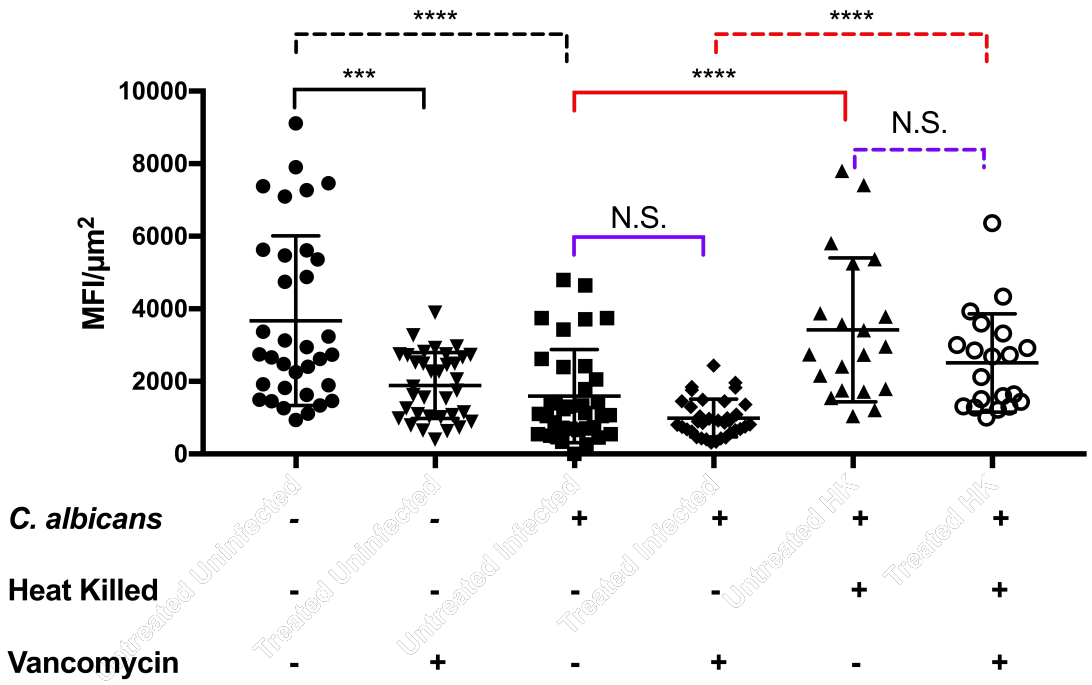
We also sought to determine whether vancomycin affected membrane potential within the macrophage, as this would indicate a shift in the ratio of oxidative phosphorylation to glycolysis, which may have a detrimental effect in the control of infection and fungal killing. To do this, membrane potential was measured by combining a mitochondrial dye MitoSpy orange (which localises to mitochondria based on membrane potential) with fluorescence microscopy and subsequent quantification of the mean fluorescence intensity (MFI). Our data showed that vancomycin downregulated the membrane potential of macrophage mitochondria in the absence of experimental *Candida* infection (n=2, $P<0.01$), whereas it did not significantly downregulate mitochondrial membrane potential during infection (although infection itself downregulates membrane potential as a shift towards glycolysis occurs) (n=2, $P<0.05$) (Figure 14). *C. albicans* infection downregulated mitochondrial membrane potential, making determination of the effect of vancomycin on macrophage mitochondrial potential during infection hard to ascertain. To test whether vancomycin downregulated mitochondrial potential during infection, macrophages were also infected with heat killed *C. albicans.* Heat killing significantly reversed the infection-mediated decrease in mitochondrial membrane potential, (n=1, $P<0.0001$), whilst vancomycin downregulated mitochondrial membrane potential during infection with heat killed *C. albicans*, although not significantly (n=1, $P<0.05$) (Figure 14). Taken together, our data indicated that vancomycin does not affect glycolysis within the macrophage but does downregulate mitochondrial membrane potential in the absence of infection, potentially indicating a shift towards glycolysis and perturbation of oxidative phosphorylation. Whether vancomycin downregulates mitochondrial membrane potential during infection remains to be determined.

3.6 Vancomycin downregulated IL-1β production

Interleukin 1β is a potent proinflammatory cytokine responsible for neutrophil chemotaxis, phagocyte activation and co-ordination of the Th_{17} antifungal response after fungal uptake and phagocytosis, resulting in the stimulation of a stronger immune response. We sought to determine whether vancomycin affects the expression of IL-1β by macrophages, potentially explaining the

reduction in fungal killing. To do this, the expression of IL-1β was quantified by ELISA in the presence and absence of infection. Vancomycin treatment decreases the production of IL-1β in the presence and absence of *C. albicans* infection (P<0.05, n=2) (Figure 15).

A



B

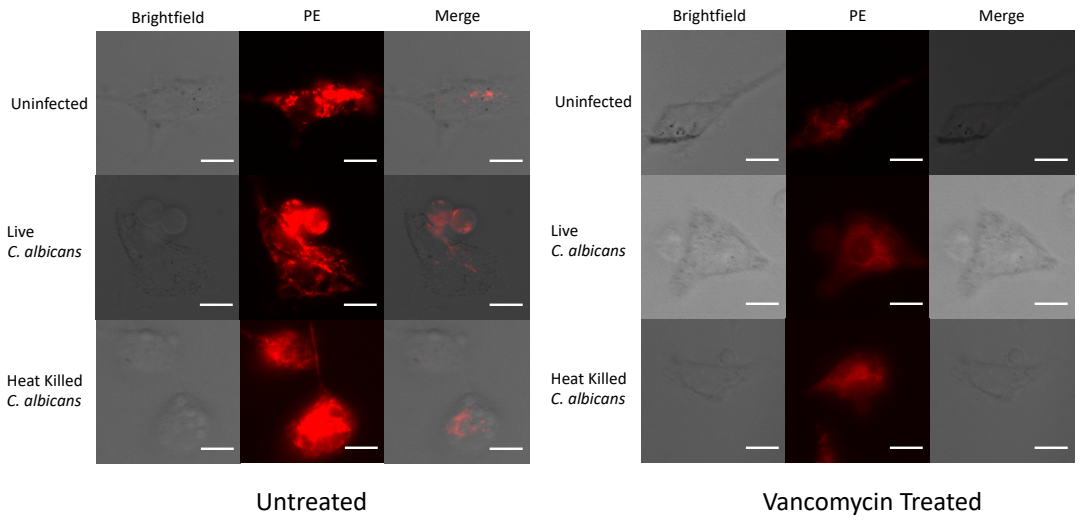


Figure 14: Vancomycin decreased mitochondrial potential in the absence of infection.
Treatment of macrophages with vancomycin decreases the membrane potential of the mitochondria in the absence of *C. albicans* infection (black, solid). Additionally, *C. albicans* infection decreases mitochondrial membrane potential (black, dashed), but heat-killing reverses this infection-mediated reduction of mitochondrial membrane potential (red solid & dashed). A: The mitochondrial potential of macrophages ± vancomycin treatment ± *C. albicans* infection (live or heat killed) was measured via microscopy (40 x objective, brightfield and PE channels) using the fluorescent dye MitoSpy Orange and the MFI per μm^2 calculated from each of 5 macrophages in 3/4 fields of view per condition. B: Representative images of macrophages ± vancomycin ± *C. albicans* (brightfield, PE and a dual merge (L-R)). Each data point represents a technical replicate for 2 independent experiment. n=2 where n represents the number of independent biological replicates obtained for this experiment (except for heat killed *C. albicans* infection which was carried out once (n=1)). Data was analysed via Mann-Whitney U-tests due to its non-parametric nature. N.S. represents non-significance at $P<0.05$, *** represents significance at $P<0.001$, **** represents significance at $P<0.0001$. A: Error bars represent the standard deviation of the data set. B: Scale bars represent 10 μM.

Of note is that the baseline IL-1β expression in the absence of infection and vancomycin was higher than expected, making IL-1β induction (the difference between non-infected and infected IL-1β levels) minimal. The reason behind this abnormally high baseline was not determined, however it may lie in the experimental procedures used to isolate and differentiate the MBMDMs stressing the cells and inducing such a response. Further repeats of this experiment would elucidate on the reasoning behind this further. Taken together, our data shows that the proinflammatory cytokine IL-1β is downregulated by vancomycin treatment, suggesting that vancomycin may perturb the cytokine response, potentially dysregulating the immune response.

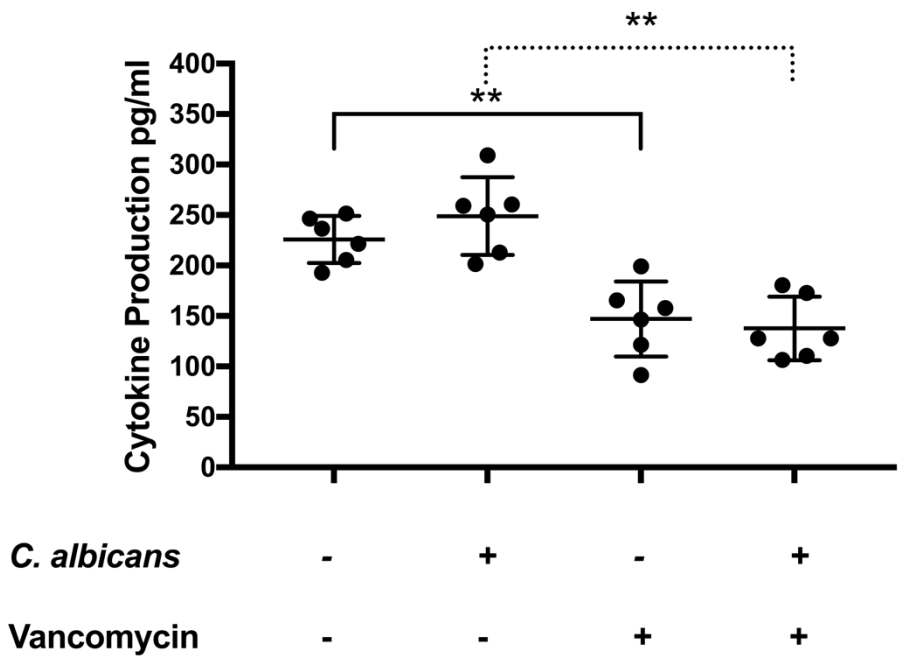


Figure 15: Vancomycin decreased IL-1β cytokine production by macrophages
Treatment of macrophages with 20 μg/ml of vancomycin decreases the production of IL-1β, a potent proinflammatory cytokine, in both the presence and absence of *C. albicans* infection. Supernatants were taken from macrophages ± vancomycin treatment ± *C. albicans* infection and IL-1β presence quantified by ELISA. Each data point represents a technical replicate for 1 biologically independent experiment (n=1). Data was analysed via Mann-Whitney U-tests due to its non-parametric nature. ** indicates significance at $P<0.01$. Error bars represent the standard deviation of the data set.

4. Discussion

4.1 Importance

Antibiotics are administered to a significant proportion of the immunocompromised population with the aim of preventing bacterial infection. However, antibiotics are a risk factor for the development of lethal fungal infections, with the underlying reasons for this remaining poorly defined. Different antibiotics have previously been shown to perturb mucosal and systemic antifungal immunity, two key components of the anti-*Candida* immune response (148,193). One antibiotic that is regularly administered to immunocompromised individuals that are at risk of infection is the glycopeptide antibiotic vancomycin. It is suggested (Drummond et al., unpublished) that glycopeptide antibiotic treatment induces a localised immune defect within host phagocytes, resulting in an insufficient immune response being mounted towards *C. albicans*, if it enters the bloodstream during catheter use, resulting in invasive candidiasis and mortality. Invasive fungal infections have a high mortality (207), and with an increasing proportion of the population becoming immunocompromised, either due to disease or immunomodulatory drug treatment, the prevalence of fungal infection is rising. This is compounded by an increased predisposition to fungal infection, mediated through the administration of long-term courses of glycopeptides and other antibiotics to already immunocompromised individuals.

Antibiotic resistance is threatening the use of antibiotics to treat even minor infections and so identification of the specific mechanisms that glycopeptides and other classes of antibiotics perturb within the antifungal immune response is needed to improve stewardship, aid the smarter clinical use of antibiotics and to combat the increasing mortality of fungal infection. Understanding the mechanisms of vancomycin-mediated susceptibility to *C. albicans* infection, a life-threatening fungal infection, will lessen the significant health burden this disease confers and will aid the wider consideration of fungal infections with regards to antibiotic stewardship and clinical practise. This study indicates a potential mechanism explaining how long-term

vancomycin treatment may be impairing the ability of macrophages to kill fungi, leading to invasive fungal infection.

In this study, we characterised the effect of vancomycin on the ability of macrophages to kill the fungus *C. albicans*. We initially determined that macrophage viability was not affected by vancomycin, so the reduced killing of *C. albicans* was not due to a lack of macrophage viability but rather a defect in macrophage function. Through a combination of flow cytometry, qPCR and microscopy, we subsequently identified a defect in Dectin-1 and Complement receptor 3 (CR3) expression, two pattern recognition receptors that are key for *C. albicans* detection. Consequently, vancomycin was shown to downregulate the membrane potential of macrophage mitochondria in the absence of infection and decrease production of the proinflammatory cytokine IL-1β.

4.2 Vancomycin negatively impacts fungal immunity

As explained previously (see Chapter 1, section 1.1.1), vancomycin is one of the antibiotics that is used as part as a long-term course of antibiotic therapy given to individuals on ICU wards, undergoing organ transplantation or immunomodulatory therapy, or those with disease-mediated immune defects. Our data show that vancomycin causes a decrease in killing of *C. albicans* by murine macrophages. This adds to our knowledge of the underlying predisposition to fungal infection seen in immunocompromised individuals undergoing long-term antibiotic treatment. This is in agreement with other studies, that show that certain classes of antibiotics (quinolones, aminoglycosides and β-lactams) have impacted mucosal and systemic immunity to fungal infection independently of their effects on the microbiome. In these studies, fungal killing was perturbed through a combination of detection impairment and mitochondrial dysfunction, resulting in the overproduction of reactive oxygen species (ROS) and oxidative stress (193). Building on this, glycopeptide antibiotic mediated elevation of ROS and oxidative stress has also been seen in rat kidney tissue, suggesting that

vancomycin may impart a similar oxidative stress on host phagocytes potentially impairing antifungal immunity in a similar way (149).

Elevation of ROS has long been documented in the bactericidal killing effect of these antibiotics (208–212), where the neutralisation of oxidative stress has been shown to negate bacterial killing (213,214), although the role of ROS in bacterial killing is debated by some (215). However, the contrary induction of elevated ROS generation in host immune cells is only recently being considered and its effect on phagocyte-mediated killing realised, therefore a link between treatment with any classes of antibiotic and defective phagocyte-mediated killing has not been widely reported.

Despite there being few reports of a direct link between antibiotic treatment and a fungal killing defect in host microglial cells, there is a general association between long term antibiotic treatment and a decrease in immunity to fungal infection. However, the mechanisms underpinning this immune defect vary due to the differing antibiotic structures, mechanisms of action and their effects on host cells.

4.2.1 Host microbiota-mediated effects

Perturbation of the gastrointestinal microbiota by glycopeptides and β-lactams has been shown to modulate adaptive fungal immunity through the downregulation of Th_{17} responses and upregulation of T_{reg} responses, leading to a hyporesponsive immune response towards pathogenic challenge (199,216). Conversely, glycopeptides, β-lactams and aminoglycosides have been demonstrated to shift hyporesponsive intestinal macrophages into a hyperresponsive state through the elimination of short chain fatty acid producing bacteria from the gut, resulting in overstimulation of the immune response by the commensal microbiota and sustained inflammation (196). Additional work is needed to determine how these varying classes of antibiotics impact the microbiota and fungal innate and adaptive immune responses on an individual basis (167) and to understand the importance of balance between hypo- and hyper-responsiveness to commensals in the gut.

4.2.2 Cell-specific effects on host phagocytes

Localised effects on host cells have also been seen, where aminoglycosides, β-lactams and quinolones have been shown to promote mitochondrial dysregulation (193) and ciprofloxacin has been shown to impair phagocytic killing and respiratory activity (194). Additionally, antibiotic treatment has been shown to perturb mucosal immunity, leading to development of mucosal infections such as VVC (157,159), and systemic immunity, predisposing to systemic infection by *Candida* and other fungal species (148,153).

The killing defect seen in this study is suggestive of a wider defect in the antifungal immune response, although at what level this defect is present is yet to be determined.

4.3 The importance of mitochondrial membrane potential

This study sought to probe further the mechanisms behind the vancomycin-mediated defect in fungal killing. Our data showed that vancomycin downregulated mitochondrial membrane potential in the absence of infection only, although downregulation of mitochondrial membrane potential by vancomycin during infection is also plausible, but is masked by the downregulation of mitochondrial potential during infection due to the switch to glycolysis (217). This downregulation of mitochondrial potential may have a wide range of implications for biological processes within the macrophage, including the functioning of the TCA cycle and oxidative phosphorylation (217).

4.3.1 Oxidative phosphorylation

Oxidative phosphorylation is reliant on the maintenance and utilisation of mitochondrial membrane potential by multiple protein complexes embedded in the cardiolipin of the mitochondrial inner membrane (218). The respiratory chain is primarily fuelled by NADH which enters into complex I (a NADH: ubiquinone dehydrogenase complex) and is oxidised by the transfer of two electrons to ubiquinone to form ubiquinol. Ubiquinol is then oxidised by a

ubiquinone cytochrome c oxidoreductase/ bc_1 complex (complex III) that donates the electrons to an oxygen molecule at complex IV (catalysed by a cytochrome oxidase) to form a single water molecule (218). The energy generated through this sequential oxidation and reduction of substrates is used to facilitate the transport of protons across the mitochondrial membrane from the mitochondrial matrix into the periplasmic space, resulting in the formation of a proton gradient (218). Protons then flow through an ATP synthase uncoupler protein (complex V) into the matrix of the mitochondria, powering the phosphorylation of ADP into ATP, resulting in ATP formation (218).

4.3.2 The TCA cycle

4.3.2.1 Overview of the TCA cycle

The TCA cycle is primarily responsible for the release of energy via the oxidation of large macromolecules to generate ATP and other small metabolites, such as succinate and NADH, which feed into the respiratory chain and fuel cellular respiration (217). Fungal killing is a highly energetically expensive process; synthesis and export of pattern recognition receptors into the phagosomal membrane, signal transduction upon PRR activation, phagocytosis, cytokine release and ROS generation all require large amounts of ATP to occur, without which antifungal killing is diminished, therefore the TCA cycle plays a key part in the antifungal immune response.

4.3.2.2 The role of the TCA cycle in innate immunity

The TCA cycle also plays other roles beyond ATP generation in immune functioning. Upon PRR stimulation, the TCA cycle stalls due to the downregulation of succinate dehydrogenase and upregulation of citrate synthase, resulting in the accumulation of succinate and citrate respectively (219,220). Usually succinate is fed back into the TCA cycle and is converted to other metabolites, or enters the respiratory chain at complex II (a succinate

dehydrogenase) and is oxidised and so does not accumulate in resting macrophages (220).

4.3.2.2.1 Succinate

Upon macrophage activation, succinate accumulates to a higher extent, whereby it enters the respiratory chain at complex II and is oxidised, reducing ubiquinone to ubiquinol, emulating the actions of complex I (221). However, instead of undergoing forward electron transfer and donating the electrons to complex III and IV, the electron transport chain is reversed and the electrons pass back through complex I and are donated to an oxygen molecule, forming ROS (222). This leads to the prioritisation of ROS generation over ATP synthesis; the high demand for ATP by host phagocytes is met through glycolysis-mediated ATP synthesis instead (222). ROS generated through this process subsequently stabilise hypoxia inducible factor 1α (HIF1α) which binds to the IL-1β promoter, enhancing the production of IL-1β, a key proinflammatory cytokine in the antifungal immune response (222,223). ROS form part of the oxidative killing mechanisms in phagocytosis that are responsible for microbial killing (124). Succinate accumulation therefore induces the production of ROS and IL-1β within the macrophage, both of which have important roles in the antifungal immune response.

4.3.2.2.2 Citrate

Citrate is utilised for phospholipid and fatty acid synthesis, contributing to membrane biogenesis and lysosome formation (220). Citrate is also used to generate NADPH, which may either enter the nitric oxide (NO) synthesis pathway where it donates an electron to L-arginine to form L-citrulline and NO (catalysed by the inducible nitric oxide synthase), or is utilised by NADPH oxidase which reduces oxygen to form ROS (220,224). In inactivated macrophages, arginine is depleted by arginase, limiting ROS generation (220).

4.4 Perturbation of mitochondrial membrane potential

The perturbation of mitochondrial membrane potential seen within this study can be attributed to three main causes: Disruption of the TCA cycle, a defect in electron transport across the mitochondrial inner membrane or the uncoupling and disruption of the mitochondrial inner membrane proton gradient. Each of these reasons will now be explored.

4.4.1 Disruption of the TCA cycle

The perturbation of mitochondrial membrane potential may be due to the disruption of the TCA cycle, whereby inhibition of the enzymes that catalyse it may have resulted in the accumulation of some substrates or metabolites, at the expense of others that may be required for oxidative phosphorylation, leading to a lack of metabolites entering the respiratory chain, e.g. NADH, decreasing mitochondrial membrane potential and ATP generation. Perturbation of mitochondrial membrane potential within the macrophage is likely to have a strong effect on its fungal killing capabilities. This is further supported by studies by Kalghatgi et al. which show that quinolones, aminoglycosides and β-lactams disrupt the TCA cycle and electron transport chain within mitochondria (mechanism not discussed), where they cause dysregulated overproduction of ROS and extensive oxidative damage within immune cells (193). Perturbation of membrane potential may in turn lead to a stalling of the TCA cycle which may result in accumulation of some metabolites at the expense of others, potentially leading to either a defect or an excess of citrate or succinate accumulation, and subsequent dysregulated production of ROS, IL-1β and other components of the innate immune response.

4.4.2 Uncoupling of the mitochondrial protein gradient

Alternatively, vancomycin may not be disrupting the TCA cycle, and may instead be uncoupling the proton gradient, resulting in decreased mitochondrial membrane potential and ATP synthesis. Mitochondrial membrane potential is crucial for TCA cycle and oxidative phosphorylation

function and for ATP synthesis. Some lipid soluble, phenol-ring-containing drugs may uncouple the proton gradient by integrating into and passing through the mitochondrial inner membrane, decreasing the proton gradient and thus the membrane potential (225). Vancomycin has multiple phenol rings within its structure, suggesting that the decrease in mitochondrial membrane potential seen within this study post-vancomycin treatment may be due to vancomycin integrating into the mitochondrial inner membrane, uncoupling the proton gradient and decreasing the membrane potential.

Vancomycin has been shown previously to induce mitochondrial dysfunction, whereby the ototoxicity and nephrotoxicity that occur during long-term vancomycin treatment has been attributed to mitochondrial dysfunction. Vancomycin was demonstrated to induce intracellular ROS production, leading to the depolarisation of the mitochondrial membrane and activation of caspase-9 and -3/7 mediated apoptosis pathways in ciliated ear and renal tubular cells (226). Complex I of the mitochondrial respiratory chain has also been implicated in the vancomycin-induced superoxide generation and proximal tubular epithelial cell apoptosis (226). This supports the idea that the induction of mitochondrial dysfunction through the perturbation of mitochondrial membrane potential, which has been shown to be highly detrimental in other host cells, is likely to occur in host phagocytes, supporting the link between reduced mitochondrial membrane potential and the reduced fungal killing seen in this study.

Vancomycin-induced ROS production within the mitochondria of renal tubular epithelial cells was later determined to induce cytochrome c-catalysed peroxidation of cardiolipin in the mitochondrial inner membrane (227). This permeabilises the mitochondrial inner membrane, impairing the respiratory chain at complex I (228) and triggering caspase-mediated cellular apoptosis (227,229–231). This ROS-induced apoptosis is reversible through the addition of FCCP (carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone), a well-established mitochondrial uncoupling molecule that dissipates the proton gradient across the mitochondrial inner membrane, uncoupling the respiratory chain from ATP synthesis and inhibiting superoxide production (227,232,233). FCCP also reversed the vancomycin-mediated depolarisation

of the mitochondrial membrane (227,232). This suggests that as vancomycin decreases mitochondrial membrane potential, but this effect is reversed by the addition of a known uncoupler molecule, that the vancomycin-mediated effects on mitochondrial membrane potential are not due to it being an uncoupler itself, and that vancomycin is perturbing either the TCA cycle, diminishing the supply of redox substrates into the respiratory chain, or disrupting respiratory chain complex formation.

4.4.3 Defective electron transport

Cell wall targeting antibiotics have previously been implicated in the downregulation of mitochondrial membrane potential and glycolysis; piperacillin, a broad spectrum β-lactam, has been shown to cause mitochondrial dysfunction within neuron cells, resulting in decreased respiratory rate, membrane potential and ATP generation and increased reactive oxygen species and superoxide radical generation and oxidative damage and stress responses (234). The effect of piperacillin on neuron cells was reversed via the administration of an antioxidant, suggesting that the decreased mitochondrial membrane potential and respiratory activity was due to excessive oxidative stress (234).

β-lactams have also been demonstrated to sterically inhibit the transport of carnitine, a long chain fatty acid that, when oxidised, contributing to electron transport and ATP generation, into the mitochondria (235). The decrease in ATP production would result in an impaired killing ability due to a lack of antigen presentation, signalling, fungal uptake and ROS generation. Other antibiotics, with a wide array of mechanisms of action, all exert similar effects on the mitochondria, where mitochondrial respiration, membrane potential and ATP generation are all decreased but ROS generation and oxidative damage are heavily upregulated, resulting in caspase activation, membrane permeabilisation and apoptosis (236–239), potentially due to peroxidation of cardiolipin within the mitochondrial inner membrane (227).

4.5 Antibiotics perturb mitochondrial protein synthesis

Conventional translation and protein-targeting antibiotics such as streptomycin, oxazolidinones, chloramphenicol and tetracycline have all been demonstrated to inhibit mitochondrial protein synthesis (240), likely through the perturbation of the TCA cycle and ATP generation within mitochondria (previously demonstrated in other studies). A large component of the antifungal immune response relies on the synthesis of pattern recognition receptors, cytokines and other proteins to function, making perturbation of protein synthesis within mitochondria highly likely to affect the antifungal immune response, and potentially explaining the lack of Dectin-1 and CR3 expression seen in this study.

The targeting of host mitochondria protein synthesis and function by other antibiotics has been well documented and utilised in the treatment of various forms of cancer (236–238,241–243). Due to this, the cytotoxic effects of antibiotics are well known, but implications on the immune system and on infectious disease treatment are only just beginning to be explored.

The decrease in expression of Dectin-1 and CR3 expression and IL-1β secretion seen in this study may also be caused by a downregulation of mitochondrial membrane potential and disruption of the TCA cycle, either due to a decrease in ATP production, whereby PRR expression and integration into the macrophage membrane and cytokine synthesis and release are energetically expensive processes, resulting in an insufficient energy supply for the continued functioning of these synthesis and expression pathways.

4.6 Complement receptor 3 and Dectin-1 downregulation

Our data suggests that vancomycin causes a defect in the expression of CR3 and Dectin-1 on the macrophage cell membrane, potentially accounting for the defect in fungal killing seen both in vitro, and within in vivo preliminary studies (Drummond et al., unpublished). CR3 and Dectin-1 are crucial in the anti-*Candida* immune response, as they detect non-opsonised β-glucan, the primary component of the fungal cell wall (179). Activation of CR3 and Dectin-1 is known to cause proinflammatory cytokine release, phagocytosis

and acceleration of the antifungal immune response, but the intricate mechanisms behind this signalling remain unknown (244). The importance of CR3 and Dectin-1 has previously been demonstrated to be required for proinflammatory cytokine release and formation of the IL-12 response within dendritic cells, both of which have been shown to be important in the control of *C. albicans, Aspergillus fumigatis* and *Histoplasma capsulatum* infections (245,246), where blockage or deficiency is highly detrimental due to increased dissemination of infection and Th_1 response impairment (245–247).

Antibiotic-mediated downregulation of Dectin-1 and CR3 expression has not been documented previously for any classes of antibiotic. Deficiencies and polymorphisms in Dectin-1, resulting in decreased expression, have been shown to cause decreased production of cytokines e.g. IL-17 and TNFα, giving increased susceptibility to recurrent VVC and other mucosal and cutaneous infections, but not systemic infections as fungal phagocytosis and killing was shown to be unaffected (165). This suggests that Dectin-1 plays a role in protecting against mucosal infection, but that other PRRs are responsible for defence against systemic infection.

However, extensive crosstalk occurs within the antifungal immune response, so it is important to not consider the effects on PRR expression in isolation. Dectin-1 stimulation alone is insufficient to cause proinflammatory cytokine release, NLRP3 inflammasome activation and IL-1β release (248). However, it has subsequently been shown that activation of Dectin-1 promotes the binding of CR3 to fungal β-glucan, whereby they are both required to stimulate proinflammatory cytokine release and inflammasome activation (247,249). The effects of the downregulation of CR3 and Dectin-1 expression seen in our study i.e. reduced fungal killing, look to emulate those that would occur in Dectin-1-CR3 deficiency.

There have been few established links between antibiotic treatment and downregulation of PRR expression. Ciprofloxacin has been shown to suppress TLR-4 expression, a key player in the anti-*Candida* response, ultimately resulting in decreased TNF-α production (250) and predisposition

to invasive *C. albicans* infection (251), suggesting a defect in phagocytosis or fungal killing is present, although this has not been investigated further.

4.7 IL-1β downregulation

IL-1β is a key proinflammatory cytokine in *C. albicans* infection, whereby it promotes neutrophil chemotaxis, activation and differentiation and IL-17 release, sculpting the formation of the Th_{17} response, which is key for antifungal immunity, especially at mucosal surfaces (252). In this study, IL-1β was shown to be downregulated by vancomycin, both in the presence and absence of infection. Through their actions on cytokine expression, antibiotics, such as ciprofloxacin and quinolones, have been demonstrated to have a disproportionately large effect on regulation of the antifungal immune response, as they acts on regulatory pathways that are common to multiple cytokines (253).

Activation of both CR3 and Dectin-1 coordinates the caspase-1 dependent activation of the NLRP3 inflammasome in response to β-glucan, and subsequent cleavage of pro-IL-1β into its active form (190,249,254,255), and so the decreased expression of CR3 and Dectin-1 may lead to the decrease in IL-1β production seen in our experiments. The perturbation of IL-1β expression due to vancomycin treatment is highly likely to be a downstream effect of the perturbation of PRR expression or mitochondrial membrane potential, but despite being a downstream effect it could still have a large effect on the control and prevention of infection and fungal killing.

