Investigating *Bdellovibrio* predation: novel isolates and bioinformatics studies of predatory enzymes

Joshua Yates

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Abstract

Bdellovibrio are predatory bacteria that replicate within the periplasm of their Gram-negative bacterial prey. They exhibit a biphasic lifestyle existing as free-swimming cells and inter-periplasmic growing cells. There is great interest in *Bdellovibrio spp.* due to their potential as antibacterial therapeutics in the current era of increasing levels of antibiotic resistance. These predators are ubiquitous in nature, being found in environments from freshwater, to soil, to the GI tracts of humans.

This study isolated novel *Bdellovibrio spp.* from the GI tract of farm animals and completed initial characterization of these isolates: one isolate from reindeer was found to have greater predation efficiency in a microaerophilic environment and at 37 ^{o}C, which contrasts with the well-characterized strains of *Bdellovibrio bacteriovorus* which grow best in an aerobic environment at 29 °C. This isolate represents the first reported *Bdellovibrio spp*. that preferentially undergoes a predatory lifecycle under microaerophilic conditions. This study has led us to hypothesize that isolation of novel *Bdellovibrio* isolates from the proposed environment for future applications may result in the identification of new strains that may be more optimal for future use because they are more likely to have adapted to the environmental conditions they would encounter.

The *Bdellovibrio bacteriovorus* HD100 genome encodes a large catalogue of hydrolytic enzymes which are hypothesized to play a major role in the degradation of the contents of prey cells for uptake as nutrients for growth by the *Bdellovibrio*. In this study a bioinformatics approach was used to further classify and group these enzymes by similar domain structure. Potential predatory candidates were identified based on previous transcriptomic studies to determine putative roles of each enzyme within the predatory lifecycle. This study will guide the direction of future molecular studies into the functions of the *Bdellovibrio* enzyme catalogue.

This work has combined experimental and bioinformatic approaches to study the diversity of *Bdellovibrio* and other predatory bacteria as well as the genomic complement of hydrolytic enzymes they encode. Taken together, these two approaches have highlighted the potential of *Bdellovibrio* as both an antimicrobial in itself and as a putative source of putative antibacterial enzymes.

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Abbreviations

AgNPs	Silver nanoparticles
AMR	Antimicrobial resistance
AP	Attack phase
CFU/mL	Colony forming units per millilitre
EM	Electron microscope
ETEC	Enterotoxigenic Escherichia coli
GP	Growth phase
HD	Host dependent
HI	Host independent
IKC	Infectious keratoconjunctivitis
IM	Inner membrane
LPS	Lipopolysaccharide
MCPs	Methyl-accepting chemotaxis proteins
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
OM	Outer membrane
ORF	Open reading frame
OriC	Origin of replication
PA	Protein associated domain
PAAT	Polar amino acid uptake transporter
PBPs	Penicillin binding proteins
PFU/mL	Plaque forming units per milliliter
PG	Peptidoglycan
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase PCR
SNase	Staphylococcal-like nuclease

T1SS	Type 1 secretion system
T2SS	Type 2 secretion system
T4SS	Type 4 secretion system
T5SS	Type 5 secretion system
T6SS	Type 6 secretion system
TCDB	Transporter classification database

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1.0 Introduction

In the 21st century, antimicrobial resistance (AMR) among pathogenic microbes poses a great threat, not only by increasing pressures on already struggling health care systems around the world, but by also impacting on animal health and welfare, subsequently threatening global food security (Founou, Founou and Essack, 2021). The predatory bacterium *Bdellovibrio* has been proposed as a much-needed alternative approach, with potential as a therapeutic option against many important resistant Gram-negative bacteria (Sockett, 2009). Further understanding of the biological mechanisms underlying predation and the safety of this organism *in vivo* are needed before this 'living antibiotic' can be deployed as a viable antimicrobial solution in the future.