4.8 CXCR3 upregulation

Another finding of this study was that vancomycin upregulated the expression of CXCR3 within macrophages. CXCR3 is a chemokine receptor for the proinflammatory chemokines CXCL9, CXCL10 and CXCL11, all of which have roles in phagocyte recruitment and chemotaxis (256). CXCR3 is expressed on a range of cell types, such as macrophages and T cells, whereby it mediates acute and chronic inflammatory phenotypes through its

effects on cell migration, activation, differentiation and effector function (256). Expression of CXCR3 is strongly upregulated during infection, induced by IFN-γ release and the Th_1 response (257), whereby it plays a key role in mucosal immunity (258). Expression of CXCR3 is highly regulated; an absence of expression, as seen in $CXCR3^{-/-}$ mice, is associated with increased dissemination of disease (259), whilst overexpression may result in mitochondrial dysfunction, accumulation of ROS, cytokine dysregulation and cell death (256). Our experiments demonstrated that vancomycin upregulated CXCR3 in the absence of infection, potentially resulting in an inflammatory phenotype and accounting for mitochondrial dysfunction and the defect in fungal killing. However, upregulation of CXCR3 by antibiotic treatment has not been observed in any other study to date.

Through the further study and understanding of the antifungal immune response pathway, and the further investigation of the exact point(s) of the antifungal pathway that vancomycin affects, will we be able to classify this interaction and chain of events for certain.

4.9 Limitations

Currently, this research is only applicable to an in vitro murine macrophage model of candidiasis, making the application of these results to clinical scenarios limited. The effect of vancomycin on other immune cell subsets and on the immune response as a whole requires further investigation. As documented above, antibiotics with differing modes of action can cause the same off-target effects within cells, whilst mechanistically similar antibiotics can have wide ranging implications for different diseases, so the findings of this study and future studies should be considered on an antibiotic-specific and disease-specific basis. A simplistic mechanism links these findings, that doesn't account for the extensive crosstalk and signalling that occurs within the antifungal immune response. The further probing of the mechanisms behind these findings is limited by a severe lack of understanding of the signalling pathways that initiate and sustain the antifungal response, due to it receiving considerably less research than other areas of the immune system.

Further proposed research that will expand upon the findings of this study and apply them further can be found in Chapter 4.

The findings of this study are summarised below (Figure 16):

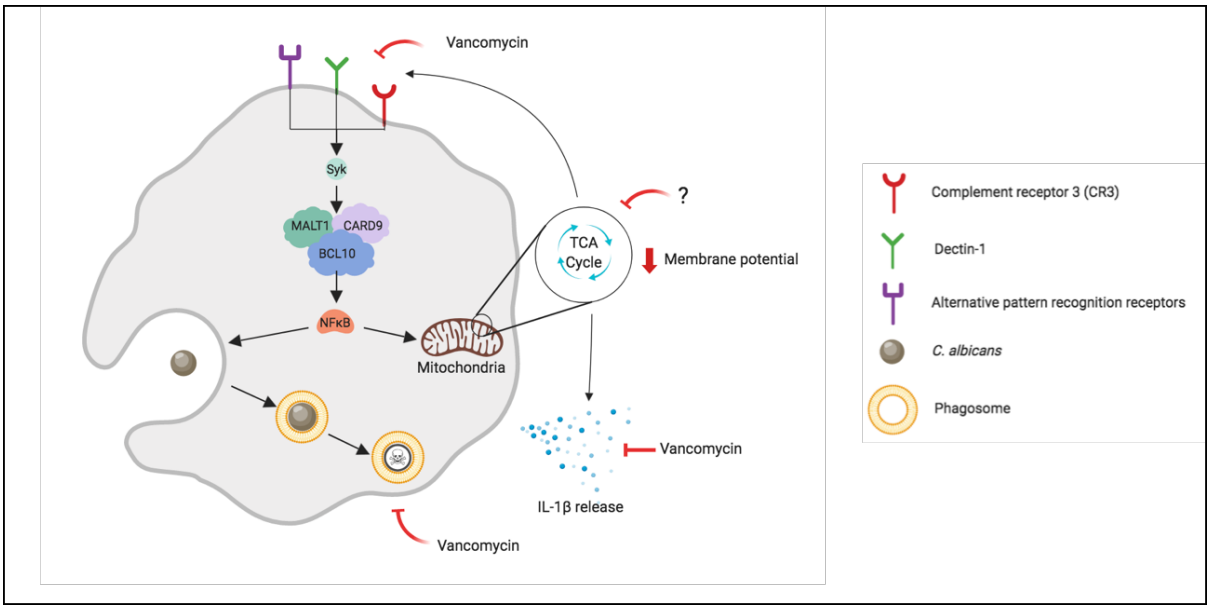


Figure 16: A mechanistic representation of how vancomycin affects *C. albicans* killing by macrophages, based on the results of this study
This study sought to determine the underlying mechanisms behind the reduced fungal killing of *C. albicans* by macrophages, when macrophage have been treated with vancomycin. This study found that vancomycin reduced the expression of Complement receptor 3 and Dectin-1 in macrophages, but phagocytic uptake was not affected (probably due to detection and induction of phagocytosis by other pattern recognition receptors). A decrease in mitochondrial membrane potential and IL-1β release was also induced by vancomycin, ultimately culminating in the reduced killing of *C. albicans* by macrophages. The order of events, and therefore whether reduced detection is responsible for the reduction in mitochondrial potential and cytokine release or vice versa is unknown, as is the effect of the reduction in mitochondrial membrane potential on TCA cycle function. Figure generated using Biorender software.

5. Concluding remarks

Vancomycin has a detrimental effect on myeloid cells within the antifungal innate immune response, leading to a defect in *C. albicans* killing, signifying a wider defect in antifungal immunity may be mediated through long-term glycopeptide antibiotic administration. The effect of glycopeptide antibiotics on the innate immune response needs prudent evaluation and is of the greatest importance, especially within immunocompromised populations who are already at increased risk of opportunistic bacterial and fungal infection and morbidity. This study explores the mechanisms underlying glycopeptide antibiotic-mediated susceptibility to lethal Candida infection, with the aim of shaping antibiotic stewardship and improving clinical practice. It found that vancomycin causes a defect in macrophage killing of the fungus *C. albicans*, and that this was likely due to a combination of the downregulation of pattern recognition receptor expression leading to altered signalling, culminating in the decrease in mitochondrial membrane potential and IL-1β expression. The treatment of infectious disease is likely to shift within the coming years due to the loss of current treatments to antimicrobial resistance. The improved usage of current antimicrobial therapies, combined with a better understanding of their effects on the immune system to minimise infection in the first instance, represent the best approaches to treating infectious disease during the coming antimicrobial resistance crisis.



CHAPTER THREE: Characterising proteins for roles in antibacterial entry and exit of Gram-negative pathogens by the predatory bacteria *Bdellovibrio bacteriovorus*

University of Nottingham rotation project

Supervised by Professor Liz Sockett

Abstract

Bdellovibrio bacteriovorus is a Gram-negative predatory bacterium that invades and replicates within the periplasm of other Gram-negative bacteria, killing them in the process. Despite decades of research, some of the underlying mechanisms behind the complex, multi-enzymatic predation process, and the cell-wall targeted enzymes that facilitate this, are still unknown. The improved understanding of the *B. bacteriovorus* predatory lifecycle will aid its potential as a treatment for antibiotic resistant pathogen-mediated disease in future, either as a “living antibiotic” or through the use of components of its predatory cycle as enzybiotics, forming a potential solution to the impending antimicrobial resistance crisis.

Previous work within the Sockett laboratory has revealed the presence of an active predatory vesicle deposited within prey during predation. However, the origin, function and components of this predatory vesicle remain unknown (and are being actively researched within the lab). In this project, bioinformatic techniques and molecular cloning were utilised alongside microscopy to fluorescently tag three of the proteins predicted by proteomics to be contained within this vesicle, with the aim of studying their distribution throughout the predatory cycle and assigning a function to them. It was discovered that Bd2439, a previously predicted cell wall anchor family protein, exhibited a dynamic localisation throughout the predatory cycle whereby it initially was distributed at both poles within attack phase *B. bacteriovorus* and subsequently was lost at one. It also co-localised with CpoB, a known vesicle component and penicillin binding protein modulator, supporting its role in incorporation into the predator cell outer membrane compartment for prey cell engagement, or a role in attachment of a vesicle within the predatory cell.

Contrastingly, Bd1815 and Bd2754, two hypothetical, unannotated proteins, were predicted to be porin-like in function, suggesting they play roles in macromolecule transport or nutrient acquisition during predation, with both exhibiting diffuse distributions within attack phase predatory cells.

Additionally, Bd2754 but not Bd1815 was found to be compartmentalised in a vesicle location being deposited on cellular debris post-predation within the predatory vesicle. These data further aid the ascription of a function to some of the components of the novel predatory vesicle. Further work will aim to further confirm the function of these proteins and apply these findings to the wider context of their role throughout predation.

1.0 Introduction

1.1 Overview

Bdellovibrio bacteriovorus is a small (0.3 μm x 1 μm), vibroid shaped, Gram-negative delta proteobacterium found in the environment within soil and water, where it swims around using a single polar flagellum, randomly colliding with and preying upon other Gram-negative bacteria (260).

The genome of *B. bacteriovorus* is disproportionately large for its predatory lifestyle (261). The genome is devoid of repeat elements, which would add a strong potential for diversity within the genome, and analysis of codon usage suggests that minimal levels of horizontal gene transfer have occurred recently (262), compared to the large amounts of horizontal gene transfer that previously occurred and heavily shaped the early evolution of *B. bacteriovorus* predation (263). This indicates that *B. bacteriovorus* has a core genome that whilst devoid of recent variation, encodes a wide array of enzymes and components vital for its highly complex predatory lifestyle (264). However, the genome of *B. bacteriovorus* is poorly annotated compared to the genomes of other bacteria, with only 55% of the predicted proteins having a homology-assigned function, making the study of these novel proteins more complex (265). It is this arsenal of hydrolytic enzymes and novel proteins that has great potential in aiding the treatment of infectious disease, owing to the need for alternative novel therapies to combat Gram-negative mediated infections.

1.2 Clinical significance

As outlined in Chapter 1, there is a desperate and urgent need to find new treatment options for infectious disease, especially those caused by the ESKAPE pathogens (where all except *S. aureus* are Gram-negative) owing to them causing a significant threat to human health (28,266), especially within a healthcare setting (267). Due to their minimally immunogenic nature (see Chapter 1: section 2.3.1.1), their ability to co-evolve alongside prey to limit resistance formation (268), the scope to modify *B. bacteriovorus* to

prevent it using nutrients from external sources such as those in wounds or serum (269) and their capability to work alongside the host immune system in the eradication of infection (see Chapter 1: section 2.3.1.1) (120), *B. bacteriovorus* represent one potential alternative therapy for the treatment of diseases caused by these organisms.

As with all strategies to combat infection, there is a need to be mindful about resistance developing to treatment. The likelihood of resistance to *B. bacteriovorus* predation forming is reduced by the ability of *B. bacteriovorus* to co-evolve alongside pathogens (268), and the targeting of several components of the prey bacterium simultaneously during predation rather than a single component. Studies of the transcriptional responses of prey during the early stages of predation also showed an absence of phage resistance gene expression, giving further support against the potential of resistance formation to this approach (270).

1.3 The lifecycle of *B. bacteriovorus*

1.3.1 Host independent lifecycle

B. bacteriovorus has two distinct lifestyles, a host-dependent, predatory lifestyle and a mutation-driven host independent lifestyle (Figure 17) (271). A host independent lifecycle occurs due to a rare, mutation driven reversible phenotypic switch, where growth mirrors that of the intraperiplasmic growth within prey (271,272). This mutation, contained within the HIT (host interaction) locus was shown to occur in *bd0108,* a gene responsible for type 4 pilus retraction regulation. Knockout mutant strains show reduced pilus extrusion, with this pilus extrusion believed to be normally acting as a cue to switch on the predatory genome, thus leaving an altered genome expression state in its absence (271). However, it is the predatory lifecycle of *B. bacteriovorus* that is of great interest to scientists for the infectious disease treatment.

1.3.2 Predatory lifecycle

The predatory lifecycle of *B. bacteriovorus* can be divided into distinct phases; attachment, invasion, prey rounding, replication and cell lysis.

1.3.2.1 Attachment

Attachment is initiated when the highly motile, non-replicative attack phase *B. bacteriovorus* is chemotactically guided towards regions of high prey abundance via flagellar-mediated motility (in liquid) or gliding motility (in biofilms) (273,274). *B. bacteriovorus's* single, polar, membrane-sheathed flagellum has been shown to be

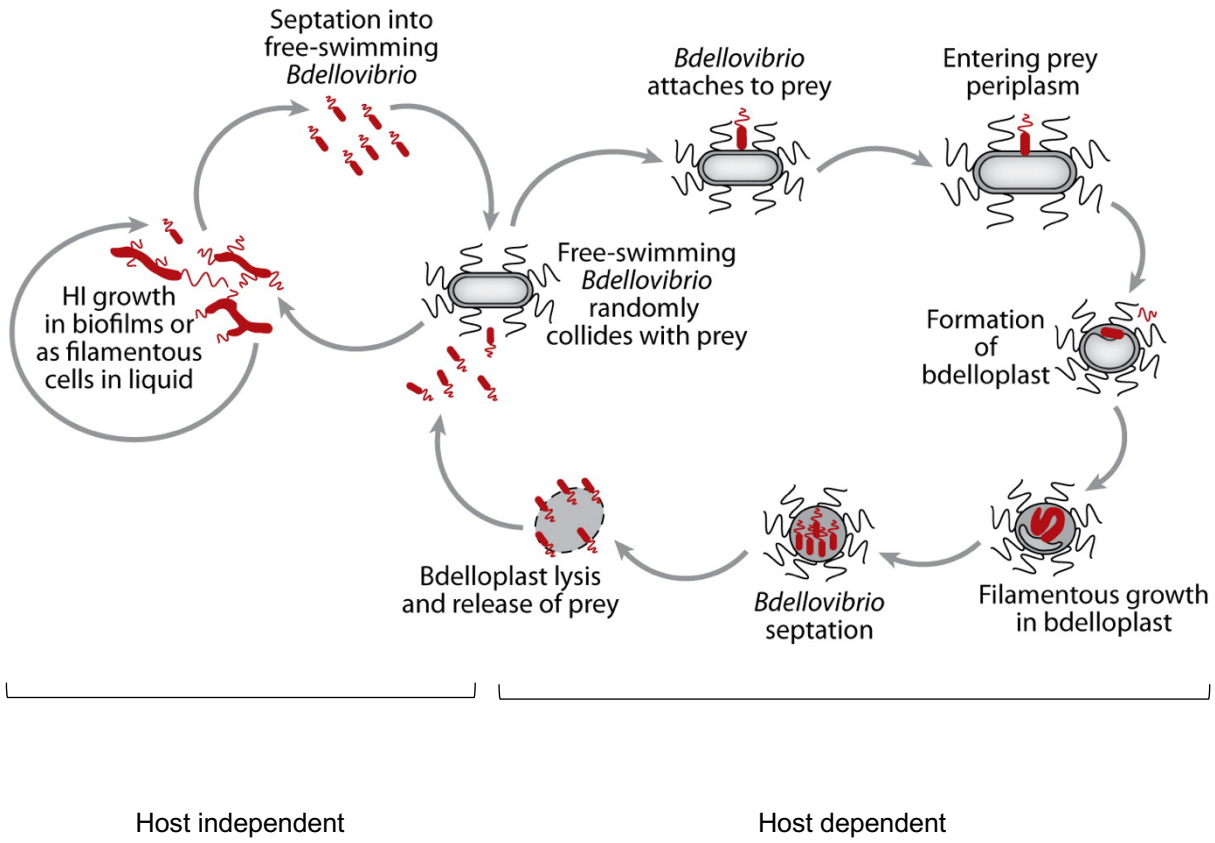


Figure 17: Schematic of the host dependent and host independent lifestyles of *B. bacteriovorus*. Host dependent: Highly motile attack phase *B. bacteriovorus* cells randomly collide with Gram-negative prey whereby they attach and enter the prey cell through the use of peptidoglycan-modifying enzymes. Upon entry, *B. bacteriovorus* further modifies the prey cell to form a rounded, osmotically stable bdelloplast whereby it can uptake nutrients and grow into a long filament. Upon nutrient exhaustion, the filament septates to form progeny attack phase cells that are then released upon prey lysis to invade further prey. Host independent: *B. bacteriovorus* may also undergo a rare, mutation driven host independent lifestyle whereby it grows in a slower, non-predatory manner. Figure taken from (Sockett, 2009).

of great importance for its predatory capabilities, where it aids prey encounters early on in the predatory cycle by facilitating random collision and subsequent binding to prey cells. Despite facilitating the collision and subsequent binding of *B. bacteriovorus* to prey cells, the flagellum is not essential for prey entry and invasion (143). Instead, the active adhesion of *B. bacteriovorus* to prey is thought to be facilitated by multiple pili, protein structures that have previously been shown to be important for protein secretion, adherence, DNA uptake and twitching motility in other bacteria (275). Attachment is thought to be facilitated by a Type 4 pilus and further accessory pili, encoded for by the HIT locus, whereby a cell wall associated protein pilus, encoded by *bd0109,* binds to cellulose within the prey cell wall (276). Upon collision with a prey cell, *B. bacteriovorus* either aborts attachment and detaches itself, or it binds in a more permanent manner using further pili and retracting its Type 4 binding pilus, ultimately bringing the membranes of the two bacteria into contact and prompting prey invasion (261,272).

1.3.2.2 Invasion

After attachment to prey, the next barrier to invasion is the bacterial cell wall. As mentioned in Chapter 1: section 1.1.2.1, the bacterial cell wall is comprised of peptidoglycan monomers, highly crosslinked together to form a strong, dynamically remodelled structure that is vital for cell shape and osmotic stability (3). The bacterial cell wall is indispensable for bacterial cell viability and is one of the first points of contact with the environment (second only to the outer membrane), making peptidoglycan of great interest and importance in the *B. bacteriovorus* predatory cycle also (3).

To cross the cell wall and invade the prey cell, *B. bacteriovorus* releases a series of hydrolytic enzymes including *bd0816* and *bd3459* encoded D, D endopeptidases (amongst others) that hydrolyse higher order (3, 4) D-Ala, D-Ala peptide crosslinks within peptidoglycan (see Chapter 1: section 1.1.2.1), weakening and decrosslinking the prey cell wall to allow for entry by a yet undetermined mechanism (110). As *B. bacteriovorus* enters the periplasm of

the prey cell, the flagellum is thought to detach and flagellar induced motility ceases (277). Shortly after entry *B. bacteriovorus* uses another subset of peptidoglycan modification enzymes, encoded by *bd0886* and *bd1176*, to remodel the prey peptidoglycan and cytoplasmic membrane through D, D endopeptidase-mediated modification and to alternatively re-crosslink the prey peptidoglycan, a process believed to be mediated by L, D transpeptidases but is yet to be shown (278,279). *B. bacteriovorus* then reseals the entry pore through a yet to be determined mechanism (actively researched within the Sockett lab) resulting in the formation of an osmotically stable bdelloplast (278,280,281). Modification also prevents the entry of a second *B. bacteriovorus* cell preventing wasteful secondary predation and ensuring a so called 'private dining' scenario. 20 minutes after predator entry, electron transport across the inner membrane of the prey cell ceases and the prey cell is killed (280).

1.3.2.3 Intraperiplasmic growth

Intraperiplasmic growth then begins, wherein DNA replication and cellular biogenesis occurs. Early on in the growth phase, *B. bacteriovorus* uses GlcNac and NAG deacetylases encoded by *bd0468* and *bd3279* respectively, to mark the prey cell wall for degradation (282). It also uses further deacetylase enzymes to temporarily make the bdelloplast peptidoglycan lysozyme resistant, to prevent premature lysis (283). To facilitate the nutrient uptake required for intraperiplasmic growth, *B. bacteriovorus* expresses an array of transmembrane uptake proteins and porins once it has entered the prey cell e.g. ATP binding cassette proteins and transporters belonging to the major facilitator superfamily. These uptake proteins allow for the uptake of nutrients across the prey cytoplasmic membrane and the *B. bacteriovorus* inner and outer membranes (284).

As mentioned in Chapter 1: section 2.3.1.2.2, *B. bacteriovorus* is highly efficient in its use of prey resources for intraperiplasmic growth, using as much of the prey resources by as energetically inexpensive means as possible. *B. bacteriovorus* degrades DNA and RNA once it enters the bdelloplast, incorporating the nucleic acids directly into its own genome

(144,145) and using fatty acids for membrane synthesis (285). The uptake and integration of LPS and prey outer membrane proteins has also been described whereby OmpF, and to a lesser preference OmpC of *E. coli*, are incorporated into the bdelloplast membrane (286,287). These porins are crucial for nutrient acquisition but may also play other roles in the predation process. Upon nutrient exhaustion, synchronous septation of the *B. bacteriovorus* filament occurs creating odd or even numbers of progeny dependent on the amount of nutrients that were present in the prey cell, ending with flagellum biosynthesis (288). The bdelloplast peptidoglycan is then partially digested by an unknown acetylation sensitive mechanism to form discrete pores in the bdelloplast membrane, facilitating the release of progeny attack phase cells which subsequently go on to invade further prey cells (282,283,289).

1.3.3 Unknowns

Despite decades of research into the predatory lifecycle of *B.* bacteriovorus, many questions still remain regarding the underlying mechanisms of the predatory lifecycle. Such questions include: What mechanism is responsible for the resealing of the porthole after predator entry? What are the next steps after pili binding and retraction that result in invasion? What happens to the flagellum after entry into the prey cell?

Whilst actively researching the answers to these questions, it was discovered that a vesicle of unknown composition or function forms at the predator-prey interface shortly after prey invasion, and subsequently persists throughout the predatory cycle (290). These vesicles were subsequently shown to be deposited within the prey during the predation process and persist after prey lysis attached to cellular debris (291). The contents of these vesicles and the mechanism through which they originate and pinch off from the predator's inner membrane, outer membrane or cell wall are also unknown and are being actively researched within the lab.

One potential indication of vesicle content and function is provided through the association of this vesicle with the PBP activity modulator CpoB

(Bd0635). Association with CpoB may suggest that the vesicle is synthesised upon entry into prey via CpoB-mediated modulation of prey PBPs, which is supported by the dynamic localisation of CpoB throughout the predation process, suggesting that some synthesis of membranes, and therefore vesicles, may be possible (291). The underlying mechanisms of how and where this vesicle is synthesised (i.e. at what position within the predator cell) is currently under investigation.

An alternative explanation for the source of the vesicle may be from the outer membrane sheath of the flagellum of *B. bacteriovorus*, that is thought to be partially shed upon prey entry (273,283). The flagellum is encoded by 6 clusters of flagellar synthesis and motility genes, *fliC1-6*, each of which plays a different role in flagellar structure (143,273). The expression of flagellins is highly variable throughout the predatory cycle, where during attack phase flagellin expression is highly upregulated facilitating motility and subsequent localisation and invasion of prey, and upon prey invasion, flagellin expression is greatly reduced as *B. bacteriovorus* is stationary and expends energy on biosynthesis and growth (143). The vesicle may emanate at the flagellar pole upon prey entry and so the flagellum may form part of the developing vesicle membrane. This is supported by the presence of flagellar basal motor proteins within the vesicle, that may be deposited within the vesicle when the flagellum tip is shed, whereby its membrane may form part of the vesicle membrane (Pratik Jagtap, personal communication). This flagellum-derived vesicle may then play a role in sealing the porthole in the prey cell membrane after predator entry.

It is known that CpoB is able to modulate the activity of penicillin binding proteins within non-predatory bacteria to aid the dynamic modification of peptidoglycan within bacterial cell walls to facilitate growth and to constrict the outer membrane during division, whereby it co-ordinates the activity of PBP1 based on the activation state of the Tol protein (292). Extensive cell wall remodelling occurs within both *B. bacteriovorus* and prey cell walls during the predation process, facilitating the entry and invasion of the prey cell. It is possible that CpoB may aid this cell wall modification and facilitate invasion and prey killing, making any genes associated with CpoB of great

interest in the search for novel antimicrobial approaches. The fluorescent tagging of penicillin binding proteins with mCherry tags proves lethal owing to defective cell wall formation and subsequent lysis, making the investigation of CpoB's role within the predation process difficult (290). However, isolation of CpoB associated vesicles indicated further proteins of interest that were present within the vesicle, of which many had unknown functions. The genes at the centre of this study are a subset of genes predicted via protein analysis to be present and expressed within these vesicles, identified through vesicle enrichment preparations.

1.4 Research aims

Although invasion of bacterial prey is natural, efficient and rapid, it is a complex process and only some of the cell wall activities that facilitate attachment, invasion and predation are known. For my second rotation at the University of Nottingham, I joined researchers examining the membrane dynamics during invasion.

Previous studies have successfully visualised the dynamic localisation of CpoB throughout the predation process via the addition of a fluorescent mCherry tag and subsequent microscopy (291).

To investigate the roles of these vesicle-associated proteins within the *B. bacteriovorus* predatory cycle, this study aims to emulate this approach and fluorescently C-terminally tag a subset of these vesicle-associated genes with an mNeon tag.

This will allow for the visualisation of the dynamic localisation of these proteins to be seen via microscopy throughout the whole predation process.

The further understanding of the roles of these proteins within the predation process may provide us with potential candidates for novel antimicrobial therapies or may aid the tailoring of *B. bacteriovorus* for the use as a potential 'living' antibiotic in the future treatment of infectious disease.

2. Materials and methods

2.1 Bacterial strain culture and maintenance

Escherichia coli DH5α and S17-1 for gene cloning and prey purposes were cultured in YT broth (5 g/l Difco yeast extract, 8 g/l Difco tryptone, 5 g/l NaCl, pH 7.5), at 37°C, 200 rpm for 18-24 hours, or on YT agar (YT broth composition plus 10 g/l agar (Sigma)) at 37°C overnight. To provide selection for plasmid constructs, 50 µg/ml of kanamycin sulphate was added to media when needed.

Bdellovibrio bacteriovorus wild type strain HD100 was cultured in 10 ml Ca/HEPES (0.295 g/l $CaCl_2{\cdot}2H_2O$, 5.94 g/l HEPES free acid, pH 7.6) containing 600 µl *E. coli* S17-1 prey (grown in YT broth at 29°C for 24 hours prior to use) and an inoculum of 200 µl of an overnight (minimum 16 hours) culture grown in the same way previously (typically containing 9.5 $x10^7$ plaque forming units/ml). Alternatively, *B. bacteriovorus* was cultured on YPSC overlay plates (0.25 g/l $MgSO_4$, 0.5 g/l NaOAc, 1 g/l broadbean peptone, 1 g/l yeast extract, 0.25 g/l $CaCl_22H_2O$, pH 7.6 with 10 g/l agar (YPSC-bottom plates) or 6 g/l agar (YPSC-top agar plates) at 29°C until plaques appeared. To provide selection for plasmid constructs, 50 µg/ml of kanamycin sulphate was added to media when needed.

Full details of the strains and primers used in this study can be found in Appendix: Supplementary Table 3 & 4 respectively.

2.2 Construction of mNeon fusion strains

To study the localisation of the proteins encoded by these genes during the predatory cycle, fluorescently tagged genes were required. To do this the *B. bacteriovorus* genes *bd1815*, *bd2439* and *bd2754* were cloned into a pk18*mobsacB* suicide vector plasmid (293) in combination with an mNeon gene being added at the 3' end, via NEB Gibson assembly using the primers detailed in Appendix: Supplementary Table 3. The plasmid was introduced into the *B. bacteriovorus* genome by homologous recombination in a single

crossover event generating a genome containing an mNeon C-terminally tagged gene that will fluoresce within the cytoplasm of the cell (271).

2.2.1 Primer design

Primers for Gibson assembly were designed to encompass our gene of interest plus 600 bp of flanking region to facilitate recombination of our gene into the genome (a minimum of 1 kb is sufficient to allow for crossover into the genome). Full details of primer sequences can be found in Appendix: Supplementary Table 5.

2.2.2 PCR amplification of DNA fragments for Gibson Assembly

To generate mNeon tagged genes, DNA fragments containing our gene of interest and the mNeon tag were amplified via PCR. PCR reactions (100 µl total volume) were comprised of 0.5 µl each of forward and reverse primers (100 µM stock), 2 µl of 100 µM dNTPs (VWR Life Science), 20 µl of 5 x Q solution, 1 µl of Phusion polymerase (Thermo Scientific), 20 µl of 5 x Phusion High Fidelity buffer, 1 µl of DNA template and 55 µl of ultrapure Analar water. PCR reactions were then incubated under the following thermocycling conditions: Initial denaturation at 98°C for 3 minutes, annealing at 60°C for 30 seconds and subsequent extension at 72°C for 4.5 minutes for 30 cycles and final extension at 72°C for 5 minutes. This 100 µl PCR reaction was then ran on an agarose gel (0.8% agarose, 10.8 g/L Tris, 5.48 g/L Boric Acid and 0.6% 0.375 M Na_2EDTA) measured against a 1 kb ladder (NEB) at 100 V for 1 hour and then bands were visualised under UV light, their correct size confirmed and then excised from gel under UV light visualisation.

2.2.3 Gibson Assembly

PCR products were extracted and purified from gel slices using the GenElute Gel Extraction Kit in accordance with manufacturer's

instructions (Sigma). DNA fragments were cloned into the vector pK18*mobsacB* (digested with *EcoRI* and *HindIII*) using the NEBuilder HiFi DNA Assembly Kit (NEB) at a ratio of 7:1 and incubated at 50°C for 1 hour to promote assembly.

2.2.4 Plasmid verification

To verify that the plasmid contained the correct gene fragments, 2 µl of product from the Gibson Assembly was transformed into 50 µl of chemically competent *E. coli* DH5α cells as per manufacturer's instructions (NEB). Through a blue-white selection approach, successfully transformed colonies containing our plasmid of interest were isolated (appearing white on YT agar plates containing 50 µg/ml kanamycin sulphate, 40 µg/ml X-Gal and 40 µg/ml IPTG). These colonies were further screened via restriction digest of the isolated plasmid, followed by transformation of Sigma preparations of validated plasmids into 200 µl of chemically competent *E. coli* S17-1 cells and further restriction digests on the re-isolated plasmids. Further details regarding the restriction enzymes used for these digests and the resulting fragment sizes can be found in Appendix: Supplementary Table 6.

Plasmids were further verified by sequencing whereby genomic DNA from *B. bacteriovorus* containing either *bd1815mNeon*, *bd2439mNeon* or *bd2754mNeon* were amplified using the screening primers listed in Appendix: Supplementary Table 5 to amplify the fragment of plasmid containing our gene of interest and the mNeon fragment via PCR. Screening primers were designed to bind 100 bp upstream of the start of the gene of interest and 100 bp downstream of the end of the *mNeon* gene. The PCR product was purified through gel extraction and sent for sequencing (Source Bioscience).

2.2.5 Conjugation into B. bacteriovorus HD100

To conjugate the plasmid constructs into *B. bacteriovorus* HD100 wild type and obtain a single crossover integrant of the tagged gene into the genome, 10 ml of *E. coli* S17-1 containing the construct and 10 ml of wild-type *B. bacteriovorus* HD100 lysate were centrifuged at 5100 rpm for 10 minutes and resuspended either in residual supernatant (*B. bacteriovorus)* or 50 µl YT broth (*E.coli* S17-1) and applied to a nitrocellulose membrane secured on a SPY agar plate (10 g/l broadbean peptone, 3 g/l Difco yeast extract, 10 g/l Sigma select agar, pH adjusted to 6.8). SPY plates were incubated at 29°C overnight before being resuspended in 1 ml Ca/HEPES and spread onto YPSC overlay plates (comprised of YPSC bottom plates and YPSC top plates containing 150 µl *E. coli* S17-1 pZMR100 prey per 5 ml YPSC top). Overlay plates were incubated at 29°C until plaques appeared (approximately 5 days) after which plaques were picked into 2 ml of predatory cultures containing *E. coli* prey and cultured for 48 hours before being validated via microscopy where fluorescence was indicative of successful conjugation. Alternatively, the fluorescently tagged genes were conjugated into a *B. bacteriovorus* HD100 strain containing a double crossover integrated mCherry-tagged CpoB (Bd0635) gene in the same way as above (Sockett lab, unpublished; Capeness *et al.*, 2013).

2.3 Microscopy and image analysis for bacteria

Microscopy was carried out on a Nikon Eclipse Ti-E inverted fluorescence microscope equipped with a 100 x Phase 3 objective lens using phase contrast (exposure: 100 ms), the GFP channel (Exposure: 20 s, Gain: 1, Excitation: 400-440 nm, Emission: >470 nm), used to detect mNeon fluorescence, and the mCherry channel (Exposure: 10 s, Gain: 1, Excitation: 550-600 nm, Emission 610-665 nm), used to detect mCherry fluorescence.

2.3.1 Distribution of fluorescently tagged proteins during attack phase

Initially, predatory cultures that had been incubated overnight (containing attack phase *B. bacteriovorus* only as all prey cells had been preyed upon and lysed) were immobilised on a bed of Ca/HEPES containing 1% agarose and mounted on glass slides. 10 fields of view (each field containing at least 50 bacteria) were imaged, for three independent predatory cultures, for each of our three genes of interest. Images were processed using the MicrobeJ plugin (294) on ImageJ to determine where fluorescence and therefore protein localisation occurs within attack phase cells, from which heatmaps were generated.

2.3.2 Synchronous predation timecourse

To determine the location and chronology of fluorescently tagged protein expression during the predatory cycle, exconjugant *B. bacteriovorus* containing the constructs of interest were observed via fluorescence microscopy. Synchronous predation of *E. coli* S17-1 PZMR100 prey by *B. bacteriovorus* HD100 (± CpoBmCherry) were set up by combining a 10x concentrated *B. bacteriovorus* predatory culture with *E. coli* S17-1 (standardised to an OD_{600} of 1) and Ca/HEPES at a ratio of 5:4:3 respectively. 10 µl samples were then taken at 0 hour, 15 minute, 30 minute, 45 minute, 1 hour, 2 hour, 3 hour and 4 hour timepoints and immobilised in a bed of Ca/HEPES containing 1% agarose and mounted on glass slides.

Due to time constraints, a single time-course experiment was conducted for each gene whereby 10 fields of view were taken for each timepoint to characterise all stages of predation. ImageJ was then used to select and process representative images for each gene of interest at each stage throughout the predatory cycle. Selections from images at each stage of predation (100 pixels by 100 pixels) were selected from in focus regions and images of individual channels were generated i.e. phase, GFP only, mCherry only, colour merge

(GFP & mCherry) and merge (all channels) for each stage. Images were then processed using 1 smooth and 1 sharpen filter and scale bars were overlaid onto each image. Panels for each time-course were then constructed.

Images were taken using Nikon NIS software and analysed using ImageJ software.

2.4 Statistical analysis

Due to the qualitative nature of the data within this study, combined with only single biological repeats for the time-course experiments (due to time limitations), it was deemed that statistical analysis of the data generated by this study was not appropriate.

3. Results

3.1 Bioinformatic analysis

Bd1815 is a predicted gene product 179 amino acids in length with a predicted molecular weight of 19 kDa. Bd2754 is a predicted gene product 173 amino acids in length with a predicted molecular weight of 18.7 kDa. Both gene products have not been previously studied and do not have an ascribed function and so they are classed as hypothetical proteins. Bd2439 is a predicted gene product 1107 amino acids in length with a predicted molecular weight of 112 kDa that is predicted by GenBank to be an uncharacterised cell wall surface anchor family protein due to it containing a cell wall surface anchor family domain.

3.1.1 Protein homology

An initial search for homologues of these proteins, with the aim of elucidating on a possible function through the comparison to proteins present in other bacterial species, gave few results. Bd2754 has homologues, each of which is present as a single copy, within the laboratory strains of invasive *B. bacteriovorus* HD100 and 109J and within the environmental invasive strain *B. bacteriovorus* strain *Tiberius,* all of which were classed as hypothetical proteins, but not within other *Bdellovibrio* bacteria outside of this species or within other bacteria in general (BLAST). Bd1815 also has hypothetical protein homologues within invasive *B. bacteriovorus* HD100 and 109J strains including one partial homologue that was annotated as a NAD-dependent epimerase/dehydratase (an energy providing domain for cellular processes) (BLAST). Bd2439 had homologues within *B. bacteriovorus* and *B. bacteriovorus Tiberius,* most of which were characterised as hypothetical proteins. A small number of homologues were identified as cell wall anchor family proteins and tail fibre domain containing proteins, both of which had S74 peptidase domains predicted within their structures, but had no further annotation or analysis carried out on them. All three proteins lack genomic homologues outside of the species, making determination of protein function more challenging.

3.1.2 Domain prediction

We can begin to assign a role to these proteins by studying where and when they localise within cells and how this varies based on the predatory activities of the cell, especially whether this differs between attack phase, invasion, pore resealing and exit within *B. bacteriovorus.* To determine where and when these proteins may localise both within the *B. bacteriovorus* cell, and outside of *B. bacteriovorus,* inside the prey cell, the amino acid sequences of Bd1815, Bd2439 and Bd2754 were screened against the SignalP 4.0 database (295), a program that predicts the presence of signal peptides. The presence of a signal peptide within the protein sequences would indicate that the protein may be translocated over the cytoplasmic membrane and so would localise within the periplasm of the *B. bacteriovorus* cell, or be translocated across the predator outer membrane and localise within the prey periplasm, or outside of the prey cell altogether. However, there is insufficient information to comment on this further potential translocation using bioinformatic analysis. SignalP 4.0 analysis predicted the presence of a signal peptide within the N-terminus of Bd1815 and Bd2439, indicating it may be trafficked across the cytoplasmic membrane into the periplasm, possibly via the Sec-dependent secretion system (Appendix: Supplementary Figure 3C). SignalP 4.0 did not detect the presence of a signal peptide within the amino acid sequence of Bd2754 (Appendix: Supplementary Figure 3C). This contradicts with my preliminary investigations of Bd2754 on the UniProt database that suggests a signal peptide is present at the N-terminus of Bd2754 (predicted by the positive outcome of at least 2 of the following analyses: Phobius, Predotar, SignalP and TargetP).

The protein sequences of Bd1815, Bd2754 and Bd2439 were also screened against the Pfam database, a database the predicts the presence of known domains within the protein structure (296). This also confirmed the prediction of signal peptides at the N-terminus of all three of these proteins, as well as predicting a small number of regions of disorder or low complexity, and so failed to highlight any further recognisable domains within the protein structure that could suggest a potential known function of these proteins (Appendix: Supplementary Figure 3A). As these genes are unique to

predatory bacteria, and domain characterisation by PFam is based on known, well studied bacteria (of which *B. bacteriovorus* is not), this explains the lack of domain identification within our proteins. Screening of these protein sequences in the KEGG database also suggested the presence of a pilin domain in the middle of the Bd2439 protein and an outer membrane protein beta barrel domain in Bd2754, that may also aid the assignment of a function to these proteins (Appendix: Supplementary Figure 3B) (KEGG).

3.1.3 Structural analysis

The structure of all three of these proteins remains unknown. To get an approximate impression of their structure, amino acid sequences were screened against the ITASSER server, a coalition of multiple structural databases combined into one predicted structural output (297). Initial secondary structure analysis of Bd1815 and Bd2754 reveals large areas of coils and some minor helical regions within the secondary structure, although no further recognisable domains were detected (ITASSER, JPRED, PHYRE). Both proteins also contain a large number of buried, minimally accessible residues, supporting the idea of a highly coiled, barrel like structure (ITASSER). Bd2754 was also seen to share some structural homology to the *E. coli* outer membrane proteins OmpA (PBD: 1BXW) (Tm score 0.75, where 1 is a perfect match) and OmpW (PDB:2FLT) (Tm score 0.763) (Figure 18B), and to *Neisseria* surface protein A (NspA) (PDB: 1P4T) (Tm score 0.750) (ITASSER), whilst Bd1815 had a good alignment with the *E. coli* outer membrane proteins OmpA (PDB: 1BXW) (TM score 0.681) (Figure 18A), *Neisseria* surface protein A (NspA) (PDB: 1P4T) (TM score 0.653), *E. coli* OmpW (PDB:2F1T) (Tm score 0.646), and a variety of other *P. aeruginosa* outer membrane proteins OprH (PDB: 2LHF), OprF (PDB: 4RLC) and OprG (PDB: 2X27) (ITASSER). Initial secondary structure analysis of Bd2439 revealed a more helical structure with large stretches of coiled regions bordered by a large number of highly buried residues, although no further recognisable domains were found (ITASSER, JPRED, PHYRE). Bd2439 loosely aligned to *Shigella flexneri* serine protease SepA (PBD: 5J44) (Tm score 0.877) (Figure 18C), and the *E. coli* proteins haemoglobin binding

protease (PDB: 1WXR) (Tm score 0.800) and serine protease EspP (PDB: 3SZE) (Tm score 0.705) but was shown to have very few structurally similar proteins otherwise (ITASSER).

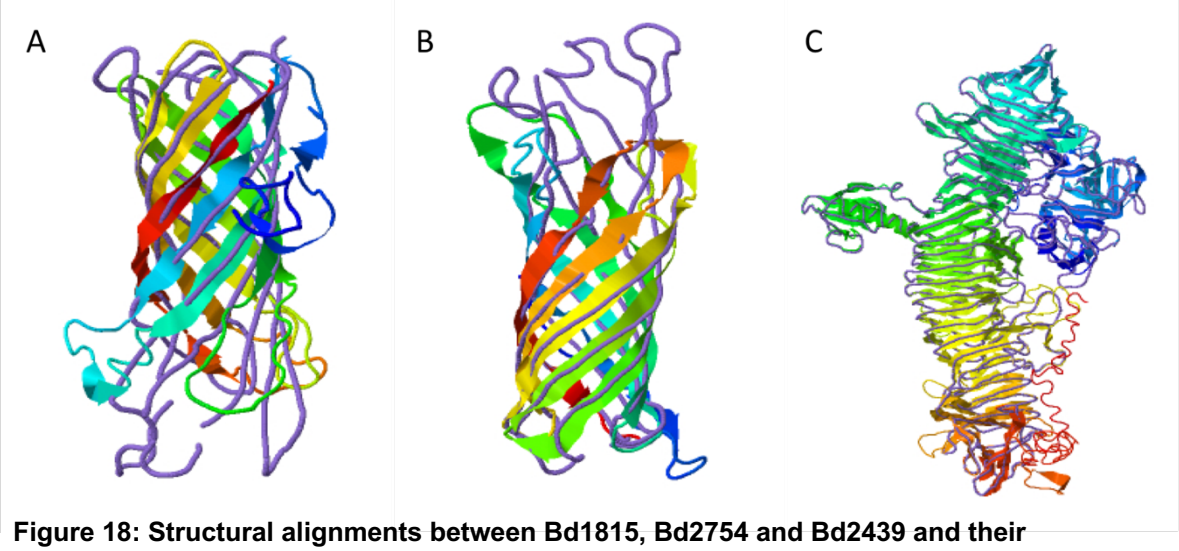


Figure 18: Structural alignments between Bd1815, Bd2754 and Bd2439 and their closest structural homologues
Structural alignments between the 3D model of the predicted protein structure of A: Bd1815, B: Bd2754 and C: Bd2439 depicted as multicoloured threads, and their closest structural homologues *E. coli* OmpA (PDB: 1BXW), *E. coli* OmpW (PDB: 2FLT) and *Shigella flexneri* serine protease (SepA) (PDB: 5J44) respectively depicted as purple threads. Each alignment has template modelling scores of 0.681, 0.783 and 0.877 respectively, where 1 indicates an identical match. 3D models of each protein and their corresponding alignments were

3.1.4 Sequence homology to closest structural homologues

The tertiary structure of these proteins shows some homology to other non-*Bdellovibrio* proteins. To determine how the secondary structures and amino acid sequences compare between our proteins and their closest structural homologues, further sequence threading and alignments were carried out. Protein sequences for Bd1815, Bd2754 and Bd2439 were screened using Promals3D, an alignment tool, alongside the protein sequences for the closest structural homologues found above, and a novel *B. bacteriovorus* porin CAE47737.1 (286). Bd1815 and OmpA showed a strong alignment initially, with few conserved individual amino acids but a strongly conserved consensus amino acid sequence and nature, which later breaks down owing to OmpA being twice the size of Bd1815. Bd1815 also shows a strong alignment to the *B. bacteriovorus* porin CAE47737.1, especially within the first 20 amino acids where the signal peptide is known to be encoded. This

novel *B. bacteriovorus* porin also shows conserved consensus amino acid sequence with the *E. coli* porin OmpA suggesting that all three proteins have a highly conserved consensus amino acid sequence and secondary structure, but a dissimilar amino acid sequence (Appendix: Supplementary Figure 4). Bd2754 and OmpW showed a strong alignment throughout, despite there being few conserved amino acids, as the nature of the amino acids was highly similar. CAE47737.1 also showed strong conservation with Bd2754 and the *E. coli* porin OmpW, once again suggesting a similar secondary structure for all 3 proteins (Appendix: Supplementary Figure 5). On the other hand, the alignment between Bd2439 and SepA was considered poor. Whilst having individual conserved amino acids throughout the alignment, the sequences highly differed between Bd2439 and SepA and large regions of the sequence were omitted to make the alignment fit, suggesting that despite having similar predicted 3D structures, the two are highly dissimilar on a sequence level (Appendix: Supplementary Figure 6). This trend of similar secondary structure and nature but dissimilar amino acid sequence seen with Bd1815 and Bd2754 is further suggested by the 3D modelling of Bd2754 and Bd1815 against the *E. coli* OmpA porin (Gift from Dr Andrew Lovering, University of Birmingham). Comparison of the amino acid sequences of our genes of interest to OmpA shows very low similarity, but comparison of the nature of the amino acids reveals a similar structure, giving a highly similar arrangement of coils and helices (Dr Andrew Lovering, Personal Communication) (Appendix: Supplementary Figure 5). The alignment of both of these proteins with outer membrane proteins and porins from other bacterial species, combined with the further threading and 3D modelling of Bd1815 and Bd2754 to the *E. coli* porin OmpA is highly suggestive of these two proteins belonging to a class of porins.

However, this contrasts with the poor sequence alignment with known porins from *E. coli* and *B. bacteriovorus.* OmpA and OmpW are regular porins present in the LPS of the well-studied bacterium *E. coli,* and so their amino acid sequence and biophysical properties will favour their localisation within the outer membrane LPS. Bd1815 and Bd2754, the predicted porins in this study, are hypothesised to be found in a novel vesicle structure made by an

atypical predatory bacterium *B. bacteriovorus*, which has an unknown composition and structure. Due to this, these novel *B. bacteriovorus* porins may have entirely different biophysical properties and amino acid sequences as they are found in an entirely different membranous environment, potentially explaining the lack of similarity between amino acid sequences of *E. coli* porins OmpA and OmpW and the predicted *B. bacteriovorus* porins Bd1815 and Bd2754, despite their highly similar, porin-indicative tertiary structure.

Bd1815 and Bd2754 have a highly similar tertiary structure to known porins in *E. coli* and *B. bacteriovorus*. As true porins, Bd1815 and Bd2754 should be present in the outer membrane of the predator cell and should be exported from the cytoplasm of the cell where they are synthesised (supported by the presence of signal peptides encoded within their amino acid sequences and their diffuse distribution within attack phase cells where they may be present throughout the outer membrane). The signal sequence present within these two proteins is highly similar to the signal peptide present within novel OMPs found in *B. bacteriovorus* (study: Beck *et al.*, 2004) which are highly similar in structure to the *E. coli* porins OmpA and OmpW, further confirming the likelihood that Bd1815 and Bd2754 are porins.

3.2 Constructions of mNeon fusion strains and transfer into *B. bacteriovorus*

To determine the chronology and localisation of protein expression, Bd1815, Bd2439 and Bd2754 were C-terminally tagged with a fluorescent mNeon tag. To generate mNeon tagged *bd1815*, *bd2754* and *bd2439* genes, DNA fragments containing our gene of interest and the mNeon tag were amplified via PCR using genomic DNA from *B. bacteriovorus* HD100 as a template for amplification of the gene fragments or the plasmid pAKF220 as a template for mNeon fragment amplification. These fragments were then ligated into the suicide vector pK18*mobsacB* via Gibson assembly (Figure 19A) and transformed into *E. coli* DH5α. Restriction digests were used to validate that the gene fragments had correctly integrated into the plasmid and that the

fusion of the gene with mNeon was successful. Sigma preparations of these plasmids were then transformed into *E. coli* S17-1, where they underwent further validatory restriction digests (Figure 19B) and, if correct, were then conjugated into *B. bacteriovorus* HD100 wildtype or *B. bacteriovorus* HD100 containing an mCherry tagged CpoB gene (introduced via double crossover) (Sockett lab, unpublished) to generate single crossover integrant strains containing mNeon tagged fluorescent *bd1815*, *bd2754* or *bd2439* genes.

To validate the identity of the plasmids generated in this study, genomic DNA was isolated from *B. bacteriovorus* HD100 strains containing *bd1815mNeon, bd2754mNeon* and *bd2439mNeon* genes and the region containing the gene of interest and mNeon fragment was amplified by PCR using the screening primers detailed in Appendix: Supplementary Table 5. This PCR product was purified by gel extraction and sequenced (Source Bioscience). Further validation of correctly mNeon tagged proteins could be achieved through the purification of mNeon-tagged proteins through Western Blot using primary antibodies specific to the WT (untagged) protein and an mNeon-specific antibody, coupled to a secondary antibody e.g. goat-anti-rabbit that is linked to a horseradish peroxidase molecule. These purified proteins may then be run on an SDS page gel and compared to demonstrate the mNeon tagged gene is approximately 711 bases (the size of the mNeon protein) bigger than the WT protein.

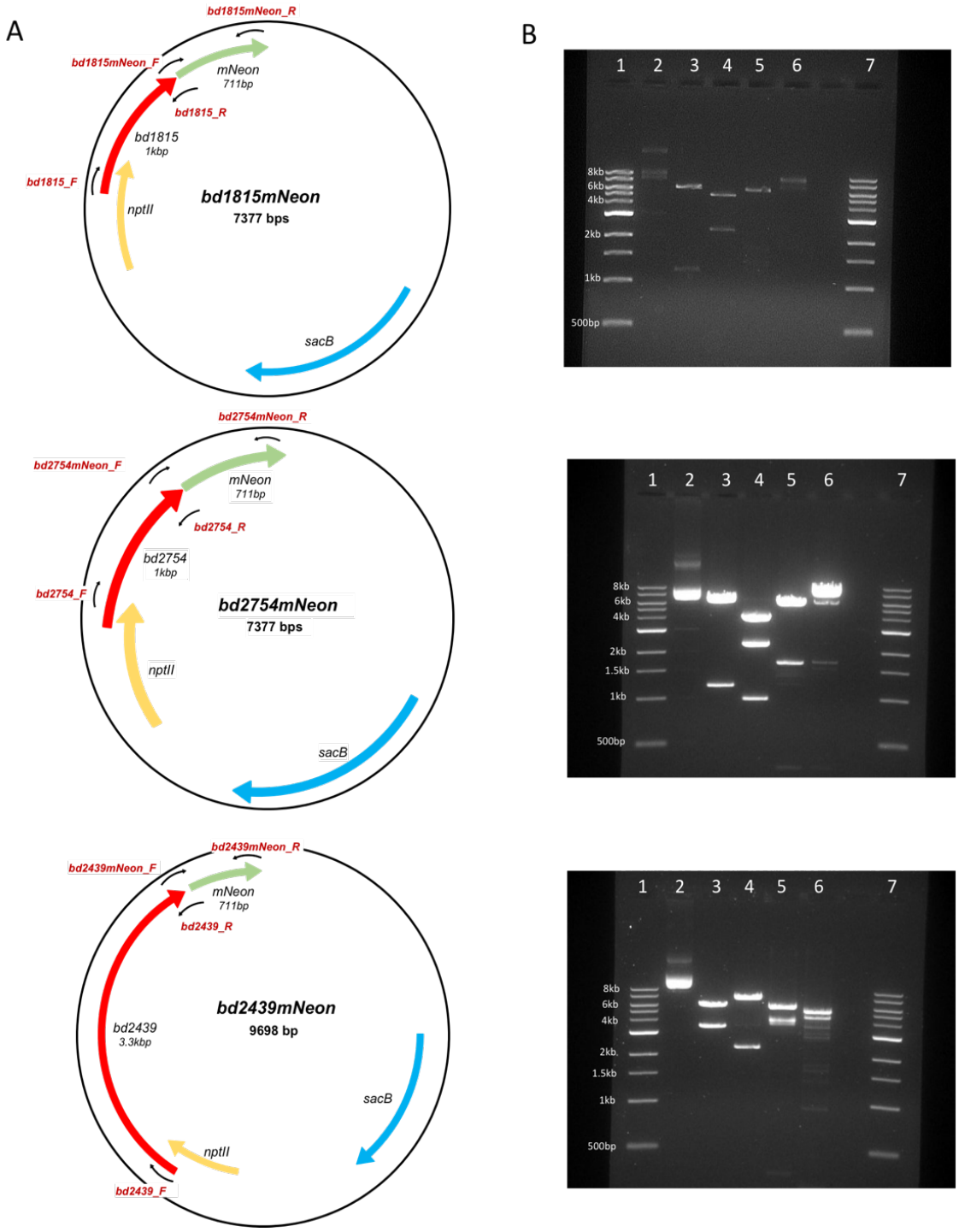


Figure 19: Plasmid maps showing A: gene and fluorescent tag locations and B: restriction digests for *bd1815, bd2754* and *bd2439*

A: Three plasmids containing *mNeon* C-terminal tagged *bd1815, bd2754* and *bd2439* genes within a pk18*mobsacB* suicide vector. *sacB* encodes a levansucrase, an enzyme that converts sucrose into a toxic product. *nptII* represents a neomycin phosphotransferase that confers resistance to neomycin and kanamycin providing a selection pressure for generation of the above plasmids. Arrows labelled with the suffix "_F/R" represent forward and reverse primers used for amplification of the gene fragment or the *mNeon* fragment. B: Representative restriction digests for each gene validating the identity and composition of the plasmid. Plasmids containing our *mNeon* tagged gene of interest were digested and ran against a 1 kb NEB ladder (Lanes 1 & 7). Plasmids were uncut (2), cut with NheI (3), cut with SacI (4), cut with BamHI-BglII (5) or cut with restriction enzymes where the restriction site was not present within the plasmid (6). Expected band sizes can be found in Appendix: Supplementary Table 6.

3.3 Characterisation of fluorescently tagged protein distribution during predation

This study focused on the roles of three proteins, thought to be associated with a CpoB-containing vesicle that is deposited within the prey after predation, where both the proteins and the vesicle have unknown functions. The chronology and location of protein expression, and where they localise at certain stages of the predatory lifecycle can help the assignment of potential functions to some hypothetical proteins. In this study, the timing and localisation of these hypothesised vesicular proteins at different stages throughout the predatory cycle was visualised via the means of a fluorescent mNeon tag and subsequent microscopy.

3.3.1 Determination of fluorescent protein distribution during attack phase

To determine where these proteins localise within *B. bacteriovorus,* attack phase cells from predatory cultures were imaged via fluorescence microscopy and their fluorescence mapped on an artificially rod-shaped heatmap, aligned by cell polarity, using the ImageJ plug-in MicrobeJ (294). Within attack phase *B. bacteriovorus,* Bd1815 and Bd2754 were distributed diffusely throughout the cell, with distribution resembling that of an mCherry tagged Bd0064 PilZ protein, a known cytoplasmically distributed protein as it has no signal peptide present (Figure 20A) (Gift from Emma Banks, University of Nottingham). However, as Bd1815 and Bd2754 have signal peptides within their protein sequences, it is likely that they are studded into the outer membrane of the *B. bacteriovorus* attack phase cell, giving a diffuse distribution instead of being present in the cytoplasm. The distribution of Bd1815 and Bd2754 varies slightly, despite both showing diffuse distributions, due to their amino acid sequences varying and not showing high similarity suggesting they may localise slightly differently. Bd2439 exhibited a bipolar distribution within attack phase cells (Figure 19A).

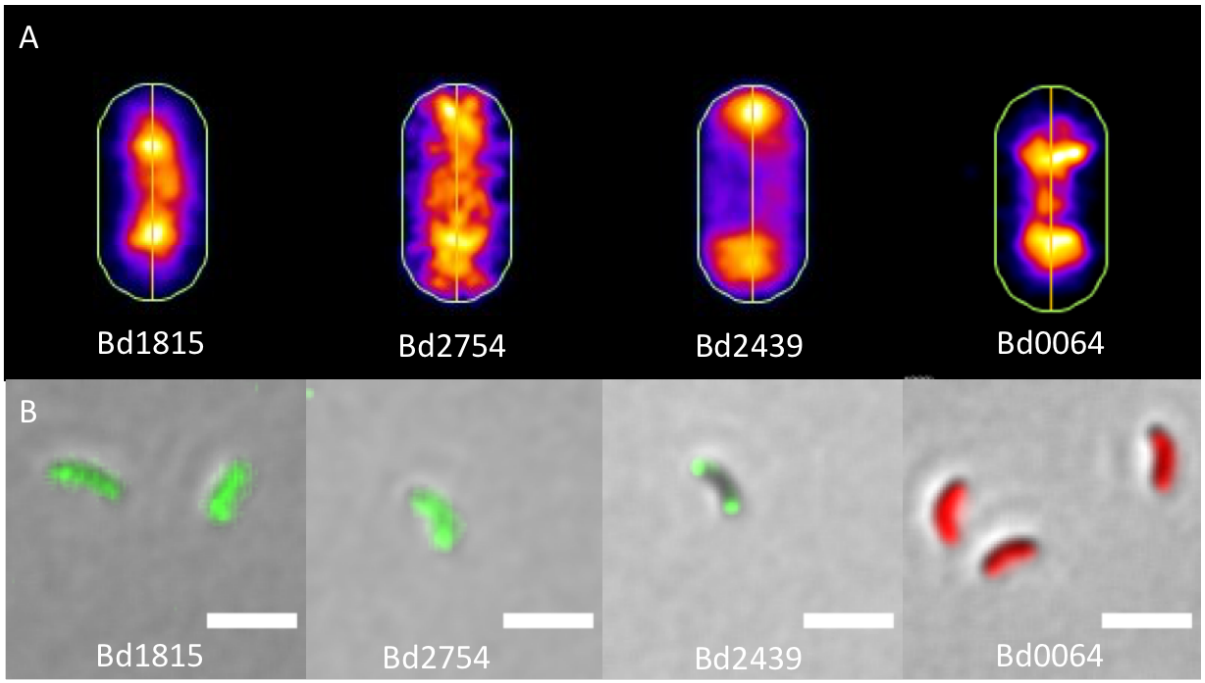


Figure 20: Fluorescent distribution of Bd1815, Bd2754, Bd2439 and Bd0064 in attack phase *B. bacteriovorus.*
The proteins Bd1815 and Bd2754 show a diffuse localization within attack phase *B. bacteriovorus* and show a similar localization pattern to Bd0064, a known cytoplasmically distributed protein, whereas the protein Bd2439 shows a bipolar distribution within attack phase cells. A: A minimum of 100 bacteria from each of at least 10 fields of view for each of three independent predations were analysed for GFP fluorescence (a proxy for expression of either *bd1815mNeon*, *bd2754mNeon* or *bd2439mNeon*) or for mCherry fluorescence (a proxy for *bd0064mCherry* expression) and the maxima/foci of fluorescence was visualized via heatmap using the MicrobeJ plugin of ImageJ (Ducret et al., 2016). B: Representative images of the distribution of Bd1815, Bd2754, Bd2439 and Bd0064. Scale bars represent 2 μM. Phase: Exposure 100 ms, GFP: excitation 400-440 nm, emission > 470 nm, Gain 1, exposure time 20 s. mCherry: excitation 550-600 nm, emission 610-665 nm, Gain 1, exposure time 10 s.

3.3.2 Determination of fluorescent protein distribution throughout predation

We next sought to determine whether the distribution of these proteins varied throughout the course of the predatory cycle, as any change in localisation may aid the functional annotation of these proteins. To do this, fluorescent protein expression was visualised throughout the predation cycle, with 10 fields of view being imaged at t0, 15 minute, 30 minute, 45 minute, 1 hour, 2 hour, 3 hour and 4 hour timepoints within the CpoBmCherry containing *B. bacteriovorus* strain to simultaneously determine where the proteins localise throughout the predatory cycle and whether protein expression co-localises with CpoB expression, or within the WT *B. bacteriovorus* HD100 strain in the case of Bd1815 time-courses.

Bd2439mNeon initially exhibits a bipolar distribution within attack phase cells (as previously shown in Figure 20) at t0. Upon attachment (t 15 minutes), Bd2439mNeon remains at the poles of the cell whilst CpoBmCherry remains diffusely distributed throughout the cytoplasm. During early entry (t 30 minutes and 45 minutes), Bd2439mNeon remains at both poles, and co-localises with CpoBmCherry at one pole. Later in intraperiplasmic growth (t 1 hour and 2 hours), the second non-CpoBmCherry associated protein accumulation disappears, leaving Bd2439mNeon distributed at one pole and associated with CpoBmCherry within the growing filament. At 3 hours, late in intraperiplasmic growth, Bd2439mNeon becomes more diffusely distributed within the growing filament, as well as remaining highly localised at one pole and associated with CpoBmCherry. Finally, at 4 hours, prey lysis has occurred and the *B. bacteriovorus* filament has septated to form attack phase cells once more, all of which have a bipolar distribution of Bd2439mNeon (Figure 21). At 4 hours, Bd2439mNeon also co-localises with the CpoBmCherry-associated vesicles that are deposited on cellular debris post-prey lysis, confirming their interaction further (Figures 21 and 25).

In contrast, Figure 20 shows that Bd2754mNeon and Bd1815mNeon initially exhibit a diffuse distribution throughout the attack phase cell. Upon attachment and entry into the cell (t 15 minutes and 30 minutes), the fluorescence of Bd2754mNeon and Bd1815mNeon is occluded by the autofluorescence of the prey cell, making any judgement on where either protein localises throughout the duration of the predatory cycle impossible (Figure 22 and 23 respectively). After prey lysis, at 4 hours, Bd2754mNeon and Bd1815mNeon exhibit a diffuse distribution as before. Of note is the co-localisation of Bd2754mNeon with CpoBmCherry within some vesicles deposited on cellular debris at the 4hr timepoint, suggesting that Bd2754mNeon may co-localise with CpoBmCherry at some point during the predatory cycle, and that this is obscured by the autofluorescence of the prey (Figure 22). The majority of these cellular-debris associated vesicles contain CpoBmCherry alone, suggesting

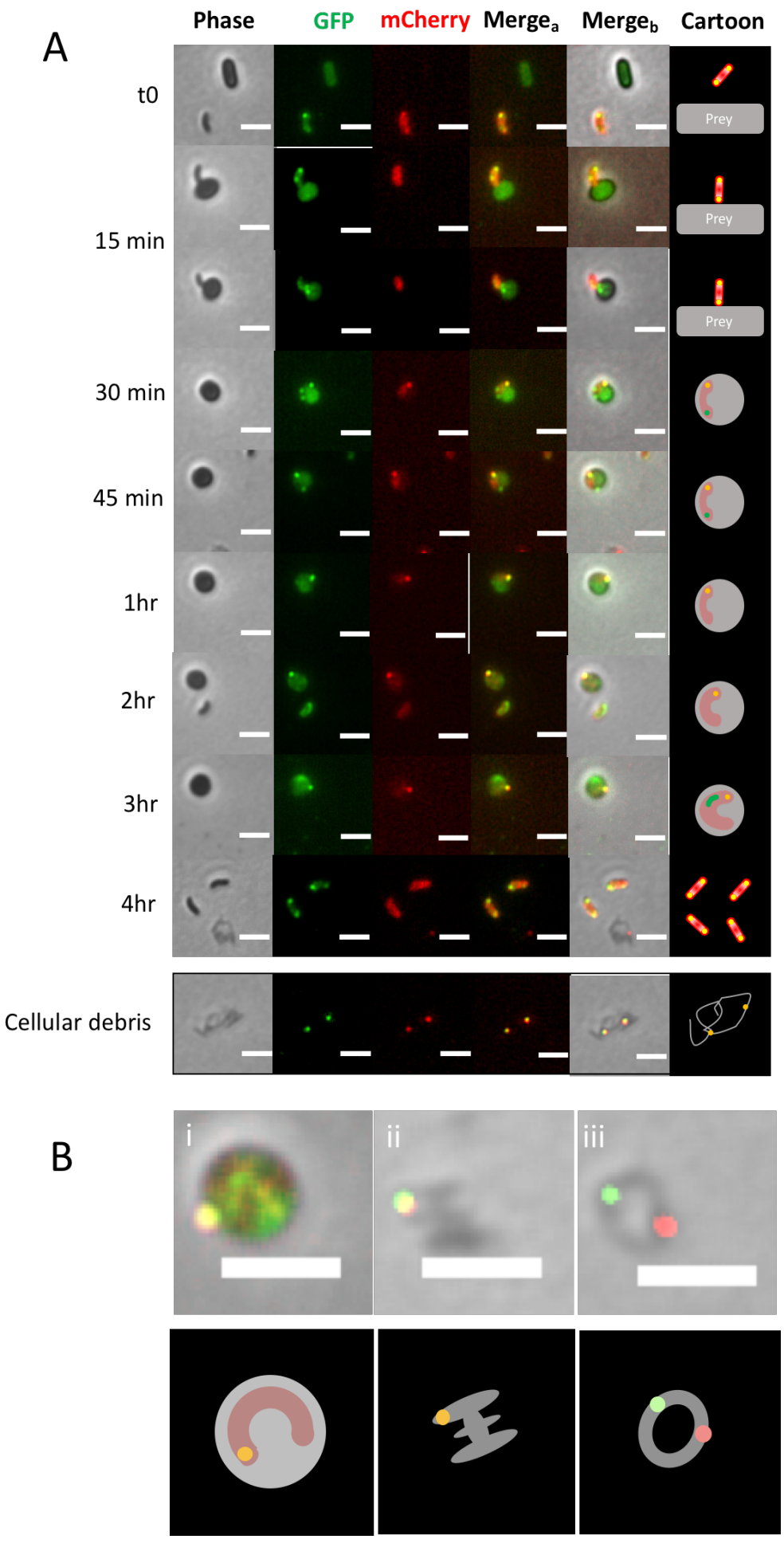

A
Phase
GFP
mCherry
Merge$_a$
Merge$_b$
Cartoon
t0
15 min
30 min
45 min
1hr
2hr
3hr
4hr
Cellular debris
Prey
Prey
Prey
B
i
ii
iii

Figure 21: Expression and co-localization of Bd2439mNeon with CpoBmCherry throughout the *B. bacteriovorus* predatory cycle.
A: Representative images taken at t0, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 3 hours and 4 hours of the expression of the fluorescent mNeon tagged protein Bd2439, within CpoBmCherry tagged *B. bacteriovorus,* preying upon *E. coli* S17-1 pZMR100 prey. Also shown is cellular debris after prey lysis associated with both Bd2439mNeon and CpoBmCherry. B: Close-up images of protein compartments at the 3 hour timepoint, i: within the bdelloplast showing co-localisation of Bd2439mNeon and CpoBmCherry in a distinct compartment at the end of the growing *B. bacteriovorus* filament, and ii: vesicles present on cellular debris containing either Bd2439mNeon and CpoBmCherry together, or iii: in separate, distinct vesicles. At least 10 fields of view were taken for each timepoint. Phase: Exposure 100 ms. GFP: excitation 400-440 nm, emission > 470 nm, Gain 1, exposure time 20 s. mCherry: excitation 550-600 nm, emission 610-665 nm, Gain 1, exposure time 10 s. White scale bars represent 2 µM.

Bd2754mNeon is not present or has been degraded, but a portion of them do contain Bd2754mNeon co-localised with CpoBmCherry (Figure 26). Bd1815mNeon did not co-localise with any CpoBmCherry-associated vesicles after prey lysis (Figure 23).

To determine how frequently Bd2439mNeon co-localised with CpoBmCherry later on in intraperiplasmic growth, when the second accumulation of Bd2439mNeon at the other pole had disappeared, bdelloplasts from each field of view at the 3 hour timepoint were characterised based on whether the foci indicating protein accumulation was green (indicating Bd2439mNeon localisation only), red (indicating CpoBmCherry localisation only) or yellow (indicating co-localisation of both Bd2439mNeon and CpoBmCherry) (Figure 24A). The majority of Bd2439mNeon foci were co-localised with CpoBmCherry at 3 hours into predation (Figure 24B).

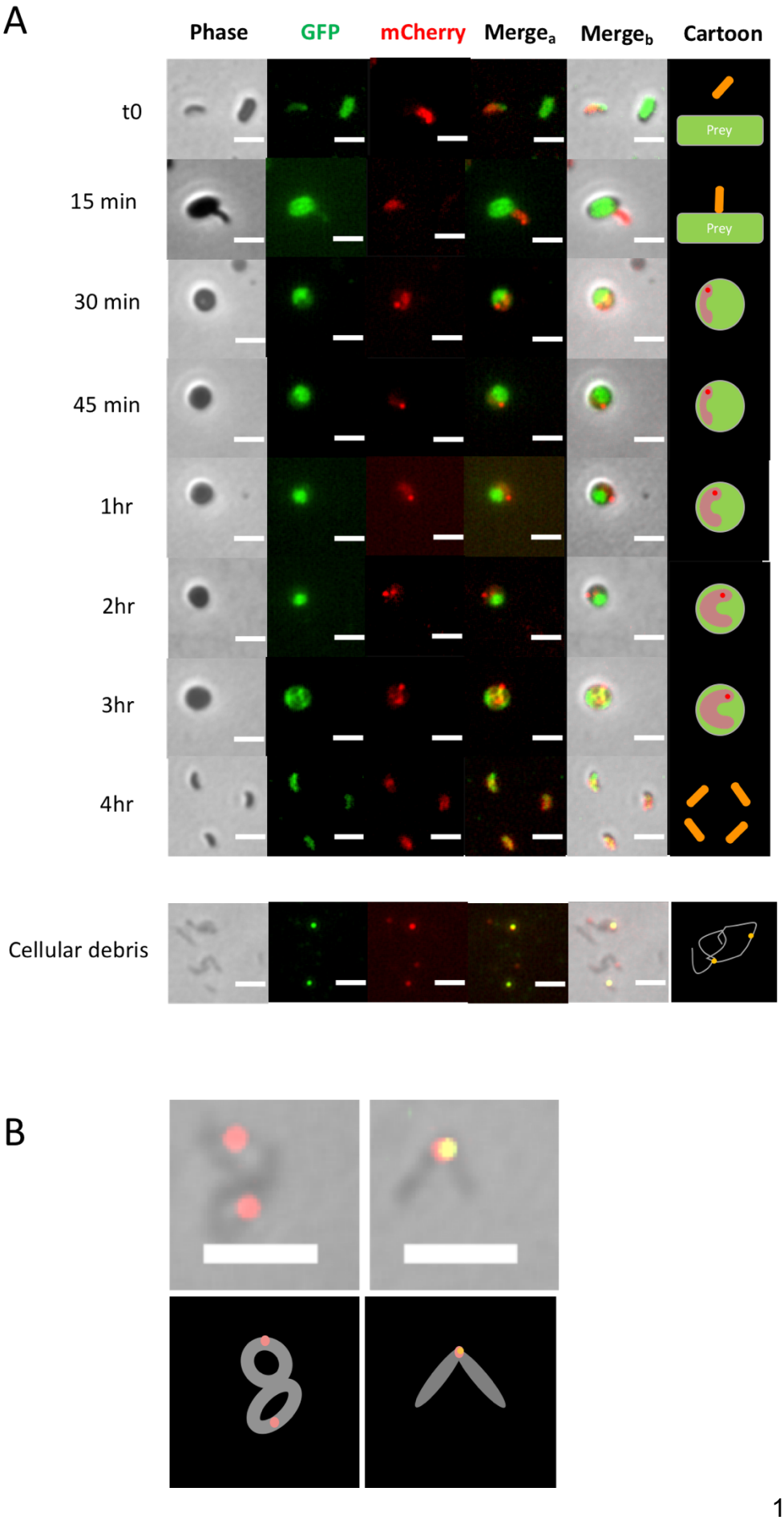

A
Phase
GFP
mCherry
Merge$_a$
Merge$_b$
Cartoon
t0
15 min
30 min
45 min
1hr
2hr
3hr
4hr
Prey
Prey
Cellular debris
B

Figure 22: Expression and co-localization of Bd2754mNeon with CpoBmCherry throughout the *B. bacteriovorus* predatory cycle.
A: Representative images taken at t0, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 3 hours and 4 hours of the expression of the fluorescent mNeon tagged protein Bd2754, within CpoBmCherry tagged *B. bacteriovorus,* preying upon *E. coli* S17-1 pZMR100 prey. Also shown is cellular debris after prey lysis associated with both Bd2754mNeon and CpoBmCherry. B: Close up images of vesicle-associated debris 4 hours into predation where red indicates association with CpoBmCherry and yellow indicates association with CpoBmCherry and Bd2754mNeon. At least 10 fields of view were taken for each timepoint. Phase: Exposure 100 ms. GFP: excitation 400-440 nm, emission > 470 nm, Gain 1, exposure time 20 s. mCherry: excitation 550-600 nm, emission 610-665 nm, Gain 1, exposure time 10 s. White scale bars represent 2 μM.

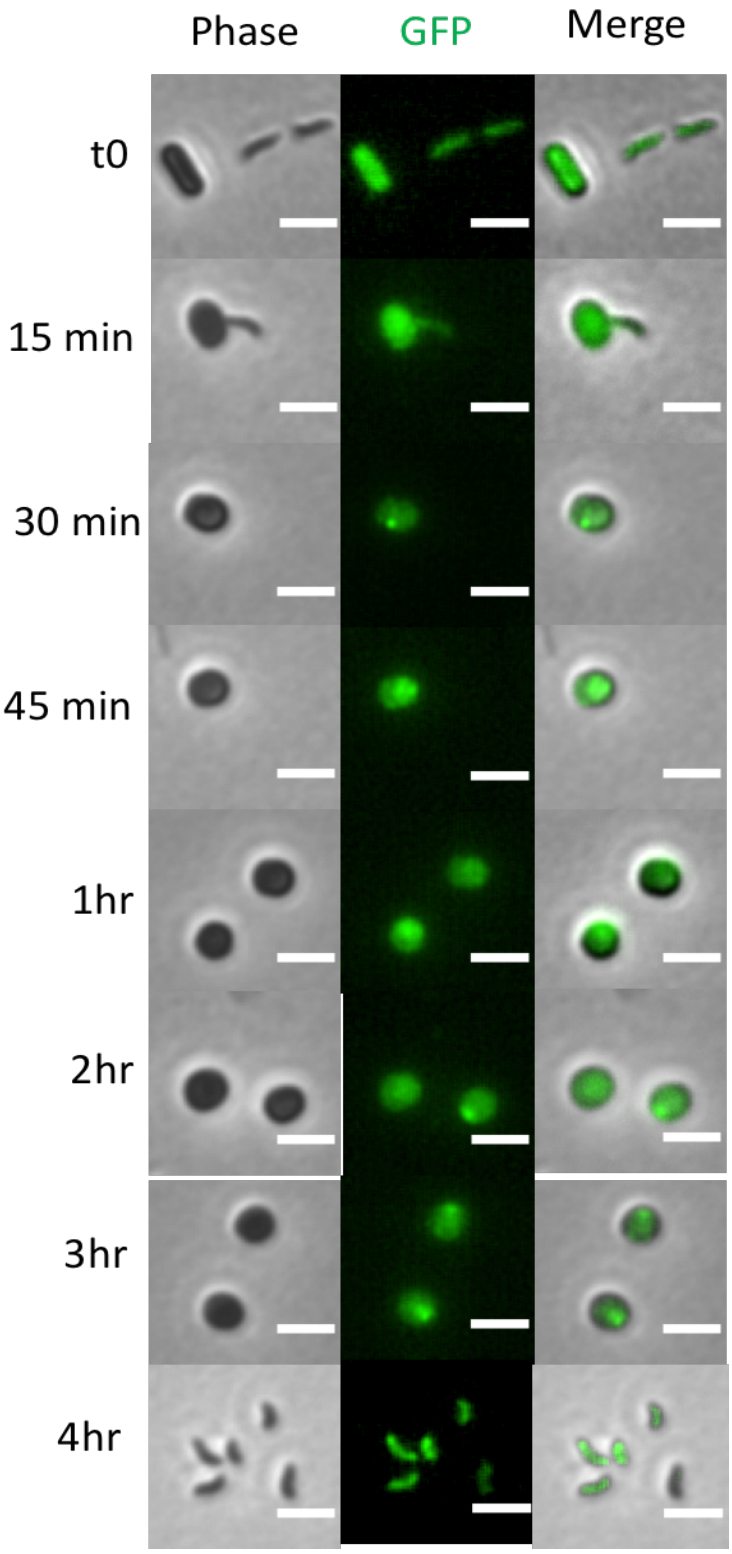


Figure 23: Expression and localization of Bd1815mNeon throughout the *B. bacteriovorus* predatory cycle.
Representative images taken at t0, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 3 hours and 4 hours of the expression of the fluorescent mNeon tagged protein Bd1815 within WT *B. bacteriovorus* preying upon *E. coli* S17-1 pZMR100 prey. Due to high levels of prey autofluorescence in the GFP channel, Bd1815mNeon fluorescence was occluded upon prey attachment and entry, making determination of the localisation and timing of Bd1815mNeon expression throughout the predation cycle undiscernible. At least 10 fields of view were taken for each timepoint. Phase: Exposure 100 ms. GFP: excitation 400-440 nm, emission > 470 nm, Gain 1, exposure time 20 s. White scale bars represent 2 μM.

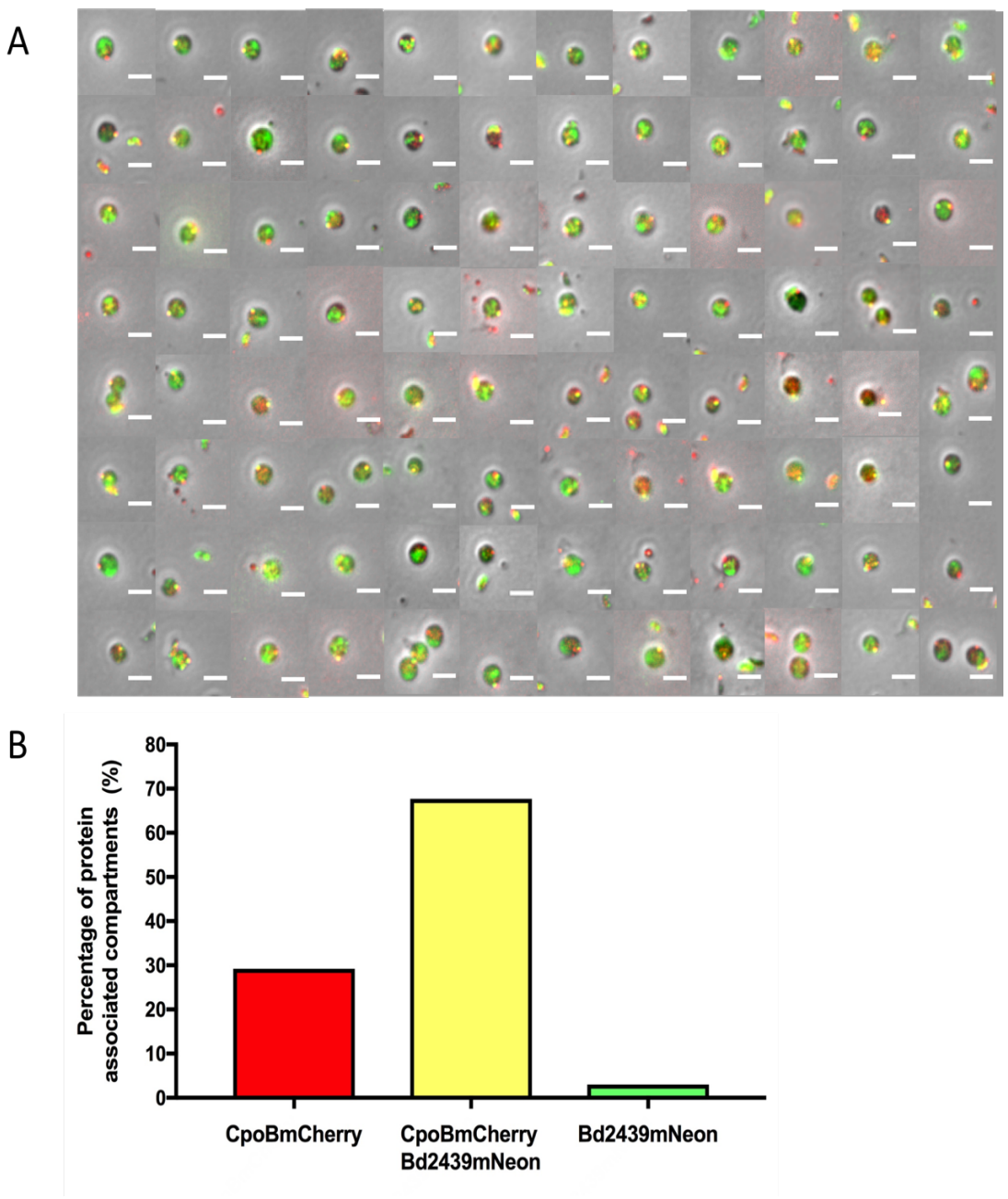


Figure 24: Gallery of expression and co-localization of Bd2439mNeon with CpoBmCherry in bdelloplasts at the 3 hour timepoint of the *B. bacteriovorus* predatory cycle.
A: Representative merge images of bdelloplasts taken at 3 hours into the predatory cycle visualizing the expression of the fluorescent mNeon tagged protein Bd2439 within CpoBmCherry tagged *B. bacteriovorus* preying upon *E. coli* S17-1 pZMR100 prey. Images taken from 10 fields of view. Phase: Exposure 100 ms. GFP: excitation 400-440 nm, emission > 470 nm, Gain 1, exposure time 20 s. mCherry: excitation 550-600 nm, emission 610-665 nm, Gain 1, exposure time 10 s. Scale bars represent 2 μM. B: Bd2439mNeon and CpoBmCherry colocalize within the majority (approximately 70%) of protein compartments 3 hours into predation. The colours of the vesicles present within the bdelloplasts were scored as red (indicating the presence of CpoBmCherry but not Bd2439mNeon), green (indicating the presence of Bd2439mNeon but not CpoBmCherry) or yellow (indicating the co-localization of both CpoBmCherry and Bd2439mNeon) and graphically depicted. Due to time constraints a single dataset was collected, and so standard errors were not calculated. Calculation of standard errors will be carried out after two further independent experiments.

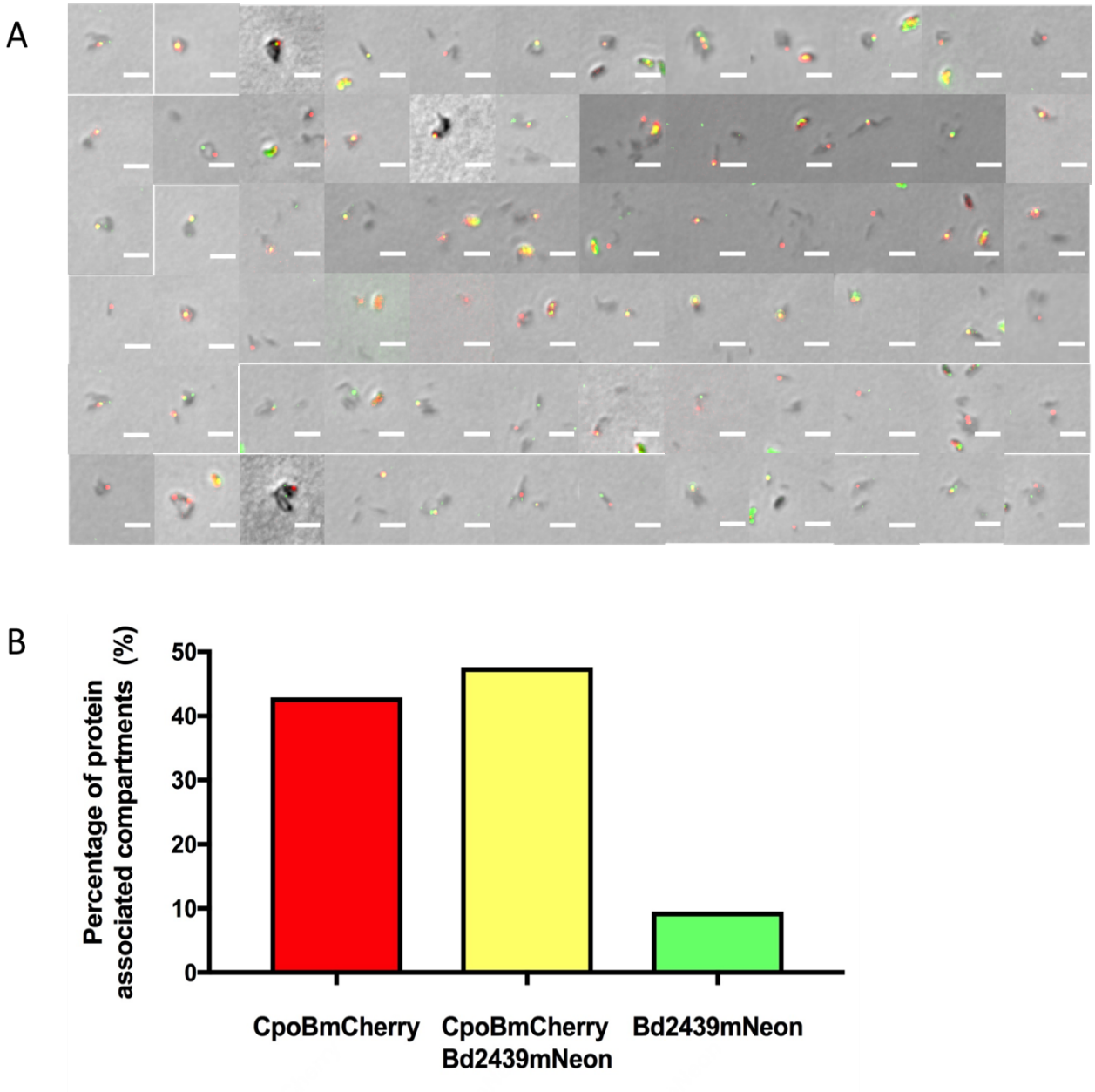
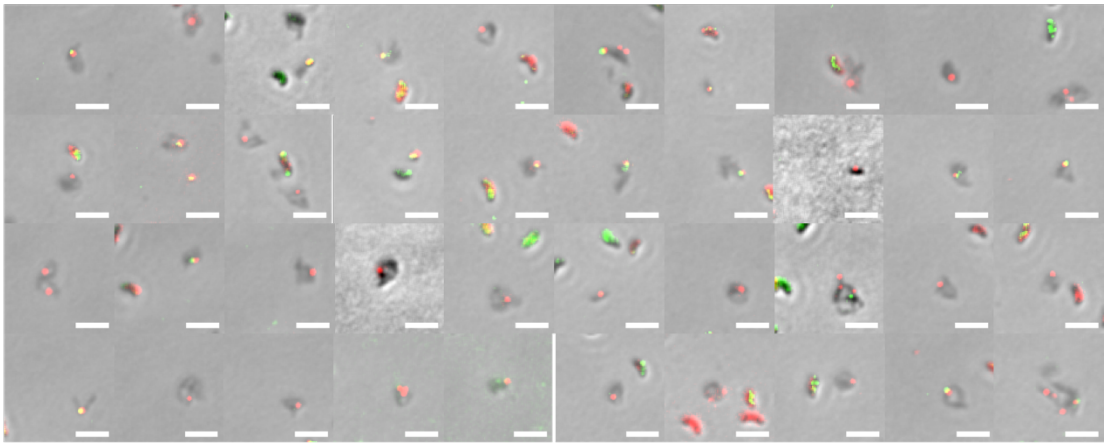


Figure 25: Gallery of expression and co-localization of Bd2439mNeon with CpoBmCherry within cellular debris-associated vesicles 4 hours into the *B. bacteriovorus* predatory cycle. A: Representative merge images of cellular-debris associated vesicles taken at 4 hours into the predatory cycle visualizing the expression of the fluorescent mNeon tagged protein Bd2439 within CpoBmCherry tagged *B. bacteriovorus* preying upon *E. coli* S17-1 pZMR100 prey. Images taken from 10 fields of view. Phase: Exposure 100 ms. GFP: excitation 400-440 nm, emission > 470 nm, Gain 1, exposure time 20 s. mCherry: excitation 550-600 nm, emission 610-665 nm, Gain 1, exposure time 10 s. Scale bars represent 2 μM. B: Bd2439mNeon and CpoBmCherry colocalize within a similar number of vesicles 4 hours into predation, as CpoBmCherry alone. The colours of the vesicles present within the vesicles were scored as red (indicating the presence of CpoBmCherry but not Bd2439mNeon), green (indicating the presence of Bd2439mNeon but not CpoBmCherry) or yellow (indicating the co-localization of both CpoBmCherry and Bd2439mNeon) and graphically depicted. Due to time constraints a single dataset was collected, and so standard errors were not calculated. Calculation of standard errors will be carried out after two further independent experiments.

A



B

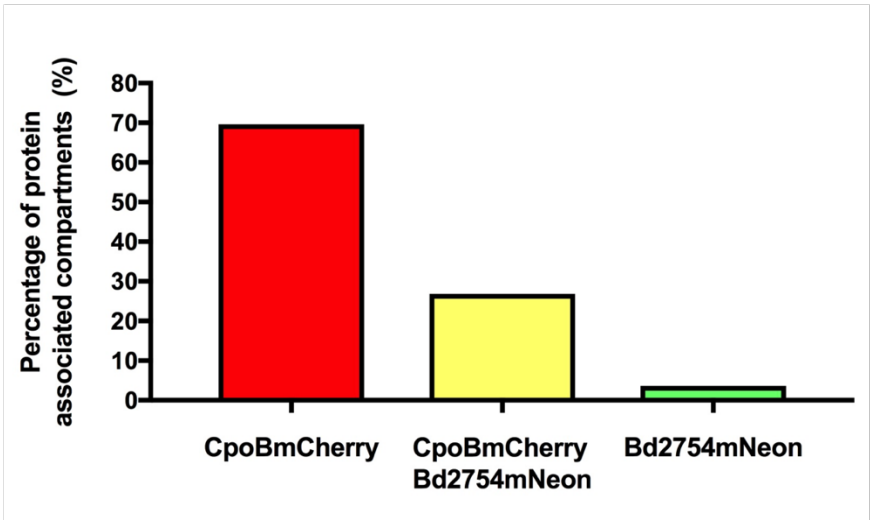


Figure 26: Gallery of expression and co-localization of Bd2754mNeon with CpoBmCherry <u>within cellular debris-associated vesicles</u> 4 hours into the *B. bacteriovorus* predatory cycle.
A: Representative merge images of cellular-debris associated vesicles taken at 4 hours into the predatory cycle visualizing the expression of the fluorescent mNeon tagged protein Bd2754 within CpoBmCherry tagged *B. bacteriovorus* preying upon *E. coli* S17-1 pZMR100 prey. Images taken from 10 fields of view. Phase: Exposure 100 ms. GFP: excitation 400-440 nm, emission > 470 nm, Gain 1, exposure time 20 s. mCherry: excitation 550-600 nm, emission 610-665 nm, Gain 1, exposure time 10 s. Scale bars represent 2 μM. B: Bd2754mNeon and CpoBmCherry colocalize within a smaller number of vesicles 4 hours into predation, than CpoBmCherry alone. The colours of the vesicles present within the vesicles were scored as red (indicating the presence of CpoBmCherry but not Bd2754mNeon), green (indicating the presence of Bd2754mNeon but not CpoBmCherry) or yellow (indicating the co-localization of both CpoBmCherry and Bd2754mNeon) and graphically depicted. Due to time constraints a single dataset was collected, and so standard errors were not calculated. Calculation of standard errors will be carried out after two further independent experiments.

4.0 Discussion

4.1 Importance

There is a desperate need for novel antimicrobial therapies to treat human diseases caused by the Gram-negative members of the ESKAPE pathogens, due to the loss of other antimicrobial therapies to antimicrobial resistance. The use of *B. bacteriovorus* as a “living antibiotic”, or the enzymatic components of its predatory lifestyle as enzybiotics represent two potential alternatives. This will only be possible by advancing understanding of the intricacies of the predatory process. One aspect of predation that is currently being actively studied by our lab, and which is poorly understood is the role and composition of a predatory vesicle that is deposited during predation. The mechanism of synthesis, function and composition of the vesicle remains unclear. Identifying the proteins present in this vesicle, and their role in predation will facilitate further understanding of the role of the vesicle within the predatory cycle and may yield new antimicrobial proteins.

To investigate the role of the vesicle within predation, namely its components and their individual roles in predation, this study fluorescently tagged three proteins, previously identified proteomically by Dr Carey Lambert in our lab, to be found in purified predation-associated vesicles. This vesicle was previously discovered by Sam Greenwood and Asmaa Al Bayati in our lab as containing the regulatory protein CpoB – a known controller of peptidoglycan-modifying enzyme PBP1B. An mNeon tag was fused at the C-terminus of each protein, allowing the visualisation of the dynamic localisation and expression of these proteins throughout the predatory cycle via microscopy.

4.2 Bd2439 is a potential facilitator of attachment to prey

Bd2439 is a cell wall anchor family protein with unknown function, that contains a pilin domain (KEGG). Cell wall anchor family proteins have been speculated to provide reversible and irreversible modes of attachment to aid in predator-prey interactions during prey recognition, attachment and

invasion (261,262). In this study, we found that Bd2439 localises at both poles within attack phase *B. bacteriovorus.* Bd2439 may therefore act as an initiator of attachment to the prey cell, whereby it can both detach, or stimulate further attachment to prey via other pili. Close structural homology to the serine endopeptidases *S. flexneri* SepA, *E. coli* EspP and *E. coli* HBP, all of which are translocated to the outer membrane and have protease activity to allow for the cleaving of peptide bonds, may suggest a similar role of Bd2439 whereby it may promote reversible attachment to a surface and so plays a role in attachment and invasion. A role in attachment is also supported by the presence of a pilin domain within the protein, which may suggest possible attachment at a second site, allowing for a closer attachment and bringing the cell membranes into contact to facilitate the next stages of invasion.

Further study of Bd2439 at Birmingham found that it belonged to the 4UW8 bacteriophage T5 fibre family of proteins (Dr Andrew Lovering, personal communication). These proteins have an exposable carbohydrate domain on their tip which recognises oligomannose on the bacterial cell surface prior to infection, facilitating attachment (117).

The bipolar distribution of Bd2439 is subsequently lost approximately 1 hour into predation, suggesting that it is only required at one end of the cell and so is turned over and lost at the other, giving a sense of polarity. This bipolar distribution is subsequently restored within the attack phase progeny cells released post-prey lysis, when attack phase cells are seeking out new prey and have a need for attachment and invasion.

Co-localisation of Bd2439 with CpoB occurred 30 minutes into predation, and persisted until prey lysis at 4 hours, and beyond within the vesicles deposited on cellular debris. There has been no documented evidence of cell wall anchor family proteins associating with CpoB in current literature. CpoB has been shown to be responsible for the modulation of penicillin binding protein 1B activity during peptidoglycan synthesis in non-predatory bacteria and for invagination of the outer membrane through its interactions with the Tol-Pal system (292,298). Possibly, the close association of CpoB and Bd2439

throughout the intraperiplasmic growth phase of the filamentous *B. bacteriovorus* cell may facilitate the incorporation of Bd2439 into the outer membrane of the growing filament, supported by the presence of a diffuse streak at 3 hours (Figure 21), whereby Bd2439 is both localized diffusely along the length of the filament and tightly associated with CpoB at the pole in a tight foci. The majority of foci at this 3hr stage of predation contain both CpoB and Bd2439, supporting their close association (Figure 24). Upon nutrient exhaustion and septation of the cell, Bd2439 would be present within the outer membrane and able to facilitate immediate binding to further prey cells upon progeny release.

The mechanism behind the invasion of the prey cell remains unknown. Bd2439 may play a role in the attachment and invasion process. Future work would aim to confirm this and will be detailed in Chapter 4.

4.3 Bd1815 and Bd2754 are potential novel porins

Bd1815 and Bd2754 are hypothetical proteins with unknown functions. They show a diffuse distribution within attack phase *B. bacteriovorus.* Bioinformatic analysis of the amino acid sequence of both proteins revealed that they were likely outer membrane proteins (OMPs) or porins due to their close structural homology to novel OMPs in *B. bacteriovorus* and to OMPs outside of the species e.g. OmpA of E.coli and OprH of *P. aeruginosa* (Beck *et al.*, 2004; ITASSER). The similarity with these porins suggests that Bd1815 and Bd2754 may play roles in nutrient acquisition or molecular transport into or out from the vesicle or the predator cell during predation. Although the sequence similarity and alignment is poor between these proteins and their structural homologues (potentially explaining the lack of BLAST homologues), the nature of their structures and comprising amino acids is similar so in a role where structure defines function, these proteins have a similar structure to known *E. coli* porins and therefore I inferred a similar function also.

The localisation of Bd1815 and Bd2754 was not determined beyond attack phase in the predatory cycle due to a technical difficulty of the fluorescence

of the mNeon tagged proteins being obscured by the autofluorescence of the prey *E. coli* cells, although attack phase progeny released after prey lysis also showed the same diffuse, cytoplasmic distribution. This study also found that Bd2754, but not Bd1815, was deposited in the CpoB-associated vesicles deposited on cellular debris after predation. This suggests that some interaction between CpoB and Bd2754 may occur during the predatory cycle, but that it is occluded by the prey cell autofluorescence, making further investigation of this interaction prudent. TolA, part of the Tol-Pal system responsible for outer membrane invagination during septation in other bacteria, has previously been shown to interact with CpoB and *E. coli* outer membrane proteins e.g. OmpC, making interactions between CpoB and other OMPs also likely (298,299). The interaction between CpoB and OmpC in *E. coli* was not characterised but it is likely to involve the incorporation of OmpC into the outer membrane during peptidoglycan and outer membrane synthesis. The Tol-Pal system is speculated to bring the membranes into closer contact, aiding the incorporation of outer membrane components such as OMPs (300). It may also catalyse porin biogenesis or aid regulation of porin activity through interactions with TolA and TolB (299). The specific role of CpoB in these protein complexes is yet to be determined but its interaction with Bd2754 is likely to be regulatory or to aid incorporation into the outer membrane.

Very little is known about the potential roles of porins within *B. bacteriovorus* other than for typical nutrient acquisition during intraperiplasmic growth. As nutrient acquisition is such a vital part of the predatory lifecycle, the locations of porins throughout the predatory cycle is of great interest. The architecture and arrangement of porins throughout the predatory cycle is keenly debated. One theory suggests that the localisation or porins within both predator and prey cytoplasmic and outer membranes is highly co-ordinated and regulated, to form a continuous channel between the prey cell cytoplasm and the predator cytoplasm (via 3 cellular membranes), facilitating the intraperiplasmic growth of *B. bacteriovorus* and the death of the prey cell (301).

The source of these porins, i.e. whether they originate from the outer membrane of the predator or the prey cell, is highly contested with some suggesting that *B. bacteriovorus* induces the insertion of OmpF (another *E. coli* OMP) into the prey cell membrane when it preys upon *E.coli* (287,302,303) and others suggesting it is a predator derived OmpF homolog that is inserted (301,304). This difference has been suggested to be due to the *B. bacteriovorus* and prey strains used. Irrespective of this, the presence of porins within both predator and prey membranes has been shown and is likely to be of vital importance for the predatory cycle (305) and so it is logical that they would be present within a vesicle derived of one or both of these membranes, irrespective of what their role within the vesicle and predation may be.

Preliminary time-course results suggest that Bd1815 does not form part of the vesicular proteome, as it does not appear to be expressed within the vesicle that is present at the predator-prey interface during predation or within the vesicle deposited within cellular debris upon prey lysis. Bd1815 does not appear to be vesicular in its distribution, despite being identified within vesicle preparations as being present within the vesicle. This disparity may be due to the vesicle isolation process in the first instance. Vesicles were isolated both by filtration through 0.22 nm filters to isolate purified vesicles and limit the presence of cellular debris or separated by percol gradient (which may contain a mixture of purified vesicles and vesicles with membrane and cellular debris attached). This associated membrane may belong to *B. bacteriovorus* (either cytoplasmic or outer membrane) or prey *E. coli* (cytoplasmic or outer membrane) and may be present due to lysis of both predator and prey in vesicle preps. Bd1815 was identified as being a predicted vesicular protein as it was present in both of these vesicle preparations. However, Bd1815 may be present within the vesicular prep but not vesicular due to contamination of the vesicle preparations with membrane components, where these porin-like proteins are likely to have been previously incorporated into.

Very little is known about the porins that *B. bacteriovorus* uses for nutrient acquisition within its predatory lifestyle, making the ascribing of a porin-like

function to Bd2754 and Bd1815 more difficult. The NCBI database contains 15 porin family proteins belonging to *B. bacteriovorus*, all of which have had no further studies conducted on them (at time of writing). Beck et al, 2004 researches 3 porins of *B. bacteriovorus* in detail which are highly conserved throughout their sequence when aligned (Promals3D). Upon comparison to Bd1815 and Bd2754, approximately the first 20 residues align well, a region that is known to comprise the signal peptide for these porin proteins, but the remainder did not align as well. Further study revealed that regions of sequence did align well regarding conserved amino acid nature, but not sequence identity itself. The poor overall alignment is suggested to be due to Bd1815 and Bd2754 being approximately half the size of the outer membrane proteins within Beck et al.'s study, a theme that is consistent with other porins e.g. OmpA whereby our two proteins of interest are approximately half the size. Further consultation of the NCBI protein database shows that porin family proteins of *B. bacteriovorus* range from 170 amino acids in length to 532 amino acids, placing our predicted porins at the smaller end of this range (NCBI) and potentially explaining the lack of homology with other proteins.

It was observed that both Bd1815 and Bd2754 were diffusely distributed within attack phase *B. bacteriovorus,* and due to the presence of a signal peptide in each protein they were concluded to not be cytoplasmically distributed, instead studded into the outer membrane. However, they are not uniformly distributed throughout the cell. The absence of fluorescent protein expression at the periphery of the cell and its high expression towards the middle of the cell is due to the distribution of the *B. bacteriovorus* genome and cellular assembly machinery. The *B. bacteriovorus* genome is of comparable size to the *E. coli* genome (3.85 Mb c.f. 4.7 Mb), and yet it is contained within a cell approximately a quarter of the size, making the cellular machinery and genome highly compact within the cell. This influences the fluorescence distribution of memrane proteins away from the edges of the cell (where the ribosomes are found) and towards the centre where the light passing through the cell is less scattered by the genome than it is by the ribosomes (306,307). If they did show a cytoplasmic distribution,

this may suggest that the porin has a degenerate signal peptide and is not successfully translocated across the cytoplasmic membrane.

4.4 Limitations

The major limitation of this study is the lack of biological repeats of the timecourse experiments due to time constraints, preventing any firm conclusions being drawn from this study without the need for further experimental confirmation. One further regrettable limitation is the inability to discern the distribution of Bd1815 and Bd2754 throughout the predatory cycle due to the lack of fluorescent signal intensity compared to highly GFP autofluorescent prey, although alternative prey was tested including *Klebsiella pneumoniae, E. coli* K12 and *Serratia marscens*.

Possible explanations for this may be that the expression of this protein is highly diffuse throughout the cell and so we do not get a strong, localised expression pattern, or the fluorescent tag may be interfering with expression in some way. Future work would aim to remedy this and is further detailed in Chapter 4.

The main findings of this study are summarised in Figure 27.

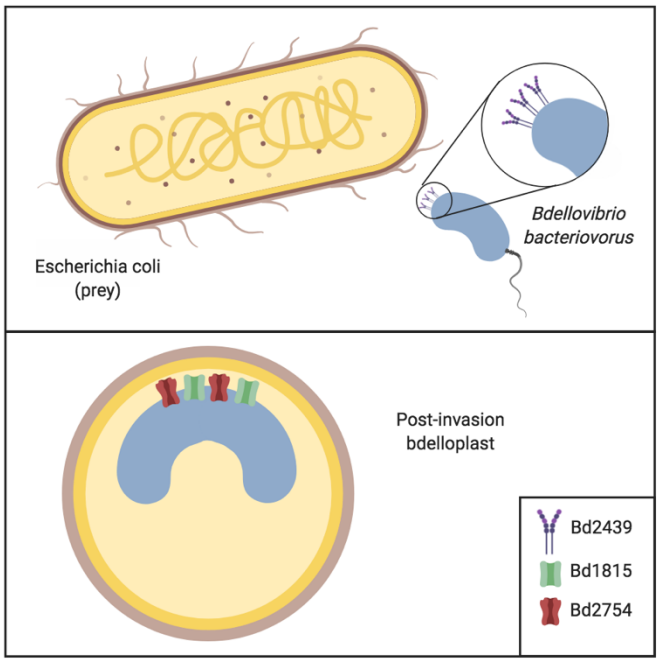


Figure 27: A mechanistic representation of the roles of Bd1815, Bd2754 and Bd2439, the three proteins investigated in this study
In this study, the roles of Bd2439, Bd1815 and Bd2754, three previously unannotated proteins of unknown function, were investigated. A: This study found that Bd2439 likely plays a role in attachment of the predator, or components of its predatory cycle, to the prey cell. B: Bd1815 and Bd2754 were suggested to be outer membrane proteins or porins, making a role in nutrient acquisition within the post-invasion bdelloplast highly likely. Figure generated using Biorender software.

5.0 Concluding remarks

This project aimed to investigate the function of three proteins predicted to be present within an active vesicle deposited in prey after predation by *B. bacteriovorus* HD100, namely Bd1815, Bd2439 and Bd2754. Fluorescent tagging of Bd2439, a cell surface anchor family protein with potential roles in reversible attachment to prey, revealed that it localises in a bipolar fashion within attack phase cells and subsequently co-localises in a vesicle with CpoB later on in the predatory cycle. Bd2439 may act as a potential facilitator of initial attachment to prey during attack phase, after which it then co-localises with CpoB to ensure its incorporation into the membrane of the growing *B. bacteriovorus* filament or into the vesicle. Bioinformatic analyses of Bd1815 and Bd2754 protein structures reveals them to be two novel outer membrane porins, with potential roles in nutrient acquisition or macromolecule transport within or between cells. Bd1815 and Bd2754 are thought to act as porins or outer membrane proteins for the transport of nutrients or other macromolecules between the growing *B. bacteriovorus* filaments cytoplasm and the prey cell cytoplasm. Future experiments will aim to elucidate on the localisation of these porins and conclusively determine the role of all three proteins, with the aim of answering what the role of these proteins, and the vesicle, is during predation.

Chapter 4: Future directions

1. Summary of findings

1.1 Overview of MRes findings

Within this MRes thesis, I have explored the importance of the consideration of the host immune response when administering conventional antimicrobial therapies, and when contemplating novel therapies. Not only does the immune system work synergistically with some novel antimicrobial therapies such as the vital co-ordination between the predatory bacterium *Bdellovibrio bacteriovorus* and the leukocytes of the zebrafish larvae immune response in the resolution of antibiotic resistant *S. flexneri* infection (120), a feature that should be fully utilised in the future administration of *B. bacteriovorus* therapy, this thesis outlines the potential problems that arise when the host immune response is not considered i.e. the vancomycin-mediated defect in antifungal immunity that leads to an increased predisposition to lethal fungal infection.

This thesis has explored the impact of vancomycin, one of the many antibiotics administered to patients that are immunocompromised or at increased risk of bacterial and fungal infection, on predisposition to systemic fungal infection through the *in-vitro* characterisation of the previously observed phagocytic killing defect present within murine systemic candidiasis infection. This stresses the importance of the consideration of the host immune response when administering antimicrobial treatments, a failure of which predisposes to further morbidity and mortality. This is an ever-growing problem due to the combined loss of antimicrobial therapies to antimicrobial resistance and the increase in the proportion of immunocompromised individuals present within an ageing population.

This thesis has also explored proteins important to the predatory process which may be utilised if *B. bacteriovorus* is applied as a novel antimicrobial therapy, an approach that may have a wide-spanning impact on the treatment of a range of Gram-negative, antibiotic resistant infections in the

future. The use of *B. bacteriovorus* as a “living” antimicrobial therapy would be a sharp contrast to the use of evolutionarily static, chemical, conventional antibiotics that succumb to the development of resistance formation due to their static nature, something that is unlikely to occur (and has not been seen to form within *Bdellovibrio*-prey bacteria tested so far). With *B. bacteriovorus* treatment, due to its scope to co-evolve with the pathogen to negate resistance development, and due to its multi-target predation approach, resistance is unlikely. However, the introduction of a live, therapeutic bacterium into a host that is already compromised by bacterial infection will require thorough study before it can be implemented, including study of the interactions that will occur between *B. bacteriovorus* and the host immune system (something that is already beginning to be explored by our lab). It is therefore essential that we fully understand the mechanisms underlying the predation process before we can begin to consider the use of *B. bacteriovorus* as an antimicrobial treatment in humans.

This thesis presents the findings from two short rotation projects, the first characterising the negative, immunomodulatory effects that antimicrobial treatments can have on the host, and the further dangers they may pose if not considered in the future, and the second exploring the promising potential of *B. bacteriovorus* as a novel treatment for antibiotic resistant Gram-negative infections, which will be aided by the increased understanding of the mechanisms underlying the predation process (something this rotation project aimed to achieve).

2. Future work

2.1 University of Birmingham rotation project

In my first rotation, based at the University of Birmingham, I investigated the effect of vancomycin, a regularly administered bactericidal antibiotic, on the ability of murine macrophages to kill the fungus *Candida albicans* due to an observed increase in mortality seen with vancomycin treatment for invasive *C. albicans* infection (Drummond et al, unpublished). I hypothesised that this increase in mortality was due to a vancomycin-induced immune defect within the host. Investigation of this, using murine primary bone marrow derived macrophages, revealed the presence of a fungal killing defect associated with vancomycin treatment. This was later compounded by a decrease in Dectin-1 and Complement receptor 3 expression, two pattern recognition receptors important for *C. albicans* detection, and a decrease in expression of the proinflammatory cytokine IL-1β. These effects were attributed to a decrease in mitochondrial membrane potential seen within vancomycin treated macrophages, whereby previous studies have shown that membrane potential perturbation can result in increased and dysregulated production of reactive oxygen species and extensive oxidative damage to the host, but have not applied them to the context of an fungal infection immune defect.

Future work would aim to elucidate the exact mechanism and point of the fungal detection and killing pathway that is being affected by vancomycin (likely to be associated with mitochondrial membrane potential), and the downstream effects of this. Initial studies would seek to characterise whether an increase in ROS generation is present in vancomycin treated macrophages, which would align our findings with previous studies, through the use of fluorescent ROS and superoxide dyes and the probing of oxidative damage markers such as lipid peroxidation within host macrophages.

Another effect of the decreased mitochondrial membrane potential seen within other studies could be a decrease in ATP synthesis. As fungal killing involves the ATP-dependent synthesis and export of a wide array of receptors and cytokines, as well as cytoskeleton manipulation for phagocytosis and killing, it is therefore a highly energetically expensive

process. Any decrease in ATP generation is likely to have a large impact on fungal killing and the immune response, therefore the study of the effect of vancomycin on ATP synthesis through the use of an ATP luciferase assay would be encouraged, as this may potentially explain the other defects seen in this study (PRR downregulation and decreased IL-1β expression) and uncover further defects.

Once an overarching mechanism is proposed for this immune defect, further investigation within other phagocyte types such as neutrophils, which are also one of the main cell types involved in the antifungal immune response, and human primary phagocytes to see if we observe a similar decrease in fungal killing and mitochondrial membrane potential (via the same investigations carried out in this study) would also improve the scope of these findings further. Application to *ex-vivo* gut models and murine *in-vivo* models to test potential modulators of this effect, and the expanding effects of this immune system on the adaptive immune response would also be important.

Overall determination of the specific point of the antifungal immune response that vancomycin affects would aid the improved stewardship surrounding antibiotic use and educate clinical practise further to encourage the consideration of increased fungal infection risk in patients in the future.

2.2 University of Nottingham rotation project

In my second rotation, based at the University of Nottingham, I investigated the function of three hypothetical proteins that were proteomically predicted to be associated with a predatory vesicle of unknown composition and function. We hypothesised that these proteins would play a role in the predatory cycle of *B. bacteriovorus* as they were identified by Dr Carey Lambert in a proteomic analysis of a vesicle that is deposited within prey during predation and remains after the completion of predation, but could not predict a role for them as they were unannotated and had never previously been worked on. By fluorescently tagging these proteins and studying their distribution throughout the predator cell during predation, combined with bioinformatic analysis, we found that Bd2439 was likely to play a role in the

attachment of the vesicle to the prey cell during predation, and possibly in the transfer from predator to prey.

Future work would aim to characterise the importance of Bd2439 in attachment during predation through the generation of a gene knockout mutant and subsequent phenotyping through microscopic analysis of any predatory cycle, or its stalling point, in the absence of Bd2439. If predation was altered in any discernible way, this would confirm our hypothesis that Bd2439 plays a role in the predatory cycle. Other future work would consist of the use of a bacterial-two-hybrid assay, an assay that reveals protein-protein interactions, to determine what other counterparts of predation Bd2439 interacts with, after which the elaboration of the roles of those proteins could be undertaken (if unknown) or the further confirmation of the role of Bd2439 in predation could be achieved (if Bd2439 interacts with proteins of known function).

In this study, we also found that Bd1815 and Bd2754, identified in the vesicle-enriched proteome, were likely to be porins involved in nutrient acquisition of macromolecule transport between the predator cell and either the prey cell or the external environment. The distribution of these proteins was not determined after attack phase due to technical difficulties involving a loss of detectable mNeon fluorescent signal due to highly autofluorescent prey with similar wavelengths. As previously mentioned, alternative prey species were tested but the fluorescent protein distribution was still indeterminable. Future work would aim to fluorescently tag Bd1815 and Bd2754 with an alternative fluorescent protein, such as mCherry, which have a stronger fluorescent signal, within a channel that prey will be less autofluorescent in. Not only will this aid the determination of the distribution of these two proteins within the predatory cycle, hopefully aiding the annotation of a function also, it will also tell us whether the addition of the mNeon tag within this study interfered with the accumulation or expression of these proteins. Once the distribution of these proteins are known, further studies may determine the importance of these proteins within predation via

the means of gene knockout and phenotyping, or the tagging of other proteins such as other predicted porins, to further confirm their function.

Due to time limitations, each timecourse experiment was only carried out once. It is therefore imperative that subsequent repeats of all time-courses experiments are carried out to confirm these preliminary findings, before further experiments to elucidate the roles of these proteins are carried out.

Ultimately, the determination of the roles of these predicted vesicular proteins will improve our understanding of the role and composition of this novel predatory vesicle, in turn providing us with a further understanding of the mechanisms that underlie the predation process and taking us one step closer to the use of *B. bacteriovorus,* or components thereof, as novel antimicrobial therapies in the future.

3. Reflection and future perspectives

Through the completion of these two research projects, I have gained not only an understanding of immunology, molecular biology, microscopy and molecular cloning within non-model cell types, i.e. primary cells and an atypical and understudied bacterium, but also the tools to continue and expand upon this research, which has potential to impact the future treatment of infectious disease, within an exceptionally supportive environment.

My PhD project will indirectly build on the overall findings of this thesis by further characterising the immunological response to *Bdellovibrio bacteriovorus* predation of clinically relevant pathogens and within host phagocytes, aiding the wider understanding of the potential implications of application of *B. bacteriovorus* as a novel antimicrobial therapy. As demonstrated in this thesis, a lack of consideration is given towards the administration of some antimicrobial therapies with regards to their off-target effects on the host and the immune system, something that should not recur with the potential application of *B. bacteriovorus* as an antimicrobial treatment. This makes the understanding of the interactions between host and *B. bacteriovorus* of increasing importance.

Innovative research has already been carried out by our lab demonstrating the importance of the immune response in the optimal resolution of infection, through the synergy of *B. bacteriovorus* treatment with the host immune response (120). My PhD project will aim to build on this work, by further characterising the immune response both towards predator and prey post-predation and by tailoring the relationship between the immune response and predation to improve infection outcome further.

4. Concluding remarks

Antimicrobial resistance not only threatens the treatment of infectious disease, including the not so well-researched fungal diseases that are increasing in prevalence year-on-year due to an ageing and increasingly immunologically susceptible population, but also the treatment of other diseases such as cancer and organ failure (without antibiotics, routine interventions for these diseases would not be possible). AMR is a multifaceted issue that cannot simply be solved by the discovery of a new antimicrobial therapy, or the education of a single country. It will take a global and continuous effort to enable the continued use of antimicrobial therapies as interventions for infectious disease, meaning that although AMR can be slowed and tackled, it will never be “solved”. Important considerations, both for the continued and extended use of current, conventional antimicrobial therapies, and the implementation of novel therapies such as the predatory bacterium *B. bacteriovorus* hinge around several points:

- The careful and considered use of antimicrobial therapies, as a lack of stewardship surrounding antimicrobial use will only cause an acceleration or a recurrence of this issue. This includes the seeking of alternatives to long-term antibiotic therapies that are given to individuals who are at increased risk of infection, such as the immunocompromised.
- The increased consideration of the off-target effects that antibiotic treatment may be causing, which ultimately have culminated in the emergence of other classes of infectious disease such as systemic fungal infection.
- The increased support that is needed to encourage others to think outside the well-defined box of evolutionarily-static compounds such as natural, chemical antibiotics that are undermined by resistance, even before their use in clinical treatment- a resource that has been exhausted in the last few decades, leading to the current AMR crisis.

The two rotation projects contained within this thesis have discussed the role of the immune system in fighting infectious disease, and how our efforts to combat infectious disease must combine with the host immune response, not hinder it. One approach that has been demonstrated to work alongside the immune response is the use of the predatory bacterium *Bdellovibrio bacteriovorus*, and so this thesis has stressed the importance of and aided the further understanding of the mechanisms underlying the predatory cycle of this atypical bacterium, with the hope of one day aiding the implementation of *B. bacteriovorus* as a antimicrobial treatment of the future. Much future work is needed, as we can only begin to understand the interactions between immunity and predation, two highly complex but fascinating processes, when we understand each system on its own. Together, through the increased understanding of immunity and implementation of this in antimicrobial therapy development, AMR may one day be a scientific curiosity rather than a crisis.

References

1. Chain E, Florey HW, Gardner AD, Heatley NG, Jennings MA, Orr-Ewing J, et al. Penicillin As a Chemotherapeutic Agent. *The Lancet*. 1940;1(1172):226–8.

2. Clark C, Drummond R. The Hidden Cost of Modern Medical Interventions: How Medical Advances Have Shaped the Prevalence of Human Fungal Disease. *Pathogens*. 2019;8(2):45.

3. Vollmer W, Blanot D, De Pedro MA. Peptidoglycan structure and architecture. *FEMS Microbiology Reviews*. 2008;32(2):149–67.

4. Vollmer W, Bertsche U. Murein (peptidoglycan) structure, architecture and biosynthesis in *Escherichia coli*. *Biochimica et Biophysica Acta - Biomembranes*. 2008;1778(9):1714–34.

5. Schleifer KH, Kandler O. Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriological reviews*. 1972;36(4):407–77.

6. Typas A, Banzhaf M, Gross CA, Vollmer W. From the regulation of peptidoglycan synthesis to bacterial growth and morphology. *Nature Reviews Microbiology*. 2012 Feb 28;10(2):123–36.

7. de Kruijff B, van Dam V, Breukink E. Lipid II: a central component in bacterial cell wall synthesis and a target for antibiotics. *Prostaglandins, leukotrienes, and essential fatty acids.* 2008 Sep 1;79(3–5):117–21.

8. Sauvage E, Kerff F, Terrak M, Ayala JA, Charlier P. The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. *FEMS Microbiology Reviews*. 2008;32(2):234–58.

9. Vollmer W, Joris B, Charlier P, Foster S. Bacterial peptidoglycan (murein) hydrolases. *FEMS Microbiology Reviews.* 2008;32(2):259–86.

10. Yocum RR, Waxman DJ, Rasmussen JR, Strominger JL. Mechanism of penicillin action: penicillin and substrate bind covalently to the same active site serine in two bacterial D-alanine carboxypeptidases. *Proceedings of the National Academy of Sciences.* 1979 Jun 1;76(6):2730–4.

11. Yocum RR, Rasmussen JR, Strominger JL. The mechanism of action of penicillin. Penicillin acylates the active site of *Bacillus stearothermophilus* D-alanine carboxypeptidase. *The Journal of biological chemistry.* 1980 May 10;255(9):3977–86.

12. Hiramatsu K. Vancomycin-resistant *Staphylococcus aureus* : a new model of antibiotic resistance. *The Lancet Infectious Diseases*. 2001 Oct;1(3):147–55.

13. Stone KJ, Strominger JL. Mechanism of Action of Bacitracin: Complexation with Metal Ion and C55-Isoprenyl Pyrophosphate. *Proceedings of the National Academy of Sciences*. 1971 Dec 1;68(12):3223–7.

14. Epand RM, Walker C, Epand RF, Magarvey NA. Molecular mechanisms of membrane targeting antibiotics. *Biochimica et Biophysica Acta (BBA) - Biomembranes*. 2016 May;1858(5):980–7.

15. Drlica K, Zhao X. DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiology and molecular biology reviews : MMBR*. 1997 Sep;61(3):377–92.

16. Roca J. The mechanisms of DNA topoisomerases. *Trends in biochemical sciences*. 1995 Apr;20(4):156–60.

17. Cavalleri B, Turconi M, Tamborini G, Occelli E, Cietto G, Pallanza R, et al. Synthesis and biological activity of some derivatives of rifamycin. *Journal of medicinal chemistry*. 1990 May;33(5):1470–6.

18. Yoshizawa S. Structural origins of gentamicin antibiotic action. *The EMBO Journal*. 1998 Nov 16;17(22):6437–48.

19. Carter AP, Clemons WM, Brodersen DE, Morgan-Warren RJ, Wimberly BT, Ramakrishnan V. Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. *Nature*. 2000 Sep 21;407(6802):340–8.

20. Schlünzen F, Zarivach R, Harms J, Bashan A, Tocilj A, Albrecht R, et al. Structural basis for the interaction of antibiotics with the peptidyl transferase centre in eubacteria. *Nature*. 2001 Oct 25;413(6858):814–21.

21. Long KS, Porse BT. A conserved chloramphenicol binding site at the entrance to the ribosomal peptide exit tunnel. *Nucleic Acids Research*. 2003 Dec 15;31(24):7208–15.

22. Lobanovska M, Pilla G. Penicillin's Discovery and Antibiotic Resistance: Lessons for the Future? Vol. 90, *YALE JOURNAL OF BIOLOGY AND MEDICINE*. 2017.

23. Rammelkamp CH, Maxon T. Resistance of *Staphylococcus aureus* to the Action of Penicillin. *Experimental Biology and Medicine*. 1942 Dec 1;51(3):386–9.

24. Lowy FD. Antimicrobial resistance: the example of *Staphylococcus aureus*. *Journal of Clinical Investigation.* 2003 May 1;111(9):1265–73.

25. Hartman B, Tomasz A. Altered penicillin-binding proteins in methicillin-resistant strains of *Staphylococcus aureus*. *Antimicrobial agents and chemotherapy*. 1981 May;19(5):726–35.

26. Matsuhashi M, Song MD, Ishino F, Wachi M, Doi M, Inoue M, et al. Molecular cloning of the gene of a penicillin-binding protein supposed to cause high resistance to beta-lactam antibiotics in *Staphylococcus aureus*. *Journal of Bacteriology*. 1986 Sep;167(3):975–80.

27. Spellberg B, Powers JH, Brass EP, Miller LG, Edwards JE. Trends in Antimicrobial Drug Development: Implications for the Future. *Clinical Infectious Diseases*. 2004 May 1;38(9):1279–86.

28. Pendleton JN, Gorman SP, Gilmore BF. Clinical relevance of the ESKAPE pathogens. *Expert Review of Anti-infective Therapy*. 2013 Mar 10;11(3):297–308.

29. Timsit J-F, Bassetti M, Cremer O, Daikos G, de Waele J, Kallil A, et al. Rationalizing antimicrobial therapy in the ICU: a narrative review. *Intensive Care Medicine*. 2019;(September 2018).

30. McMurry LM, Oethinger M, Levy SB. Triclosan targets lipid synthesis. *Nature*. 1998 Aug;394(6693):531–2.

31. Heath RJ, Yu YT, Shapiro MA, Olson E, Rock CO. Broad spectrum antimicrobial biocides target the FabI component of fatty acid synthesis. *The Journal of biological chemistry*. 1998 Nov 13;273(46):30316–20.

32. Schweizer HP. Triclosan: A widely used biocide and its link to antibiotics. *FEMS Microbiology Letters*. 2001;202(1):1–7.

33. Levy SB. Antibiotic and antiseptic resistance: impact on public health. *Paediatric Infectious Diseases Journal.* 2000;19(10):120–2.

34. Jutkina J, Marathe NP, Flach C-F, Larsson DGJ. Antibiotics and common antibacterial biocides stimulate horizontal transfer of resistance at low concentrations. *Science of The Total Environment*. 2018 Mar 1;616–617:172–8.

35. Lu J, Jin M, Nguyen SH, Mao L, Li J, Coin LJM, et al. Non-antibiotic antimicrobial triclosan induces multiple antibiotic resistance through genetic mutation. *Environment International*. 2018 Sep 1;118:257–65.

36. Webber MA, Buckner MMC, Redgrave LS, Ifill G, Mitchenall LA, Webb C, et al. Quinolone-resistant gyrase mutants demonstrate decreased susceptibility to triclosan. *Journal of Antimicrobial Chemotherapy*. 2017 Oct 1;72(10):2755–63.

37. Poole K. Mechanisms of bacterial biocide and antibiotic resistance. J*ournal of Applied Microbiology Symposium Supplement*. 2002;92:55–64.

38. Sasatsu M, Shirai Y, Hase M, Noguchi N, Kono M, Behr H, et al. The origin of the antiseptic-resistance gene ebr in *Staphylococcus aureu*s. *Microbios*. 1995;84(340):161–9.

39. Russell AD. Plasmids and bacterial resistance to biocides. *Journal of Applied Microbiology*. 1997 Jul 30;83(2):155–65.

40. McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action, and resistance. *Clinical microbiology reviews*. 1999 Jan;12(1):147–79.

41. Tattawasart U, Maillard JY, Furr JR, Russell AD. Development of resistance to chlorhexidine diacetate and cetylpyridinium chloride in *Pseudomonas stutzeri* and changes in antibiotic susceptibility. *The Journal of hospital infection*. 1999 Jul 1;42(3):219–29.

42. Tattawasart U, Hann AC, Maillard J-Y, Furr JR, Russell AD. Cytological changes in chlorhexidine-resistant isolates of *Pseudomonas stutzeri*. *Journal of Antimicrobial Chemotherapy*. 2000 Feb 1;45(2):145–52.

43. Winder CL, Al-Adham ISI, Abdel Malek SMA, Buultjens TEJ, Horrocks AJ, Collier PJ. Outer membrane protein shifts in biocide-resistant *Pseudomonas aeruginosa* PAO1. *Journal of Applied Microbiology*. 2000 Aug 1;89(2):289–95.

44. Jones M V, Herd TM, Christie HJ. Resistance of *Pseudomonas aeruginosa* to amphoteric and quaternary ammonium biocides. *Microbios*. 1989;58(234):49–61.

45. Damier-Piolle L, Magnet S, Brémont S, Lambert T, Courvalin P. AdeIJK, a resistance-nodulation-cell division pump effluxing multiple antibiotics in *Acinetobacter baumannii*. *Antimicrobial agents and chemotherapy*. 2008 Feb 1;52(2):557–62.

46. Magnet S, Courvalin P, Lambert T. Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. *Antimicrobial agents and chemotherapy*. 2001 Dec 1;45(12):3375–80.

47. Su X-Z, Chen J, Mizushima T, Kuroda T, Tsuchiya T. AbeM, an H+-coupled *Acinetobacter baumannii* multidrug efflux pump belonging to the MATE family of transporters. *Antimicrobial agents and chemotherapy.* 2005 Oct 1;49(10):4362–4.

48. Mima T, Joshi S, Gomez-Escalada M, Schweizer HP. Identification and characterization of TriABC-OpmH, a triclosan efflux pump of *Pseudomonas aeruginosa* requiring two membrane fusion proteins. *Journal of bacteriology*. 2007 Nov 1;189(21):7600–9.

49. McMurry LM, Oethinger M, Levy SB. Overexpression of *marA, soxS* , or *acrAB* produces resistance to triclosan in laboratory and clinical strains of *Escherichia coli*. *FEMS Microbiology Letters*. 1998 Sep 1;166(2):305–9.

50. Chuanchuen R, Beinlich K, Hoang TT, Becher A, Karkhoff-Schweizer RR, Schweizer HP. Cross-Resistance between Triclosan and Antibiotics in *Pseudomonas aeruginosa* Is Mediated by Multidrug Efflux Pumps: Exposure of a Susceptible Mutant Strain to Triclosan Selects nfxB Mutants Overexpressing MexCD-OprJ. *Antimicrobial*

Agents and Chemotherapy. 2001 Feb 1;45(2):428–32.

51. Bamber AI, Neal TJ. An assessment of triclosan susceptibility in methicillin-resistant and methicillin-sensitive *Staphylococcus aureus*. *Journal of Hospital Infection*. 1999 Feb 1;41(2):107–9.

52. Suller MT, Russell AD. Antibiotic and biocide resistance in methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus*. *The Journal of hospital infection*. 1999 Dec 1;43(4):281–91.

53. WEBSTER J, FAOAGALI JL, CARTWRIGHT D. Elimination of methicillin-resistant *Staphylococcus aureus* from a neonatal intensive care unit after hand washing with triclosan. *Journal of Paediatrics and Child Health*. 1994 Feb 1;30(1):59–64.

54. McMurry LM, McDermott PF, Levy SB. Genetic evidence that InhA of *Mycobacterium smegmatis* is a target for triclosan. *Antimicrobial agents and chemotherapy*. 1999 Mar;43(3):711–3.

55. Cookson BD, Farrelly H, Stapleton P, Garvey RP, Price MR. Transferable resistance to triclosan in MRSA. *Lancet*. 1991 Jun 22;337(8756):1548–9.

56. Arzanlou M, Chai WC, Venter H. Intrinsic, adaptive and acquired antimicrobial resistance in Gram-negative bacteria. *Essays in biochemistry*. 2017 Mar 28;61(1):49–59.

57. Munita JM, Arias CA. Mechanisms of Antibiotic Resistance. *Microbiology Spectrum*. 2016 April; 4 (2)

58. Pagès J-M, James CE, Winterhalter M. The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. *Nature Reviews Microbiology*. 2008 Dec 10;6(12):893–903.

59. Piddock LJ V. Clinically Relevant Chromosomally Encoded Multidrug Resistance Efflux Pumps in Bacteria. *CLINICAL MICROBIOLOGY REVIEWS*. 2006;19(2):382–402.

60. Poole K. Efflux-mediated antimicrobial resistance. *Journal of Antimicrobial Chemotherapy*. 2005 Jul 1;56(1):20–51.

61. Roberts MC. Update on acquired tetracycline resistance genes. *FEMS Microbiology Letters.* 2005 April 15; 245 (2):195-203

62. Visalli MA, Murphy E, Projan SJ, Bradford PA. AcrAB Multidrug Efflux Pump Is Associated with Reduced Levels of Susceptibility to Tigecycline (GAR-936) in *Proteus mirabilis*. *ANTIMICROBIAL AGENTS AND CHEMOTHERAPY*. 2003;47(2):665–9.

63. Connell SR, Tracz DM, Nierhaus KH, Taylor DE. MINIREVIEW Ribosomal Protection Proteins and Their Mechanism of Tetracycline Resistance. *ANTIMICROBIAL AGENTS AND CHEMOTHERAPY*. 2003;47(12):3675–81.

64. Li W, Atkinson GC, Thakor NS, Allas Ü, Lu C-C, Chan K-Y, et al. Mechanism of Tetracycline Resistance by Ribosomal Protection Protein Tet(O). *Nature Communications*. 2013;4:1477.

65. Dönhöfer A, Franckenberg S, Wickles S, Berninghausen O, Beckmann R, Wilson DN. Structural basis for TetM-mediated tetracycline resistance. *Proceedings of the National Academy of Sciences.* 2012 Oct 16; 109 (42): 16900-5

66. Rodríguez-Martínez JM, Velasco C, Pascual Á, Cano ME, Martínez-Martínez L, Martínez-Martínez L, et al. Plasmid-mediated quinolone resistance: an update. *Journal of Infection and Chemotherapy*. 2011 Apr;17(2):149–82.

67. Aldred KJ, Kerns RJ, Osheroff N. Mechanism of Quinolone Action and Resistance. *Biochemistry*. 2014 Mar 18;53(10):1565–74.

68. Floss HG, Yu T-W. Rifamycin: Mode of Action, Resistance, and Biosynthesis. *Chemical Reviews*. 2005 Feb;105(2):621–32.

69. Hooper DC. Fluoroquinolone resistance among Gram-positive cocci. T*he Lancet Infectious diseases*. 2002 Sep;2(9):530–8.

70. Leclercq R. Mechanisms of Resistance to Macrolides and Lincosamides: Nature of the Resistance Elements and Their Clinical Implications. *Clinical Infectious Diseases*. 2002 Feb 15;34(4):482–92.

71. Hiramatsu K, Ito T, Tsubakishita S, Sasaki T, Takeuchi F, Morimoto Y, et al. Genomic Basis for Methicillin Resistance in *Staphylococcus aureus*. *Infection & chemotherapy*. 2013 Jun;45(2):117–36.

72. Bonnet R. Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. *Antimicrobial agents and chemotherapy*. 2004 Jan;48(1):1–14.

73. Poirel L, Lartigue M-F, Decousser J-W, Nordmann P. ISEcp1B-Mediated Transposition of bla CTX-M in Escherichia coli. *ANTIMICROBIAL AGENTS AND CHEMOTHERAPY*. 2005;49(1):447–550.

74. Du D, Wang-Kan X, Neuberger A, van Veen HW, Pos KM, Piddock LJ V., et al. Multidrug efflux pumps: structure, function and regulation. *Nature Reviews Microbiology*. 2018 Sep 12;16(9):523–39.

75. Blair JMA, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ V. Molecular mechanisms of antibiotic resistance. *Nature Reviews Microbiology*. 2015 Jan 1;13(1):42–51.

76. Cheng G, Dai M, Ahmed S, Hao H, Wang X, Yuan Z. Antimicrobial Drugs in Fighting against Antimicrobial Resistance. Antimicrobial Drugs in Fighting against Antimicrobial Resistance *Frontiers Microbiology*. 2016;1:470.

77. Singh S, Singh SK, Chowdhury I, Singh R. Understanding the Mechanism of Bacterial Biofilms Resistance to Antimicrobial Agents. *The Open Microbiology Journal*. 2017;11.

78. Rousham EK, Islam MA, Nahar P, Lucas PJ, Naher N, Ahmed SM, et al. Pathways of antibiotic use in Bangladesh: qualitative protocol for the PAUSE study. *BMJ open*. 2019 Jan 25;9(1):e028215.

79. Manyi-Loh C, Mamphweli S, Meyer E, Okoh A. Antibiotic Use in Agriculture and Its Consequential Resistance in Environmental Sources: Potential Public Health Implications. *Molecules*. 2018 Mar 30;23(4).

80. Jansen KU, Knirsch C, Anderson AS. The role of vaccines in preventing bacterial antimicrobial resistance. *Nature Medicine.* 2018;24(1):10–20.

81. González-Bello C. Antibiotic adjuvants – A strategy to unlock bacterial resistance to antibiotics. *Bioorganic & Medicinal Chemistry Letters*. 2017 Sep;27(18):4221–8.

82. WRIGHT G. Bacterial resistance to antibiotics: Enzymatic degradation and modification. *Advanced Drug Delivery Reviews*. 2005 Jul 29;57(10):1451–70.

83. O'Neill, Jim. Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations. 2014, Dec

84. Jansen KU, Anderson AS. The role of vaccines in fighting antimicrobial resistance (AMR). 2018; Human Vaccine Immunotherapy. 2018; 14 (9): 2142-2149

85. Doerr HW, Berger A. Vaccination against infectious diseases: What is promising?

Medical Microbiology and Immunology. 2014 Dec 27;203(6):365–71.

86. Adam HJ, Richardson SE, Jamieson FB, Rawte P, Low DE, Fisman DN. Changing epidemiology of invasive *Haemophilus influenzae* in Ontario, Canada: Evidence for herd effects and strain replacement due to Hib vaccination. *Vaccine*. 2010 May 28;28(24):4073–8.

87. Hoban D, Felmingham D. The PROTEKT surveillance study: antimicrobial susceptibility of *Haemophilus influenzae* and *Moraxella catarrhalis* from community-acquired respiratory tract infections. *Journal of Antimicrobial Chemotherapy*. 2002 Sep 1;50(suppl 2):49–59.

88. Heilmann KP, Rice CL, Miller AL, Miller NJ, Beekmann SE, Pfaller MA, et al. Decreasing prevalence of beta-lactamase production among respiratory tract isolates of *Haemophilus influenzae* in the United States. *Antimicrobial agents and chemotherapy*. 2005 Jun;49(6):2561–4.

89. Rasko DA, Sperandio V. Anti-virulence strategies to combat bacteria-mediated disease. *Nature Reviews Drug Discovery*. 2010 Feb 18;9(2):117–28.

90. Warrener P, Varkey R, Bonnell JC, DiGiandomenico A, Camara M, Cook K, et al. A novel anti-PcrV antibody providing enhanced protection against *Pseudomonas aeruginosa* in multiple animal infection models. *Antimicrobial agents and chemotherapy.* 2014 Aug 1;58(8):4384–91.

91. Qiu J, Luo M, Dong J, Wang J, Li H, Wang X, et al. Menthol diminishes *Staphylococcus aureus* virulence-associated extracellular proteins expression. *Applied Microbiology and Biotechnology*. 2011 Apr 2;90(2):705–12.

92. Starkey M, Lepine F, Maura D, Bandyopadhaya A, Lesic B, He J, et al. Identification of Anti-virulence Compounds That Disrupt Quorum-Sensing Regulated Acute and Persistent Pathogenicity. Whiteley M, editor. *PLoS Pathogens*. 2014 Aug 21;10(8):e1004321.

93. Huigens III RW, Rogers SA, Steinhauer AT, Melander C. Inhibition of *Acinetobacter baumannii, Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilm formation with a class of TAGE-triazole conjugates. *Organic & Biomolecular Chemistry*. 2009;7(4):794.

94. Maura D, Ballok AE, Rahme LG. Considerations and caveats in anti-virulence drug development. *Current Opinions in Microbiology*. 2016 Oct; 33: 41-46

95. Standish AJ, Salim AA, Zhang H, Capon RJ, Morona R. Chemical Inhibition of Bacterial Protein Tyrosine Phosphatase Suppresses Capsule Production. Brown SP, editor. *PLoS ONE*. 2012 May 15;7(5):e36312.

96. Defoirdt T. Quorum-Sensing Systems as Targets for Antivirulence Therapy. *Trends in Microbiology*. 2018;26(4):313–28.

97. Smyth AR, Cifelli PM, Ortori CA, Righetti K, Lewis S, Erskine P, et al. Garlic as an inhibitor of *Pseudomonas aeruginosa* quorum sensing in cystic fibrosis - A pilot randomized controlled trial. *Pediatric Pulmonology*. 2010;45(4):356–62.

98. Maeda T, García-Contreras R, Pu M, Sheng L, Garcia LR, Tomás M, et al. Quorum quenching quandary: resistance to antivirulence compounds. *The ISME Journal*. 2011;6.

99. García-Contreras R, Martínez-Vázquez M, Velázquez Guadarrama N, Villegas Pañeda AG, Hashimoto T, Maeda T, et al. Resistance to the quorum-quenching compounds brominated furanone C-30 and 5-fluorouracil in *Pseudomonas aeruginosa* clinical isolates. *Pathogens and Disease*. 2013 Jun 1;68(1):8–11.

100. Martinez JL, Baquero F. Mutation frequencies and antibiotic resistance. *Antimicrobial agents and chemotherapy*. 2000 Jul 1;44(7):1771–7.

101. Heras B, Scanlon MJ, Martin JL, Oxon P. Targeting virulence not viability in the search for future antibacterials. *British Journal of Clinical Pharmacology.* 2015 Feb; 79 (2): 208-15;

102. Kong C, Neoh H-M, Nathan S. toxins Targeting Staphylococcus aureus Toxins: A Potential form of Anti-Virulence Therapy. *Toxins.* 2016 March; 8 (3)

103. Resch A, Rosenstein R, Nerz C, Götz F. Differential gene expression profiling of *Staphylococcus aureus* cultivated under biofilm and planktonic conditions. *Applied and environmental microbiology*. 2005 May 1;71(5):2663–76.

104. Domingo-Calap P, Delgado-Martínez J. Bacteriophages: Protagonists of a Post-Antibiotic Era. *Antibiotics.* 2018 Jul 27; 7 (3).

105. Hermoso JA, García JL, García P. Taking aim on bacterial pathogens: from phage therapy to enzybiotics. *Current Opinion in Microbiology*. 2007 Oct 1;10(5):461–72.

106. Bikard D, Euler C, Jiang W, Nussenzweig PM, Goldberg GW, Duportet X, et al. Development of sequence-specific antimicrobials based on programmable CRISPR-Cas nucleases. *Nature Biotechnology*. 2014;32(11):1146–50.

107. O'Sullivan L, Buttimer C, McAuliffe O, Bolton D, Coffey A. Bacteriophage-based tools: recent advances and novel applications. *F1000 Research*. 2016;5(0):2782.

108. Briers Y, Miroshnikov K, Chertkov O, Nekrasov A, Mesyanzhinov V, Volckaert G, et al. The structural peptidoglycan hydrolase gp181 of bacteriophage φKZ. *Biochemical and Biophysical Research Communications*. 2008 Oct 3;374(4):747–51.

109. Briers Y, Walmagh M, Lavigne R. Use of bacteriophage endolysin EL188 and outer membrane permeabilizers against *Pseudomonas aeruginosa*. *Journal of Applied Microbiology*. 2011 Mar 1;110(3):778–85.

110. Lerner T, Lovering AL, Bui NK, Uchida K, Aizawa S-I, Vollmer W, et al. Specialized Peptidoglycan Hydrolases Sculpt the Intra-bacterial Niche of Predatory *Bdellovibrio* and Increase Population Fitness. *PLoS Pathogens*. 2012;8(2):e1002524.

111. Rodríguez-Rubio L, Martínez B, Donovan DM, Rodríguez A, García P. Bacteriophage virion-associated peptidoglycan hydrolases: potential new enzybiotics. *Critical Reviews in Microbiology*. 2013 Nov 20;39(4):427–34.

112. Nelson D, Loomis L, Fischetti VA. Prevention and elimination of upper respiratory colonization of mice by group A streptococci by using a bacteriophage lytic enzyme. *Proceedings of the National Academy of Sciences of the United States of America*. 2001 Mar 27;98(7):4107–12.

113. Jado I, López R, García E, Fenoll A, Casal J, García P. Phage lytic enzymes as therapy for antibiotic-resistant *Streptococcus pneumoniae* infection in a murine sepsis model. *Journal of Antimicrobial Chemotherapy.* 2003 Nov 12;52(6):967–73.

114. Yoong P, Schuch R, Nelson D, Fischetti VA. Identification of a broadly active phage lytic enzyme with lethal activity against antibiotic-resistant *Enterococcus faecalis* and *Enterococcus faecium*. *Journal of bacteriology*. 2004 Jul 15;186(14):4808–12.

115. Zimmer M, Vukov N, Scherer S, Loessner MJ. The murein hydrolase of the bacteriophage phi3626 dual lysis system is active against all tested *Clostridium perfringens* strains. *Applied and environmental microbiology*. 2002 Nov 1;68(11):5311–7.

116. Schuch R, Nelson D, Fischetti VA. A bacteriolytic agent that detects and kills *Bacillus*

anthracis. *Nature*. 2002 Aug;418(6900):884–9.

117. Garcia-Doval C, Castón J, Luque D, Granell M, Otero J, Llamas-Saiz A, et al. Structure of the Receptor-Binding Carboxy-Terminal Domain of the Bacteriophage T5 L-Shaped Tail Fibre with and without Its Intra-Molecular Chaperone. *Viruses*. 2015 Dec 8;7(12):6424–40.

118. São-José, C, Engineering of Phage-Derived Lytic Enzymes: Improving Their Potential as Antimicrobials. *Antibiotics.* 2018 Mar 22; 7 (2)

119. Yang H, Yu J, Wei H. Engineered bacteriophage lysins as novel anti-infectives. *Frontiers in microbiology*. 2014;5:542.

120. Willis AR, Moore C, Mazon-Moya M, Krokowski S, Lambert C, Till R, et al. Injections of Predatory Bacteria Work Alongside Host Immune Cells to Treat *Shigella* Infection in Zebrafish Larvae. *Current Biology.* 2016;26(24):3343–51.

121. Raghunathan D, Radford PM, Gell C, Negus D, Moore C, Till R, et al. Engulfment, persistence and fate of *Bdellovibrio bacteriovorus* predators inside human phagocytic cells informs their future therapeutic potential. *Scientific Reports*. 2019;9(1):1–16.

122. Littman DR, Pamer EG. Role of the commensal microbiota in normal and pathogenic host immune responses. *Cell Host Microbe.* 2011 Oct 20; 10 (4): 311-23

123. Medzhitov R. Toll-like receptors and innate immunity. *Nature Reviews Immunology*. 2001 Nov;1(2):135–45.

124. Brown GD. Innate Antifungal Immunity: The Key Role of Phagocytes. *Annual Review of Immunology*. 2011;29(1):1–21.

125. Gilbert AS, Wheeler RT, May RC, Ene I V, Brunke S, Brown AJP, et al. Fungal Pathogens : Survival and Replication within Macrophages Fungal Pathogens : Survival and Replication within Macrophages. *Cold Spring Harbor Perspectives in Medicine*. 2015;5:1–14.

126. Ley K, Pramod AB, Croft M, Ravichandran KS, Ting JP. How mouse macrophages sense what is going on. *Frontiers in Immunology*. 2016;7(JUN):1–17.

127. Kabelitz D. Expression and function of Toll-like receptors in T lymphocytes. *Current Opinion in Immunology*. 2007 Feb 1;19(1):39–45.

128. Kieser KJ, Kagan JC. Multi-receptor detection of individual bacterial products by the innate immune system. *Nature Publishing Group.* 2017;17.

129. Mogensen TH. Pathogen Recognition and Inflammatory Signaling in Innate Immune Defenses. *Clinical Microbiology Reviews*. 2009 Apr 1;22(2):240–73.

130. Gazi U, Martinez-Pomares L. Influence of the mannose receptor in host immune responses. *Immunobiology*. 2009 Jul;214(7):554–61.

131. Marakalala MJ, Graham LM, Brown GD. The role of Syk/CARD9-coupled C-type lectin receptors in immunity to Mycobacterium tuberculosis infections. *Clinical & developmental immunology*. 2010 Jan 9;2010:567571.

132. Iborra S, Izquierdo HM, Martínez-López M, Blanco-Menéndez N, Reis e Sousa C, Sancho D. The DC receptor DNGR-1 mediates cross-priming of CTLs during vaccinia virus infection in mice. *The Journal of clinical investigation*. 2012 May 1;122(5):1628–43.

133. Drummond RA, Brown GD. Signalling C-Type Lectins in Antimicrobial Immunity. PLoS *Pathogens*. 2013;9(7):9–11.

134. Tyson J, Elizabeth Sockett R. Nature knows best: employing whole microbial

strategies to tackle antibiotic resistant pathogens. *Environmental Microbiology Reports*. 2017;9(1):47–9.

135. Monnappa AK, Bari W, Choi SY, Mitchell RJ. Investigating the Responses of Human Epithelial Cells to Predatory Bacteria. *Scientific Reports*. 2016;6(August):1–14.

136. Gupta S, Tang C, Tran M, Kadouri DE. Effect of predatory bacteria on human cell lines. *PLoS ONE*. 2016;11(8):1–15.

137. Shatzkes K, Tang C, Singleton E, Shukla S, Zuena M, Gupta S, et al. Effect of predatory bacteria on the gut bacterial microbiota in rats. *Scientific Reports*. 2017;7(January):1–12.

138. Shatzkes K, Singleton E, Tang C, Zuena M, Shukla S, Gupta S, et al. Examining the efficacy of intravenous administration of predatory bacteria in rats. *Scientific Reports*. 2017;7(1):1–11.

139. Shatzkes K, Singleton E, Tang C, Zuena M, Shukla S, Gupta S, et al. Predatory Bacteria Attenuate *Klebsiella pneumoniae* Burden in Rat Lungs. *mBio*. 2016;7(6):1–9.

140. Atterbury RJ, Hobley L, Till R, Lambert C, Capeness MJ, Lerner TR, et al. Effects of orally administered *Bdellovibrio bacteriovorus* on the well-being and *Salmonella* colonization of young chicks. *Applied and Environmental Microbiology*. 2011;77(16):5794–803.

141. Romanowski E, Stella N, Brothers K, Yates K, Funderburgh ML, Funderburgh JL, et al. Predatory bacteria are nontoxic to the rabbit ocular surface. *Scientific Reports*. 2016;6(1):1–9.

142. Schwudke D, Linscheid M, Strauch E, Appel B, Zähringer U, Moll H, et al. The obligate predatory *Bdellovibrio bacteriovorus* possesses a neutral lipid a containing α-D-mannoses that replace phosphate residues. Similarities and differences between the lipid As and the lipopolysaccharides of the wild type strain *B. bacteriovorus*. *Journal of Biological Chemistry*. 2003;278(30):27502–12.

143. Lambert C, Evans KJ, Till R, Hobley L, Capeness M, Rendulic S, et al. Characterizing the flagellar filament and the role of motility in bacterial prey-penetration by *Bdellovibrio bacteriovorus*. *Molecular Microbiology*. 2006;60(2):274–86.

144. Matin A, Rittenberg SC. Kinetics of deoxyribonucleic acid destruction and synthesis during growth of *Bdellovibrio bacteriovorus* strain 109D on *Pseudomonas putida* and *Escherichia coli. Journal of Bacteriology*. 1972;111(3):664–73.

145. Hespell RB, Miozzari GF, Rittenberg SC. Ribonucleic acid destruction and synthesis during intraperiplasmic growth of *Bdellovibrio bacteriovorus*. Journal of Bacteriology. 1975;123(2):481–91.

146. Clokie M, Millard A, Letarov A, Heaphy S. Phages in nature. *Bacteriophage*. 2011;1(1):31–45.

147. Mullany P, Łó M, Hinton DM, Mathews CK, Bryan D, Kutter EM, et al. Bacteriophage T4 Infection of Stationary Phase *E. coli*: Life after Log from a Phage Perspective. *Frontiers in Microbiology.* 2016; 7: 1391

148. Liu M, Huang S, Guo L, Li H, Wang F, Zhang Q, et al. Clinical features and risk factors for blood stream infections of *Candida* in neonates. *Experimental and Therapeutic Medicine*. 2015;10(3):1139–44.

149. Bruiniera F.R., Ferreira F.M., Saviolli L.R.M, Bacci M.R., Feder M., da Luz Goncalves Pedreira M, Sorgini Peterlini M.A., Azzalis L.A., Campos Junqueira V.B., Fonseca F.L.A. The use of vancomycin with its therapeutic and adverse

effects: A review. *European Review for Medical and Pharmacological Sciences*. 2015;19(4):694–700.

150. Nitanai Y, Kikuchi T, Kakoi K, Hanamaki S, Fujisawa I, Aoki K. Crystal Structures of the Complexes between Vancomycin and Cell-Wall Precursor Analogs. *Journal of Molecular Biology*. 2009 Feb 6;385(5):1422–32.

151. de Albuquerque JAT, Banerjee PP, Castold A, Ma R, Zurro NB, Ynoue LH, et al. The role of AIRE in the immunity against *Candida albicans* in a model of human macrophages. *Frontiers in Immunology*. 2018;9(MAR).

152. Drummond RA, Gaffen SL, Hise AG, Brown GD. Innate defense against fungal pathogens. *Cold Spring Harbor Perspectives in Medicine*. 2015;5(6):1–19.

153. Chin VK, Lee TY, Rusliza B, Chong PP. Dissecting *Candida albicans* Infection from the Perspective of *C. albicans* Virulence and Omics Approaches on Host-Pathogen Interaction: A Review. *International journal of molecular sciences*. 2016 Oct 18;17(10).

154. Westman J, Moran G, Hube B. *Candida albicans* Hyphal Expansion Causes Phagosomal. *mBio.* 2018;9(5):1–14.

155. Gladfelter A, Sudbery P. Septins in four model fungal systems: diversity in form and function. In: Hall PA, Russell SEH, Pringle JR, editors. The Septins. *John Wiley-Blackwell*; 2008. p. 125–46.

156. Shibata N, Suzuki A, Kobayashi H, Okawa Y. Chemical structure of the cell-wall mannan of *Candida albicans* serotype A and its difference in yeast and hyphal forms. *Biochemical Journal.* 2007 Jun 15;404(3):365–72.

157. Rudkin FM, Bain JM, Walls C, Lewis LE, Gow NAR, Erwig LP. Altered Dynamics of *Candida albicans* Phagocytosis by Macrophages. *mBio.* 2013;4(6):1–10.

158. Lowman DW, Greene RR, Bearden DW, Kruppa MD, Pottier M, Monteiro MA, et al. Novel Structural Features in *Candida albicans* Hyphal Glucan Provide a Basis for Differential Innate Immune Recognition of Hyphae *Versus* Yeast. *Journal of Biological Chemistry.* 2014 Feb 7;289(6):3432–43.

159. De Luca A, Carvalho A, Cunha C, Iannitti RG, Pitzurra L, Giovannini G, et al. IL-22 and IDO1 Affect Immunity and Tolerance to Murine and Human Vaginal Candidiasis. *PLoS Pathogens*. 2013;9(7).

160. Borghi M, De Luca A, Puccetti M, Jaeger M, Mencacci A, Oikonomou V, et al. Pathogenic NLRP3 inflammasome activity during *Candida infection* is negatively regulated by IL-22 via activation of NLRC4 and IL-1Ra. *Cell Host and Microbe*. 2015;18(2):198–209.

161. Wu SY, Huang JH, Chen WY, Chan YC, Lin CH, Chen YC, et al. Cell intrinsic galectin-3 attenuates neutrophil ROS-dependent killing of *Candida* by modulating CR3 downstream syk activation. *Frontiers in Immunology*. 2017;8(FEB).

162. Person A, Kontoyiannis DP, Alexander BD. Fungal Infections in Transplant and Oncology Patients. *Infectious Disease Clinics of North America.* 2010;24(2):1–10.

163. Alonso M, Gow NAR, Erwig LP, Bain JM. Macrophage Migration Is Impaired within *Candida albicans* Biofilms. Vol. 3, *Journal of Fungi*. 2017. p. 31.

164. Smeekens SP, van de Veerdonk FL, Kullberg BJ, Netea MG. Genetic susceptibility to *Candida* infections. *EMBO Molecular Medicine*. 2013 Jun;5(6):805–13.

165. Ferwerda B, Ferwerda G, Plantinga TS, Willment JA, van Spriel AB, Venselaar H, et al. Human Dectin-1 Deficiency and Mucocutaneous Fungal Infections. *New England*

Journal of Medicine. 2009 Oct 29;361(18):1760–7.

166. Nahum A, Dadi H, Bates A, Roifman CM. The L412F variant of Toll-like receptor 3 (TLR3) is associated with cutaneous candidiasis, increased susceptibility to cytomegalovirus, and autoimmunity. *Journal of Allergy and Clinical Immunology*. 2011 Feb;127(2):528–31.

167. Lionakis MS. New insights into innate immune control of systemic candidiasis. *Medical Mycology*. 2014;52(6):555–64.

168. Break TJ, Jaeger M, Solis N V., Filler SG, Rodriguez CA, Lim JK, et al. CX $_3$ CR1 Is Dispensable for Control of Mucosal *Candida albicans* Infections in Mice and Humans. Deepe GS, editor. *Infection and Immunity*. 2015 Mar;83(3):958–65.

169. Smith JA, Kauffman CA. Endemic fungal infections in patients receiving tumour necrosis factor-α inhibitor therapy. *Drugs*. 2009;69(11):1403–15.

170. Drummond RA, Brown GD. The role of Dectin-1 in the host defence against fungal infections. *Current Opinion in Microbiology*. 2011;14(4):392–9.

171. Gross O, Gewies A, Finger K, Schäfer M, Sparwasser T, Peschel C, et al. Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. *Nature*. 2006 Aug 12;442(7103):651–6.

172. Glocker E-O, Hennigs A, Nabavi M, Schäffer AA, Woellner C, Salzer U, et al. A Homozygous *CARD9* Mutation in a Family with Susceptibility to Fungal Infections. *New England Journal of Medicine.* 2009 Oct 29;361(18):1727–35.

173. Drummond RA, Lionakis MS. Mechanistic Insights into the Role of C-Type Lectin Receptor/CARD9 Signaling in Human Antifungal Immunity. *Frontiers in Cellular and Infection Microbiology*. 2016;6(April).

174. Drummond RA, Collar AL, Swamydas M, Rodriguez CA, Lim JK, Mendez LM, et al. CARD9-Dependent Neutrophil Recruitment Protects against Fungal Invasion of the Central Nervous System. *PLoS Pathogens*. 2015;11(12):1–32.

175. Whitney PG, Bär E, Osorio F, Rogers NC, Schraml BU, Deddouche S, et al. Syk Signaling in Dendritic Cells Orchestrates Innate Resistance to Systemic Fungal Infection. *PLoS Pathogens*. 2014;10(7).

176. Ngo LY, Kasahara S, Kumasaka DK, Knoblaugh SE, Jhingran A, Hohl TM. Inflammatory Monocytes Mediate Early and Organ-Specific Innate Defense During Systemic Candidiasis. *The Journal of Infectious Diseases*. 2014 Jan 1;209(1):109–19.

177. Lionakis MS, Swamydas M, Fischer BG, Plantinga TS, Johnson MD, Jaeger M, et al. CX3CR1-dependent renal macrophage survival promotes *Candida* control and host survival. *The Journal of Clinical Investigation*. 2013 Dec 2;123(12):5035–51.

178. Swamydas M, Gao J, Break TJ, Johnson MD, Rodriguez CA, Lim JK, et al. CXCR1-mediated Neutrophil Degranulation and Fungal Killing Promotes Candida Clearance and Host Survival. *Science Translational Medicine*. 2017;8(322):1–27.

179. Netea MG, Joosten LAB, van der Meer JWM, Kullberg B-J, van de Veerdonk FL. Immune defence against *Candida* fungal infections. *Nature Reviews Immunology*. 2015 Oct 21;15(10):630–42.

180. Khan N, Kasperkobvitz P, Timmons A, Mansour M, Tam J, Seward M, et al. Dectin-1 Controls TLR-9 trafficking to phagosomes containing β 1-3 glucan. *Journal of Immunology*. 2017;196(5):2249–61.

181. Davis SE, Hopke A, Minkin SC, Montedonico AE, Wheeler RT, Reynolds TB.

Masking of β(1-3)-Glucan in the Cell Wall of *Candida albicans* from Detection by Innate Immune Cells Depends on Phosphatidylserine. Deepe GS, editor. *Infection and Immunity.* 2014 Oct;82(10):4405–13.

182. Brown GD, Gordon S. A new receptor for β-glucans. *Nature*. 2001 Sep 6;413(6851):36–7.

183. Wellington M, Koselny K, Sutterwala FS, Krysan DJ. Candida albicans triggers NLRP3-mediated pyroptosis in macrophages. *Eukaryotic cell*. 2014 Feb 1;13(2):329–40.

184. Brown GD, Herre J, Williams DL, Willment JA, Marshall ASJ, Gordon S. Dectin-1 Mediates the Biological Effects of β-Glucans. *The Journal of Experimental Medicine*. 2003 May 5;197(9):1119–24.

185. Wagener J, Malireddi RKS, Lenardon MD, Köberle M, Vautier S, MacCallum DM, et al. Fungal Chitin Dampens Inflammation through IL-10 Induction Mediated by NOD2 and TLR9 Activation. Sil A, editor. *PLoS Pathogens*. 2014 Apr 10;10(4):e1004050.

186. Cheng S-C, van de Veerdonk FL, Lenardon M, Stoffels M, Plantinga T, Smeekens S, et al. The dectin-1/inflammasome pathway is responsible for the induction of protective T-helper 17 responses that discriminate between yeasts and hyphae of *Candida albicans*. *Journal of Leukocyte Biology*. 2011 Aug 1;90(2):357–66.

187. Agostini L, Martinon F, Burns K, McDermott MF, Hawkins PN, Tschopp J. NALP3 forms an IL-1β-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity*. 2004 Mar;20(3):319–25.

188. Gross O, Poeck H, Bscheider M, Dostert C, Hannesschläger N, Endres S, et al. Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature*. 2009 May 1;459(7245):433–6.

189. Hise AG, Tomalka J, Ganesan S, Patel K, Hall BA, Brown GD, et al. An Essential Role for the NLRP3 Inflammasome in Host Defense against the Human Fungal Pathogen *Candida albicans*. *Cell Host & Microbe*. 2009 May 8;5(5):487–97.

190. Gringhuis SI, Kaptein TM, Wevers BA, Theelen B, Van Der Vlist M, Boekhout T, et al. Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1β via a noncanonical caspase-8 inflammasome. *Nature Immunology*. 2012;13(3):246–54.

191. Robinson MJ, Osorio F, Rosas M, Freitas RP, Schweighoffer E, Groß O, et al. Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. *The Journal of Experimental Medicine*. 2009 Aug 31;206(9):2037–51.

192. Tomalka J, Ganesan S, Azodi E, Patel K, Majmudar P, Hall BA, et al. A Novel Role for the NLRC4 Inflammasome in Mucosal Defenses against the Fungal Pathogen *Candida albicans*. Filler SG, editor. *PLoS Pathogens.* 2011 Dec 8;7(12):e1002379.

193. Kalghatgi S, Spina CS, Costello JC, Liesa M, Ruben J, Slomovic S, et al. Bactericidal Antibiotics Induce Mitochondrial Dysfunction and Oxidative Damage in Mammalian Cells. *Science Translational Medicine*. 2013;5(192):1–22.

194. Yang JH, Bhargava P, McCloskey D, Mao N, Palsson BO, Collins JJ. Antibiotic-Induced Changes to the Host Metabolic Environment Inhibit Drug Efficacy and Alter Immune Function. *Cell Host and Microbe*. 2017;22(6):757–765.e3.

195. Bae H, Zmijewski J, Deshane J, Tadie J, Chaplin D, Takashima S, et al. AMP-activated protein kinase enhances the phagocytic ability of macrophages and neutrophils. *The Journal of the Federation of American Societies for Experimental Biology*. 2011;25(12):4358–68.

196. Mann ER, Andersen P, Alcon-giner C, Leclaire C, Caim S, Wessel H, et al. Antibiotics induce sustained dysregulation of intestinal T-cell immunity by perturbing macrophage homeostasis. *Science Translational Medicine*. 2018;10(464):1–15.

197. Conti HR, Gaffen SL. IL-17-mediated immunity to the opportunistic fungal pathogen *Candida albicans*. *Journal of Immunology*. 2016;195(3):780–8.

198. Puel A, Cypowyj S, Bustamante J, Wright JF, Liu L, Lim K, et al. Errors of Interleukin-17 *Immunity*. 2011;332(6025):65–8.

199. Ivanov I, Frutos R, Manel N, Yoshinaga K, Rifkin DB, Sartor B, et al. Specific microbiota direct the differentiation of Th17 cells in the mucosa of the small intestine. *Cell Host Microbe*. 2008;4(4):337–49.

200. Fahey JR, Lyons BL, Olekszak HL, Mourino AJ, Ratiu JJ, Racine JJ, et al. Antibiotic-associated manipulation of the gut microbiota and phenotypic restoration in NOD mice. *Comparative Medicine*. 2017;67(4):335–43.

201. Allonsius CN, van den Broek MFL, De Boeck I, Kiekens S, Oerlemans EFM, Kiekens F, et al. Interplay between *Lactobacillus rhamnosus* GG and *Candida* and the involvement of exopolysaccharides. *Microbial Biotechnology*. 2017;10(6):1753–63.

202. Zelante T, Iannitti RG, Cunha C, DeLuca A, Giovannini G, Pieraccini G, et al. Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. *Immunity*. 2013;39(2):372–85.

203. Ben-Ami R, Olshtain-Pops K, Krieger M, Oren I, Bishara J, Dan M, et al. Antibiotic exposure as a risk factor for fluconazole-resistant *Candida* bloodstream infection. *Antimicrobial Agents and Chemotherapy*. 2012;56(5):2518–23.

204. Francino MP. Antibiotics and the human gut microbiome: Dysbioses and accumulation of resistances. *Frontiers in Microbiology*. 2016;6(JAN):1–11.

205. Fan D, Coughlin L, Neubauer M, Kim J, Kim M, Zhan X, et al. Activation of HIF1a and LL-37 by commensal bacteria inhibits CA colonisation. *Nature Medicine*. 2015;21(7):808–14.

206. Odds FC, Davidson AD, Jacobsen MD, Tavanti A, Whyte JA, Kibbler CC, et al. *Candida albicans* strain maintenance, replacement, and microvariation demonstrated by multilocus sequence typing. *Journal of Clinical Microbiology*. 2006;44(10):3647–58.

207. Gordon D. Brown, David W. Denning, Neil A.R. Gow, StuartM. Levitz, Mihai G. Netea, Theodore C. White. Hidden killers: human fungal infections. *Science Translational Medicine*. 2012;4(165):1–9.

208. Grant SS, Kaufmann BB, Chand NS, Haseley N, Hung DT. Eradication of bacterial persisters with antibiotic-generated hydroxyl radicals. *Proceedings of the National Academy of Sciences*. 2012 Jul 24;109(30):12147–52.

209. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ. A Common Mechanism of Cellular Death Induced by Bactericidal Antibiotics. *Cell*. 2007;130(5):797–810.

210. Dwyer DJ, Kohanski MA, Hayete B, Collins JJ. Gyrase inhibitors induce an oxidative damage cellular death pathway in *Escherichia coli*. *Molecular Systems Biology*. 2007 Mar 13;3:91.

211. Wang X, Zhao X, Malik M, Drlica K. Contribution of reactive oxygen species to pathways of quinolone-mediated bacterial cell death. *Journal of Antimicrobial Chemotherapy*. 2010 Mar 1;65(3):520–4.

212. Wang X, Zhao X. Contribution of oxidative damage to antimicrobial lethality.

Antimicrobial agents and chemotherapy. 2009 Apr 1;53(4):1395–402.

213. Liu Y, Liu X, Qu Y, Wang X, Li L, Zhao X. Inhibitors of Reactive Oxygen Species Accumulation Delay and/or Reduce the Lethality of Several Antistaphylococcal Agents. *Antimicrobial Agents and Chemotherapy*. 2012 Nov;56(11):6048–50.

214. Shatalin K, Shatalina E, Mironov A, Nudler E. H2S: A Universal Defense Against Antibiotics in Bacteria. *Science*. 2011 Nov 18;334(6058):986–90.

215. Keren I, Wu Y, Inocencio J, Mulcahy LR, Lewis K. Killing by Bactericidal Antibiotics Does Not Depend on Reactive Oxygen Species. *Science*. 2013 Mar 8;339(6124):1213–6.

216. Ivanov II, Zhou L, Littman DR. Transcriptional regulation of Th17 cell differentiation. *Seminars in Immunology*. 2007;19(6):409–17.

217. O'Neill LAJ, Kishton RJ, Rathmell J. A guide to immunometabolism for immunologists. *Nature Reviews Immunology*. 2016;16(9):553–65.

218. Sharma LK, Lu J, Bai Y. Mitochondrial Respiratory Complex I: Structure, Function and Implication in Human Diseases. *Current Medicinal Chemistry.* 2009; 16 (10): 1266-77

219. Newsholme P, Curi R, Gordon S, Newsholme EA. Metabolism of glucose, glutamine, long-chain fatty acids and ketone bodies by murine macrophages. *Biochemical Journal*. 1986 Oct 1;239(1):121–5.

220. O'Neill LAJ, Pearce EJ. Immunometabolism governs dendritic cell and macrophage function. *Journal of Experimental Medicine*. 2016;213(1):15–23.

221. Noe JT, Mitchell RA. Tricarboxylic acid cycle metabolites in the control of macrophage activation and effector phenotypes. *Journal of Leukocyte Biology*. 2019;(February):1–9.

222. Mills EL, Kelly B, Logan A, Costa ASH, Varma M, Bryant CE, et al. Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages. *Cell*. 2016 Oct 6;167(2):457–470.e13.

223. Tannahill GM, Curtis AM, Adamik J, Palsson-McDermott EM, McGettrick AF, Goel G, et al. Succinate is an inflammatory signal that induces IL-1β through HIF-1α. *Nature*. 2013 Apr 24;496(7444):238–42.

224. Infantino V, Convertini P, Cucci L, Panaro MA, Di Noia MA, Calvello R, et al. The mitochondrial citrate carrier: a new player in inflammation. *Biochemical Journal*. 2011 Sep 15;438(3):433–6.

225. Rousset S, Alves-Guerra M-C, Mozo J, Miroux B, Cassard-Doulcier A-M, Bouillaud F, et al. The biology of mitochondrial uncoupling proteins. *Diabetes*. 2004 Feb 1;53 Suppl 1(suppl 1):S130-5.

226. Arimura Y, Yano T, Hirano M, Sakamoto Y, Egashira N, Oishi R. Mitochondrial superoxide production contributes to vancomycin-induced renal tubular cell apoptosis. *Free Radical Biology and Medicine*. 2012 May 1;52(9):1865–73.

227. Sakamoto Y, Yano T, Hanada Y, Takeshita A, Inagaki F, Masuda S, et al. Vancomycin induces reactive oxygen species-dependent apoptosis via mitochondrial cardiolipin peroxidation in renal tubular epithelial cells. *European Journal of Pharmacology*. 2017 Apr 5;800:48–56.

228. Paradies G, Petrosillo G, Pistolese M, Di Venosa N, Federici A, Ruggiero FM. Decrease in Mitochondrial Complex I Activity in Ischemic/Reperfused Rat Heart. *Circulation Research*. 2004 Jan 9;94(1):53–9.

229. Ott M, Gogvadze V, Orrenius S, Zhivotovsky B. Mitochondria, oxidative stress and cell death. *Apoptosis*. 2007 May 9;12(5):913–22.

230. Orrenius S. Reactive Oxygen Species in Mitochondria-Mediated Cell Death. *Drug Metabolism Reviews*. 2007 Jan 9;39(2–3):443–55.

231. Hanske J, Toffey JR, Morenz AM, Bonilla AJ, Schiavoni KH, Pletneva E V. Conformational properties of cardiolipin-bound cytochrome c. *Proceedings of the National Academy of Sciences*. 2012 Jan 3;109(1):125–30.

232. To M-S, Aromataris EC, Castro J, Roberts ML, Barritt GJ, Rychkov GY. Mitochondrial uncoupler FCCP activates proton conductance but does not block store-operated Ca2+ current in liver cells. *Archives of Biochemistry and Biophysics*. 2010 Mar;495(2):152–8.

233. Dlasková A, Hlavatá L, Ježek J, Ježek P. Mitochondrial Complex I superoxide production is attenuated by uncoupling. *The International Journal of Biochemistry & Cell Biology*. 2008 Jan;40(10):2098–109.

234. Jiang S, Li T, Zhou X, Qin W, Wang Z, Liao Y. Antibiotic drug piperacillin induces neuron cell death through mitochondrial dysfunction and oxidative damage. *Canadian Journal of Physiology and Pharmacology.* 2018 Jun; 96 (6): 562-568

235. Pochini L, Galluccio M, Scumaci D, Giangregorio N, Tonazzi A, Palmieri F, et al. Interaction of β-lactam antibiotics with the mitochondrial carnitine/acylcarnitine transporter. *Chemico-Biological Interactions*. 2008 Jun 17;173(3):187–94.

236. Song M, Wu H, Wu S, Ge T, Wang G, Zhou Y, et al. Antibiotic drug levofloxacin inhibits proliferation and induces apoptosis of lung cancer cells through inducing mitochondrial dysfunction and oxidative damage. *Biomedicine & Pharmacotherapy*. 2016 Dec 1;84:1137–43.

237. Li H, Jiao S, Li X, Banu H, Hamal S, Wang X. Therapeutic effects of antibiotic drug mefloquine against cervical cancer through impairing mitochondrial function and inhibiting mTOR pathway. *Canadian Journal of Physiology and Pharmacology*. 2017 Jan;95(1):43–50.

238. Zhao Y, Wang X, Li L, Li C. Doxycycline inhibits proliferation and induces apoptosis of both human papillomavirus positive and negative cervical cancer cell lines. *Canadian Journal of Physiology and Pharmacology*. 2016 May;94(5):526–33.

239. Salimi A, Eybagi S, Seydi E, Naserzadeh P, Kazerouni NP, Pourahmad J. Toxicity of macrolide antibiotics on isolated heart mitochondria: a justification for their cardiotoxic adverse effect. *Xenobiotica*. 2016 Jan 2;46(1):82–93.

240. Mckee EE, Ferguson M, Bentley AT, Marks TA. Inhibition of Mammalian Mitochondrial Protein Synthesis by Oxazolidinones. *ANTIMICROBIAL AGENTS AND CHEMOTHERAPY*. 2006;50(6):2042–9.

241. Esner M, Graifer D, Lleonart ME, Lyakhovich A. Targeting cancer cells through antibiotics-induced mitochondrial dysfunction requires autophagy inhibition. *Cancer Letters*. 2017 Jan 1;384:60–9.

242. Chen SCA, Lewis RE, Kontoyiannis DP. Direct effects of non-antifungal agents used in cancer chemotherapy and organ transplantation on the development and virulence of Candida and Aspergillus species. *Virulence*. 2011;2(4):280–95.

243. Chatzispyrou IA, Held NM, Mouchiroud L, Auwerx J, Houtkooper RH. Tetracycline antibiotics impair mitochondrial function and its experimental use confounds research. *Cancer research*. 2015 Nov 1;75(21):4446–9.

244. Huang JH, Lin CY, Wu SY, Chen WY, Chu CL, Brown GD, et al. CR3 and Dectin-1

Collaborate in Macrophage Cytokine Response through Association on Lipid Rafts and Activation of Syk-JNK-AP-1 Pathway. *PLoS Pathogens*. 2015;11(7):1–26.

245. Romani L., Mencacci A, Cenci E, Spaccapelo R, Toniatti C, Puccetti P, Bistoni F, Poli, V. Impaired Neutrophil Response and CD4 + T Helper Cell 1 D e v e l o p m e n t in Interleukin 6-deficient Mice Infected with *Candida albicans. Journal of Experimental Medicine*. 1996;183(4): 1345-55.

246. Cenci E, Mencacci A. Impaired antifungal effector activity but not inflammatory cell recruitment in interleukin-6-deficient mice with invasive pulmonary aspergillosis. *Journal of Infectious Diseases*. 2001;184(5):610–7.

247. Gresnigt MS, Becker KL, Smeekens SP, Jacobs CWM, Joosten LAB, van der Meer JWM, et al. *Aspergillus fumigatus*-Induced IL-22 Is Not Restricted to a Specific Th Cell Subset and Is Dependent on Complement Receptor 3. *The Journal of Immunology*. 2013;190(11):5629–39.

248. Walachowski S, Tabouret G, Foucras G. Triggering dectin-1-pathway alone is not sufficient to induce cytokine production by murine macrophages. *PLoS ONE*. 2016;11(2):1–26.

249. Ganesan S, Rathinam VAK, Bossaller L, Army K, William J, Mocarski ES, et al. Caspase-8 modulates Dectin-1 and CR3 driven IL-1β production in response to β-glucans and the fungal pathogen, *Candida albicans*. *Journal of Immunology*. 2014;193(5):2519–30.

250. Katsuno G, Takahashi H, Iwagaki H, Sugita S, Mori S, Saito S, et al. The effect of ciprofloxacin on CD14 and Toll-like receptor 4 expression on human monocytes. *Shock*. 2006;25(3):247–53.

251. Jensen JUS, Hein LH, Lundgren B, Bestle MH, Mohr T, Andersen MH, et al. Invasive *Candida* infections and the harm from antibacterial drugs in critically ill patients: Data from a randomized, controlled trial to determine the role of ciprofloxacin, piperacillin-tazobactam, meropenem, and cefuroxime. *Critical Care Medicine*. 2015;43(3):594–602.

252. Tominaga K, Yoshimoto T, Torigoe K, Kurimoto M, Matsui K, Hada T, et al. IL-12 synergizes with IL-18 or IL-1beta for IFN-gamma production from human T cells. *International Immunology*. 2000;12(2):151–60.

253. Riesbeck K, Sigvardsson M, Leanderson T, Forsgren A. Superinduction of cytokine gene transcription by ciprofloxacin. *Journal of immunology* (Baltimore, Md : 1950). 1994 Jul 1;153(1):343–52.

254. Kumar H, Kumagai Y, Tsuchida T, Koenig PA, Satoh T, Guo Z, et al. Involvement of the NLRP3 Inflammasome in Innate and Humoral Adaptive Immune Responses to Fungal -Glucan. *The Journal of Immunology*. 2009;183(12):8061–7.

255. Kankkunen P, Teirila L, Rintahaka J, Alenius H, Wolff H, Matikainen S. (1,3)- -Glucans Activate Both Dectin-1 and NLRP3 Inflammasome in Human Macrophages. *The Journal of Immunology*. 2010;184(11):6335–42.

256. Du J, Zhang X, Han J, Man K, Zhang Y, Chu ES, et al. Pro-Inflammatory CXCR3 Impairs Mitochondrial Function in Experimental Non-Alcoholic Steatohepatitis. *Theranostics*. 2017;7(17):4192–203.

257. Cole KE, Strick CA, Paradis TJ, Ogborne KT, Loetscher M, Gladue RP, et al. Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. *The Journal of experimental medicine*. 1998 Jun 15;187(12):2009–21.

258. Flier J, Boorsma DM, Van Beek PJ, Nieboer C, Stoof TJ, Willemze R, et al. Differential expression of CXCR3 targeting chemokines CXCL10, CXCL9, and CXCL11 in different types of skin inflammation. *Journal of Pathology*. 2001;194(4):398–405.

259. Lee J-H, Kim B, Jin WJ, Kim H-H, Ha H, Lee ZH. Pathogenic roles of CXCL10 signaling through CXCR3 and TLR4 in macrophages and T cells: relevance for arthritis. *Arthritis Research & Therapy*. 2017 Dec 19;19(1):163.

260. Jurkevitch E, Minz D, Ramati B, Barel G. Prey range characterization, ribotyping, and diversity of soil and rhizosphere Bdellovibrio spp. isolated on phytopathogenic bacteria. *Applied and Environmental Microbiology*. 2000;66(6):2365–71.

261. Rendulic S, Eppinger M, Baar C, Lanz C, Keller H, Meyer F, et al. A Predator Unmasked : Life Cycle of Bdellovibrio bacteriovorus from a Genomic Perspective. *Science*. 2004;689(2004):689–93.

262. Pan A, Chanda I, Chakrabarti J. Analysis of the genome and proteome composition of *Bdellovibrio bacteriovorus*: Indication for recent prey-derived horizontal gene transfer. *Genomics*. 2011 Sep 1;98(3):213–22.

263. Gophna U, Charlebois RL, Doolittle WF. Ancient lateral gene transfer in the evolution of *Bdellovibrio bacteriovorus*. *Trends in microbiology*. 2006 Feb 1;14(2):64–9.

264. Hallin PF, Ussery DW. CBS Genome Atlas Database: a dynamic storage for bioinformatic results and sequence data. *Bioinformatics*. 2004 Dec 12;20(18):3682–6.

265. Barabote RD, Rendulic S, Schuster SC, Saier MH. Comprehensive analysis of transport proteins encoded within the genome of *Bdellovibrio bacteriovorus*. *Genomics*. 2007 Oct 1;90(4):424–46.

266. Holt K, Wertheim H, Zadoks RN, Baker S, Whitehouse C, Dance D, et al. Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in *Klebsiella pneumoniae* , an urgent threat to public health. *Proceedings of the National Academy of Sciences*. 2015;112(27):E3574–81.

267. Snitkin ES, Zelazny AM, Thomas PJ, Stock F, Comparative N, Program S, et al.. Tracking a hospital outbreak of carbapenem-resistant *Klebsiella pneumoniae* with whole-genome sequencing. *Science of Translational Medicine*. 2013;4(148).

268. Negus D, Moore C, Baker M, Raghunathan D, Tyson J, Sockett RE. Predator Versus Pathogen: How Does Predatory *Bdellovibrio bacteriovorus* Interface with the Challenges of Killing Gram-Negative Pathogens in a Host Setting? *Annual Review of Microbiology*. 2017. 71: p. 441–57.

269. Hobley L, Fung R, Lambert C, Harris MATS, Dabhi JM, King SS, et al. Discrete Cyclic di-GMP-Dependent Control of Bacterial Predation versus Axenic Growth in *Bdellovibrio bacteriovorus*. *PLoS Pathogens*. 2012;8(2):e1002493.

270. Lambert C, Ivanov P, Sockett RE. A transcriptional 'scream' early response of *E. coli* prey to predatory invasion by *Bdellovibrio*. *Current Microbiology*. 2010;60(6):419–27.

271. Capeness MJ, Lambert C, Lovering AL, Till R, Uchida K, Chaudhuri R, et al. Activity of *Bdellovibrio* hit locus proteins, *bd0108* and *bd0109*, links type IVa pilus extrusion/retraction status to prey-independent growth signalling. *PLoS ONE*. 2013;8(11).

272. Evans KJ, Lambert C, Sockett RE. Predation by *Bdellovibrio bacteriovorus* HD100 requires type IV pili. *Journal of Bacteriology*. 2007;189(13):4850–9.

273. Thomashow LS, Rittenberg SC. Waveform analysis and structure of flagella and basal complexes from *Bdellovibrio bacteriovorus* 109J. *Journal of Bacteriology*.

1985;163(3):1038–46.

274. Sockett RE, Lambert C. *Bdellovibrio* as therapeutic agents: A predatory renaissance? *Nature Reviews Microbiology*. 2004;2(8):669–75.

275. Mattick JS. Type IV Pili and Twitching Motility. *Annual Review of Microbiology*. 2002;56(1):289–314.

276. Kachlany SC, Planet PJ, DeSalle R, Fine DH, Figurski DH, Kaplan JB. Flp-1, the first representative of a new pilin gene subfamily, is required for non-specific adherence of *Actinobacillus actinomycetemcomitans*. *Molecular Microbiology*. 2001;40(3):542–54.

277. Morehouse KA, Hobley L, Capeness M, Sockett RE. Three motAB stator gene products in *Bdellovibrio bacteriovorus* contribute to motility of a single flagellum during predatory and prey-independent growth. *Journal of Bacteriology*. 2011;193(4):932–43.

278. Kuru E, Lambert C, Rittichier J, Till R, Ducret A, Gray J, et al. Modifications important for *Bdellovibrio bacteriovorus* predation. *Nature Microbiology.* 2018;2(12):1648–57.

279. Lambert C, Chang C-Y, Capeness MJ, Sockett RE. The First Bite— Profiling the Predatosome in the Bacterial Pathogen *Bdellovibrio*. Herman C, editor. *PLoS ONE*. 2010 Jan 6;5(1):e8599.

280. Sockett RE. Predatory Lifestyle of *Bdellovibrio bacteriovorus* . *Annual Review of Microbiology*. 2009;63(1):523–39.

281. Lambert C, Cadby IT, Till R, Bui NK, Lerner TR, Hughes WS, et al. Ankyrin-mediated self-protection during cell invasion by the bacterial predator *Bdellovibrio bacteriovorus*. *Nature Communications*. 2015;6:1–9.

282. Lambert C, Lerner TR, Bui NK, Somers H, Aizawa SI, Liddell S, et al. Interrupting peptidoglycan deacetylation during *Bdellovibrio* predator-prey interaction prevents ultimate destruction of prey wall, liberating bacterial-ghosts. *Scientific Reports*. 2016;6(April):1–19.

283. Thomashow MF, Rittenberg SC. Intraperiplasmic Growth of *Bdellovibrio bacteriovorus* 109J: N-Deacetylation of Escherichia coli Peptidoglycan Amino Sugars. *JOURNAL OF BACTERIOLOGY*. 1978.

284. Rittenberg SC. Bdellovibrios-intraperiplasmic growth. *Experimental Microbial Ecology*. 1982

285. Kuenen JG, Rittenberg SC. Incorporation of long chain fatty acids of the substrate organism by *Bdellovibrio bacteriovorus* during intraperiplasmic growth. *Journal of Bacteriology*. 1975;121(3):1145–57.

286. Beck S, Schwudke D, Strauch E, Appel B, Linscheid M. *Bdellovibrio bacteriovorus* Strains Produce a Novel Major Outer Membrane Protein during Predacious Growth in the Periplasm of Prey Bacteria. *Journal of Bacteriology*. 2004;186(9):2766–73.

287. Diedrich DL, Portnoy Duran C, Conti SF. Acquisition of Escherichia coli outer membrane proteins by *Bdellovibrio* sp. strain 109D. *Journal of Bacteriology*. 1984;159(1):329–34.

288. Fenton A, Lambert C, Wagstaff PC, Sockett RE. Manipulating each MreB of *Bdellovibrio bacteriovorus* gives diverse morphological and predatory phenotypes. *Journal of Bacteriology*. 2010;192(5):1299–311.

289. Fenton AK, Kanna M, Woods RD, Aizawa SI, Sockett RE. Shadowing the actions of a predator: Backlit fluorescent microscopy reveals synchronous nonbinary septation of predatory *Bdellovibrio* inside prey and exit through discrete bdelloplast pores. *Journal*

of Bacteriology. 2010;192(24):6329–35.

290. Greenwood S. University of Nottingham MSci Thesis; 2016.

291. Al-Bayati AM. A Previously Cryptic Diguanylate Cyclase Can Be Active and Allows *Bdellovibrio Bacteriovorus* To Become A Selective Predator On Human Pathogens. University of Nottingham PhD Thesis; 2017.

292. Gray AN, Egan AJ, Van IL, Verheul J, Colavin A, Koumoutsi A, et al. Coordination of peptidoglycan synthesis and outer membrane constriction during *Escherichia coli* cell division. *eLIFE*. 2015;4:1–29.

293. Schäfer A, Tauch A, Jäger W, Kalinowski J, Thierbach G, Pühler A. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene*. 1994 Jul 22;145(1):69–73.

294. Ducret A, Quardokus EM, Brun Y V. MicrobeJ, a tool for high throughput bacterial cell detection and quantitative analysis. *Nature microbiology*. 2016;1(7):16077.

295. Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nature Methods*. 2011 Oct 29;8(10):785–6.

296. El-Gebali S, Mistry J, Bateman A, Eddy SR, Luciani A, Potter SC, et al. The Pfam protein families database in 2019. *Nucleic Acids Research*. 2019 Jan 8;47(D1):D427–32.

297. Zhang Y. I-TASSER server for protein 3D structure prediction. 2008;

298. Walburger A, Lazdunski C, Corda Y. The Tol/Pal system function requires an interaction between the C-terminal domain of TolA and the N-terminal domain of TolB. *Molecular Microbiology*. 2002;44(3):695–708.

299. Derouiche R, Gavioli M, Bénédetti H, Prilipov A, Lazdunski C, Lloubès R. TolA central domain interacts with *Escherichia coli* porins. *The EMBO Journal*. 1996;15(23):6408.

300. Clavel T, Germon P, Vianney A, Portalier R, Lazzaroni JC. TolB protein of *Escherichia coli* K-12 interacts with the outer membrane peptidoglycan-associated proteins Pal, Lpp and OmpA. *Molecular Microbiology*. 1998 Jul 26;29(1):359–67.

301. Tudor JJ, Karp MA. Translocation of an Outer Membrane Protein into Prey Cytoplasmic Membranes by Bdellovibrios. *Journal of Bacteriology.* 1994 Feb; 176 (4): 948-52

302. Talley BG, Mcdade RL, Diedrich DL. Verification of the Protein in the Outer Membrane of *Bdellovibrio bacteriovorus* as the OmpF Protein of Its *Escherichia coli* Prey. Vol. 169, *JOURNAL OF BACTERIOLOGY*. 1987.

303. Stein MA, Mcallister SA, Torian BE, Diedricht DL. Acquisition of Apparently Intact and Unmodified Lipopolysaccharides from *Escherichia coli* by *Bdellovibrio bacteriovorus*. Vol. 174, *JOURNAL OF BACTERIOLOGY*. 1992.

304. Rayner JR, Cover WH, Martinez RJ, Rittenberg SC. *Bdellovibrio bacteriovorus* Synthesizes an OmpF-Like Outer Membrane Protein during Both Axenic and Intraperiplasmic Growth. Vol. 163, *JOURNAL OF BACTERIOLOGY*. 1985.

305. Rittenberg SC, Shilo M. Early host damage in the infection cycle of *Bdellovibrio bacteriovorus*. *Journal of Bacteriology*. 1970;102(1):149–60.

306. Borgnia MJ, Subramaniam S, Milne J. Three-Dimensional Imaging of the Highly Bent Architecture of *Bdellovibrio bacteriovorus* by using Cryo-Electron Tomography. *Journal of Bacteriology*. 2008;190(7):2588–96.

307. Butan C, Hartnell L, Fenton A, Bliss D, Sockett R, Subramaniam S, et al. Spiral Architecture of the Nucleoid in *Bdellovibrio Bacteriovorus*. *Journal of Bacteriology*. 2011;193(6):1341–50.

308. Gillum A, Tsay E, Kirsch D. Isolation of the *Candida albicans* gene for orotidine-5′-phosphate decarboxylase by complementation of S. cerevisiae ura3 and E. coli pyrF mutations. *Molecular Genetics and Genomics*. 1984;198(1):179–82.

309. Gratacap RL, Rawls JF, Wheeler RT. Mucosal candidiasis elicits NF- κB activation, proinflammatory gene expression and localized neutrophilia in zebrafish. *Disease Models & Mechanisms*. 2013;6(5):1260–70.

310. Wheeler RT, Kombe D, Agarwala S, Fink G. Dynamic, Morphotype-Specific *Candida albicans* β- Glucan Exposure during Infection and Drug Treatment. *PLoS Pathogens*. 2008;4(12):1–12.

311. Simon R, Priefer U, Pühler A. A Broad Host Range Mobilization System for In Vivo Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria. *Bio/Technology*. 1983 Nov;1(9):784–91.

312. Hanahan D. Studies on transformation of *Escherichia coli* with plasmids. Journal of Molecular Biology. 1983 Jun 5;166(4):557–80.

313. Rogers M, Ekaterinaki N, Nimmo E, Sherratt D. Analysis of Tn7 transposition. *MGG Molecular & General Genetics*. 1986 Dec;205(3):550–6.

314. Makowski L, Trojanowski D, Till R, Lambert C, Lowry R, Sockett RE, et al. Dynamics of chromosome replication and its relationship to predatory attack lifestyles in *Bdellovibrio bacteriovorus*. *BioRxiv*. 2019;

Appendix

Supplementary Tables

Supplementary Table 1: Fungal strains used in rotation 1

Strain	Species	Genotype	Reference
Candida albicans SC5314	*C. albicans*	Wildtype Typed Strain	(308)
dTomato *C. albicans*	*C. albicans*	CAF2.1 transformed with pENO1-dTom-NATr	(309)
GFP *C. albicans*	*C. albicans*	SC5314 transformed with pENO1-eGFP_NAT	(310)

Supplementary Table 2: Primers used for the qPCR studies in rotation 1.

Gene	Forward Primer	Reverse Primer
GapdH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA
NOS2 (Nitric Oxide Synthase 2)	GTTCTCAGCCCAACAATACAAG	GTGGACGGGTCGATGTCAC
Arg1 (Arginase 1)	CTCCAAGCCAAAGTCCTTAGAG	GGAGCTGTCATTAGGGACATCA
MRC1 (Mannose receptor 1)	CTCTGTTCAGCTATTGGACGC	TGGCACTCCCAAACATAATTTGA
Msr1 (Macrophage scavenger receptor 1)	TGGAGGAGAGAATCGAAAGCA	CTGGACTGACGAAATCAAGGAA

Clqa (Complement component 1a)	TTCGGCAGAACCCAATGACG	TGGTATGGACTCTCCTGGTTG
IL10 (Interleukin 10a)	TTGTCGCGTTTGCTCCCATT	GAAGGGCTTGGCAGTTCTG
CR3 (Complement receptor 3)	GGT TGC AGC AGT CTA TGA AGG	ATC TTG AGT GCA CCA AGC C
Dectin-1/ Clec7a	GGGTACCGACGACACAATTCAGGG	GGATCCACGCGGAACCAGCAGTTCCTTCTCACAG
Mincle/ Clec4e	AAGGATCCGATCTTGGAGGATGATTAAATGGCTTGTCCTTTGAACTGGAAACATTATC	TAAAGCTTCTACTCATGCCACTCGATTTTCTGTGCTTCGAAGATGTCATTCAGTCCGTCCAGAGGACTTATTTCTGGC
CXCR3 (CXC Motif Chemokine Receptor 3)	TACCTTGAGGTTAGTGAACGTCA	CGCTCTCGTTTTCCCCATAATC
Hexokinase 2 (HK2)	ATGATCGCCTGCTTATTCACG	CGCCTAGAAATCTCCAGAAGGG
Enolase 1 (ENO1)	TGCGTCCACTGGCATCTAC	CAGAGCAGGCGCAATAGTTTTA
Pyruvate Kinase (PK)	GAACATTGCACGACTCAACTTC	CAGTGCGTATCTCGGGAC

Supplementary Table 3: Bacterial strains used in rotation 2

Strain	**Description**	**Source**
E. coli DH5α	Strain for cloning genes into plasmids	(311)
E. coli S17-1	Strain for conjugation of mobilisable plasmids into *B. bacteriovorus* HD100	(312)
E. coli S17-1 pZMR100	*E. coli* S17-1 containing the plasmid pZMR100 to confer resistance to kanamycin.	(313)

	Strain used as prey for *B. bacteriovorus*	
B. bacteriovorus HD100	Wild type *B. bacteriovorus*	(261)
B. bacteriovorus HD100 *bd0064mCherry*	*B. bacteriovorus* containing a *bd0064mCherry* gene	Gift from Emma Banks (University of Nottingham)
B. bacteriovorus HD100 *bd1815mNeon*	*B. bacteriovorus* containing a *bd1815mNeon* gene	This project
B. bacteriovorus HD100 *bd2439mNeon*	*B. bacteriovorus* containing a *bd2439mNeon* gene	This project
B. bacteriovorus HD100 *bd2754mNeon*	*B. bacteriovorus* containing a *bd2754mNeon* gene	This project
B. bacteriovorus HD100 *bd0635mCherry*	*B. bacteriovorus* containing a double crossover integrated *bd0635mCherry* gene.	Sockett lab, unpublished

Supplementary Table 4: Plasmids used in rotation 2

Plasmid	**Description**	**Source**
pK18*mobsacB*	Suicide vector used for introduction of mNeon tagged genes of interest into *B. bacteriovorus*	(293)
pAKF220	DNA template containing *mNeon* gene	(314)
pZMR100	Plasmid conferring kanamycin resistance	(313)
p*bd1815-mNeon*	pK18*mobsacB* plasmid with the addition of *bd1815* and *mNeon* genes	This project
p*bd2439-mNeon*	pK18*mobsacB* plasmid with the addition of *bd1815* and *mNeon* genes	This project

pbd2754-mNeon	pK18*mobsacB* plasmid with the addition of *bd1815* and *mNeon* genes	This project

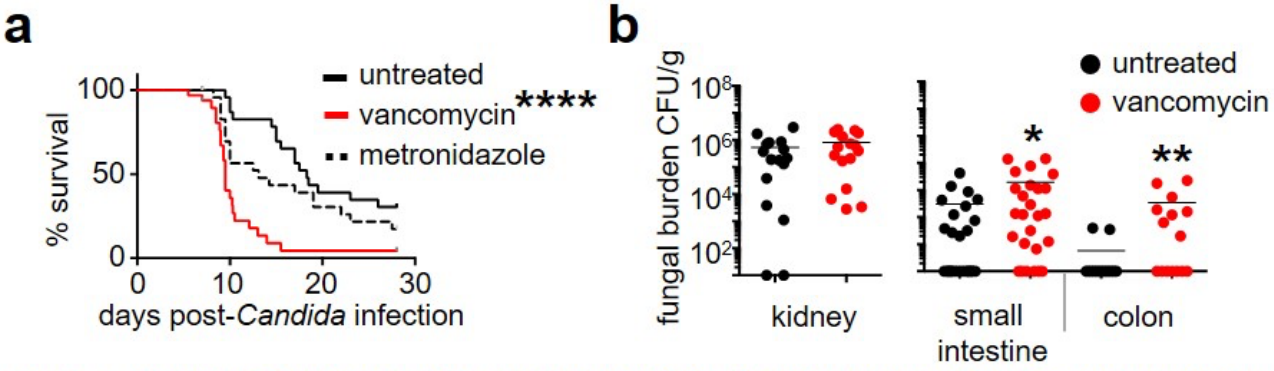
Supplementary Table 5: Primers used in rotation 2

Primer reference	Purpose	Primer sequence
Bd1815_F	*bd1815* tag Gibson assembly primers	CGTTGTAAAACGACGGCCAGTGCCAGGCGTTATTTTGTCGGGTATC
Bd1815_R		CTTTCGAAACCATGAAAATAAATCCGACCGTCAG
Bd1815mNeon_F		GGATTTATTTTCATGGTTTCGAAAGGAGAG
Bd1815mNeon_R		GGAAACAGCTATGACCATGATTACGTCACTTATAGAGTTCATCCATAC
Bd2439_F	*bd2439* tag Gibson assembly primers	CGTTGTAAAACGACGGCCAGTGCCATTGCGTGTTTTAGCTGTACC
Bd2439_R		CTTTCGAAACCATGTCCACGCGTTTGATTTG
Bd2439mNeon_F		AAACGCGTGGACATGGTTTCGAAAGGAGAG
Bd2439mNeon_R		GGAAACAGCTATGACCATGATTACGTCACTTATAGAGTTCATCCATACC
Bd2754_F	*bd2754* tag Gibson assembly primers	CGTTGTAAAACGACGGCCAGTGCCATCATACATGGGCAGGCGG
Bd2754_R		CTTTCGAAACCATGAACTCCAGAGGCAGGGATAC
Bd2754mNeon_F		CCTCTGGAGTTCATGGTTTCGAAAGGAGAG
Bd2754mNeon_R		GGAAACAGCTATGACCATGATTACGTCACTTATAGAGTTCATCCATAC
Bd1815 Forward Screening Primer	Plasmid verification: PCR amplification of genomic fragment for validatory sequencing	CCCAACCATCATCCGAGACA
Bd2439 Forward Screening Primer		GTCGCAGGTGAAATCGTGAC
Bd2754 Forward Screening Primer		CTTTCGGTAGGGTGGGACTT
mNeon Reverse Screening Primer		TCACTTATAGAGTTCATCCA

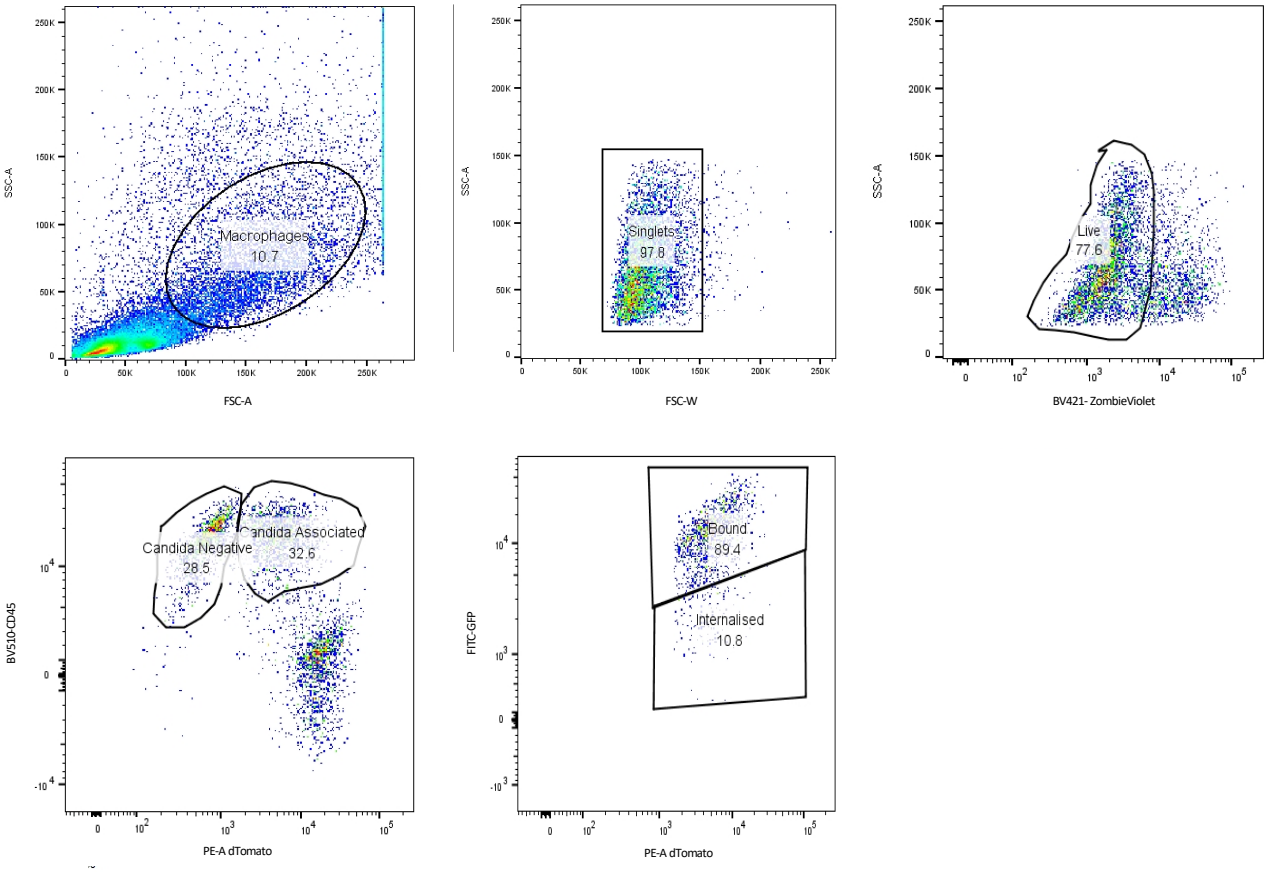
Supplementary Table 6: DNA restriction digest enzyme combinations and expected fragment sizes for validation of successful gene-*mNeon* fusion

Gene	Restriction digest enzymes	Fragment sizes (bp)
bd1815	NheI	6172, 1205
	SacI	5071, 2306
	BamHI- BglII	5699, 1678
bd2439	NheI	6172, 3526
	SacI	7392, 2306
	BamHI- BglII	5699, 3999
bd2754	NheI	6172, 1205
	SacI	4091, 2306, 980
	BamHI- BglII	5699, 1678

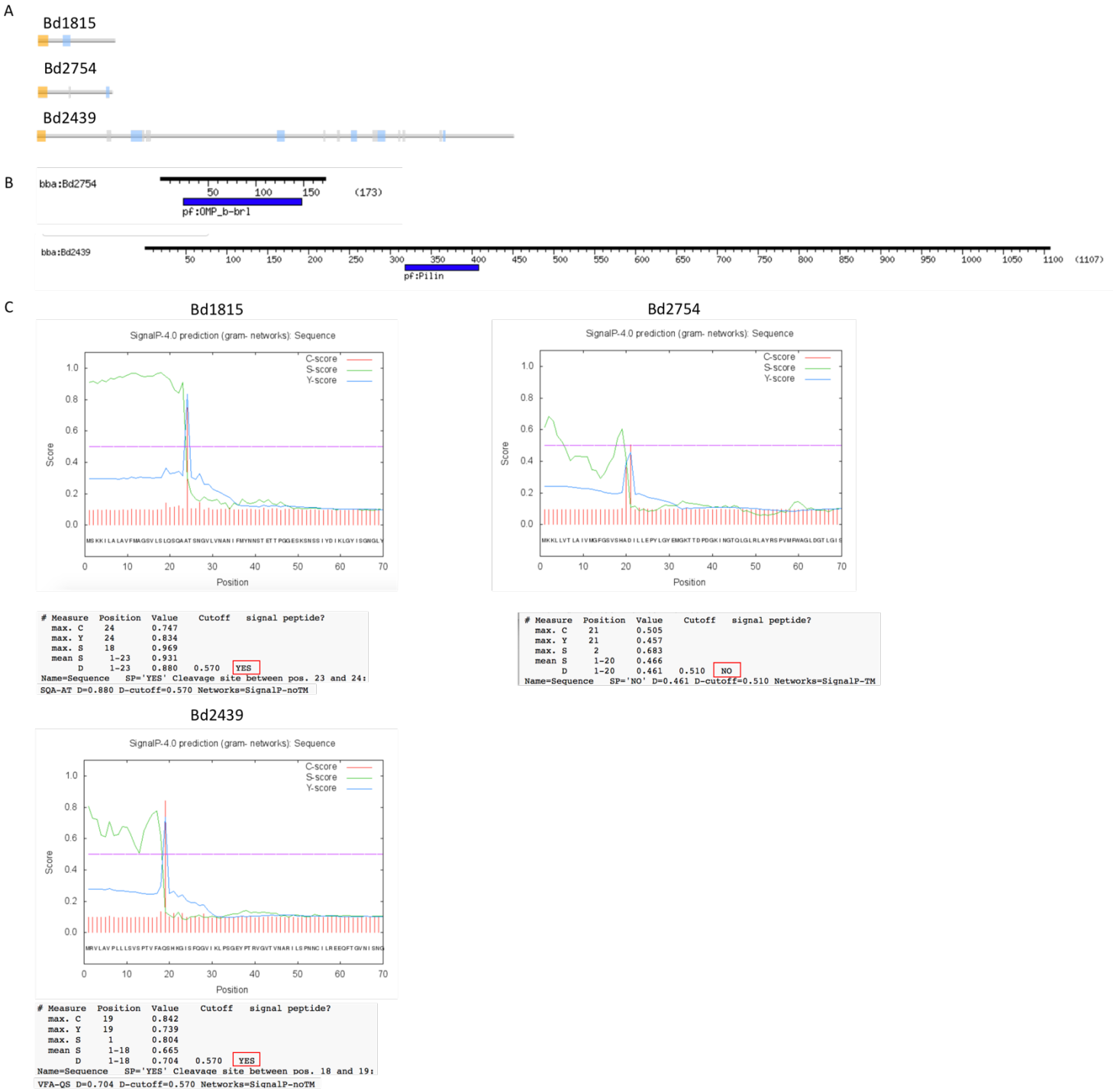
Supplementary Figures



Supplementary Figure 1: Antibiotic treatment increased susceptibility to experimental invasive candidiasis.
A: Treatment of mice with vancomycin increased mortality in an intravenous candidemia infection model, whereas treatment with metronidazole did not, and B: increased fungal burden in organs atypical of dissemination. WT mice were treated with the indicated antibiotics and infected intravenously with $1x10^5$ CFU *C. albicans* and analysed for A: survival and B: fungal burden (6 days post-infection). Data is pooled from 2-3 independent experiments and analysed by A: Log-Rank or B: Mann-Whitney U-tests. * indicates significance at $P<0.05$, ** indicates significance at $P<0.01$, **** indicates significance at $P<0.0001$. Work carried out by R. Drummond as part of (Drummond et al, unpublished).



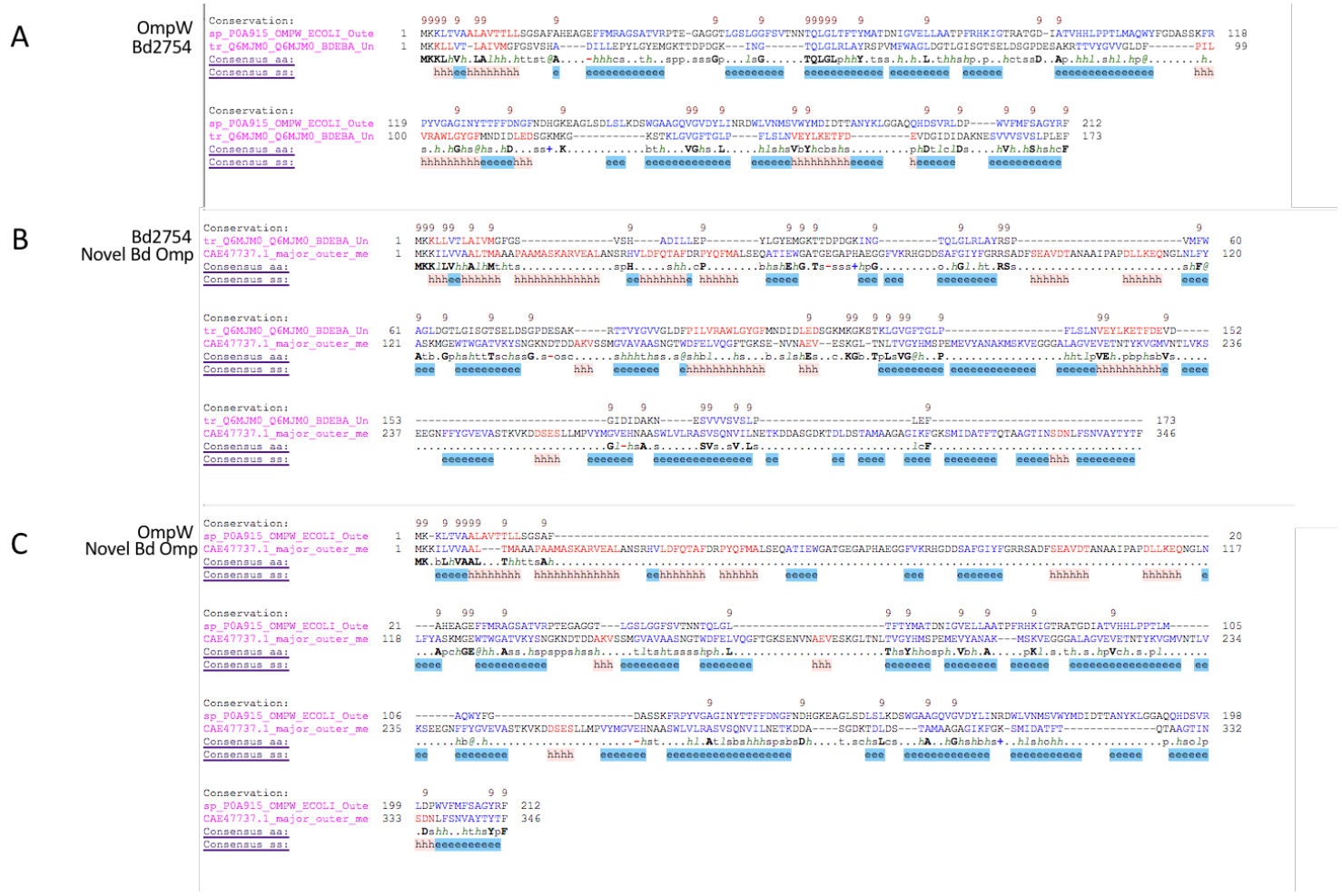
Supplementary Figure 2: Example gating strategy for the phagocytic uptake assay
A representative flow cytometry gating strategy for the phagocytic uptake assay shown in Figure 10, demonstrating how *C. albicans* was determined to be associated with macrophage cells ($CD45^+dTomato^+$), bound (associated but not internalised: $GFP^+dTomato^+$) or internalised ($GFP^-dTomato^+$). Cells were gated by forward scatter (lymphocytes); forward scatter width (singlets); $ZombieViolet^-$ (live); $CD45^+dTomato^+$ (*Candida* associated macrophage); $GFP^+dTomato^+$ (*Candida*-bound) $GFP^-dTomato^+$ (*Candida*- internalised).



Supplementary Figure 3: Predicted domain structures of Bd1815, Bd2754 and Bd2439
A: Protein sequences for each protein were ran through the Pfam database to identify potential domains within the structures of Bd1815, Bd2754 and Bd2439, where orange regions indicate signal peptides, blue regions indicate regions of low complexity and grey regions indicate regions of high levels of disorder (El-Gabali et al, 2019). B: Protein sequences were then screened against the KEGG database whereby a beta barrel domain was identified within the structure of Bd2754 and a pilin domain within Bd2439. C: Protein sequences were then screened against the SignalP 4.0 server, a programme that predicts the presence of signal peptides and gives a definitive output. Outputs from the SignalP 4.0 database indicated that Bd1815 and Bd2439 had signal domains but that Bd2754 did not, contrary to previous indications from other databases (Petersen et al, 2011).



Supplementary Figure 4: Promals3D structural alignments of Bd1815 to its closest structural homologue *E. coli* OmpA and a novel *B. bacteriovorus* porin.
Protein sequences of Bd1815, its closest structural homologue *E. coli* OmpA and the novel *B. bacteriovorus* porin CAE47737.1 were screened using the Promals3D database. A: Bd1815 and OmpA showed a strong alignment initially, with few conserved amino acids but a strongly conserved consensus amino acid sequence and nature, which later breaks down owing to OmpA being twice the size of Bd1815. B: Bd1815 and the novel *B. bacteriovorus* porin show strong similarity, especially within the first 20 amino acids (known to encode the signal peptide) and then conserved consensus amino acid sequence throughout (excluding gaps in sequence due to the difference in size of the two proteins). C: The novel *B. bacteriovorus* porin CAE47737.1 also shows strong similarity and consensus amino acid sequence to the *E. coli* OmpA porin throughout. Consensus amino acid entries: **bold** indicates conserved amino acids, non-bold indicates conserved amino acid nature. Consensus secondary structure: The letter e on a blue background represent conserved beta barrel domains, The letter h on a red background indicate conserved helical domains.



Supplementary Figure 5: Promals3D structural alignments of Bd2754 to its closest structural homologue *E. coli* OmpW and a novel *B. bacteriovorus* porin.
Protein sequences of Bd2754, its closest structural homologue *E. coli* OmpW and the novel *B. bacteriovorus* porin CAE47737.1 were screened using the Promals3D database. A: Bd2754 and OmpW showed a strong alignment, with few conserved amino acids but a strongly conserved consensus amino acid sequence and nature throughout. B: Bd2754 and the novel *B. bacteriovorus* porin CAE47737.1 show strong similarity, especially within the first 20 amino acids (known to encode the signal peptide) and then conserved consensus amino acid sequence throughout (excluding gaps in sequence due to the difference in size of the two proteins). C: The novel *B. bacteriovorus* porin CAE47737.1 also shows strong similarity and consensus amino acid sequence to the *E. coli* OmpW porin throughout. Consensus amino acid entries: **bold** indicates conserved amino acids, non-bold indicates conserved amino acid nature. Consensus secondary structure: The letter e on a blue background represent conserved beta barrel domains, The letter h on a red background indicate conserved helical domains.



Supplementary Figure 6: Promals3D structural alignments of Bd2439 and its closest structural homologue SepA. Protein sequences of Bd2439 and its closest structural homologue *S. flexneri* SepA were screened using the Promals3D database. Despite individual amino acids throughout the alignment being conserved, the sequences highly differed between Bd2439 and SepA. Another consideration is the omission of large regions of Bd2439 sequence within the alignment, undermining the alignment further. Consensus amino acid entries: **bold** indicates conserved amino acids, non-bold indicates conserved amino acid nature. Consensus secondary structure: The letter e on a blue background represent conserved beta barrel domains, The letter h on a red background indicate conserved helical domains.

Bd1815



Bd2754



Supplementary Figure 7: 3D modelling of Bd1815 and Bd2754 onto a *E. coli* OmpA template
Sequences of Bd1815 and Bd2754 were aligned onto the template of the porin *E. coli* OmpA (bottom cluster of each alignment). Red Hs indicate the presence of helices, Cs indicate the presence of extensive coiled regions and blue Es represent beta sheets. Despite a high dissimilarity in sequence identity between Bd1815/Bd2754 and OmpA, the nature and secondary structure of both proteins are high similar in nature. This alignment was a gift from Dr Andrew Lovering, University of Birmingham.