1.1 AMR and the need for new antimicrobials

1.1.1 The growing problem of AMR

Antimicrobial resistance (AMR) has been defined as "…when microorganisms such as bacteria, viruses, fungi and parasites change in ways that render the medications used to cure the infections they cause ineffective" (Anon., 2017). Mortality attributed to AMR cannot be understated. Annually, 700,000 deaths are currently reported to be attributed to AMR (Asokan et al., 2019). The O'Neill report in 2016 predicted the number of fatalities to rise to 10 million annually by 2050, a figure which eclipses current deaths by cancer (Anon., 2016). AMR will also have a significant impact on the economic status of the globe, where AMR related healthcare costs are expected to rise to between $300 billion and $1 trillion globally by 2050 (Hardie, 2020). A reduction in global GDP of 1% is also predicted due to the effects of AMR, with developing countries suffering the greatest impact, this would be an ultimate cost of $100-$200 trillion (Dadgostar, 2019). Alternatively, GDP could fall 3.8% by an earlier date of 2030 (Hardie, 2020). A common prediction made by many researchers, is that the fall in GDP will push millions of extra people into extreme poverty, leading people into a 'medical poverty trap' where they are less likely to receive the treatment needed (Ahmad and Khan, 2019; van Dongen, 2020).

Many antibiotics are produced as secondary metabolites by soil bacteria such as *Streptomyces*, exposure of other bacteria to these compounds has created profound selection pressures (Fair and Tor, 2014). Consequently, evolution has driven resistances to these antibiotics among the bacteria exposed to them, meaning resistances to 'newly discovered' drugs may already exist before their introduction as a therapeutic (Antimicrobials, 2006). In the current antibiotic pipeline 76 new antibiotics are reported to be in development, just over half of these target pathogens on the WHO priority list (Butler et al., 2022). However, only 4 of these have distinct modes of actions from those currently utilised in today's fight against pathogenic bacteria, meaning resistances are likely to occur quickly in most newly developed antibiotics leading to extremely limited treatment of many bacterial infections in the future without effective alternatives.

1.1.2 The role of agriculture and veterinary usage of antibiotics in AMR

Animals are of major concern in the wider picture of AMR. Antimicrobial usage in animals, particularly those farmed for food, is high and uncontrolled in many countries. Due to intensive farming strategies in these countries, farm animals are primed for outbreaks of pathogens, therefore antimicrobials are deployed prophylactically in an aggressive approach to solve the issue (Landers et al., 2012). In the US, approximately 80% of all antibiotic usage is focussed on animal agriculture, where most notably, 70% of these antibiotics are recognised as important to human medicine (Martin, Thottathil and Newman, 2015; Ventola, 2015). In countries such as the US and China, antibiotics are used not only for their antimicrobial properties, but are commonly used as growth promotors, although this practice has been restricted in the EU (Costa et al., 2017, Anon., 2020a). The use of antibiotics in animal agriculture not only exposes the animals themselves to these antimicrobials, but also humans through leaching into water systems, soil and through manure spreading. Crucially, antibiotics in the environment are not fully degraded, increasing exposure of bacteria to them, resulting in the mutagenic events that lead to the emergence of resistance. The acquisition of these resistances by clinically relevant pathogens further increases the appearance of multidrug resistant (MDR) bacteria (Manyi-Loh et al., 2018).

1.1.3 *Salmonella*: A key zoonotic pathogen

In the present day, bacterial infections of common livestock continue to represent a major problem in the food industry. Not only for the wellbeing of the animals themselves, but for the economic impacts they have on human society, in addition to the potential for zoonotic transmission of many human-animal shared diseases.

Salmonella spp. represent a key example of this threat, affecting farm animals and humans alike. Several *Salmonella* serovars show a strict host range, including Typhi and Paratyphi, two key human adapted pathogenic serovars (Tanner and Kingsley, 2018). Others exhibit a broad host range however; Typhimurium, Enteritidis and Gallinarum are common examples. These broad range serovars are transmissible between asymptomatic native hosts and humans, with *S.* Typhimurium and *S.* Enteritidis being the most common to be implicated with non-typhoidal human Salmonellosis (Suar et al., 2006; Crum-Cianflone, 2008; Evangelopoulou et al., 2015). *Salmonella* pose a particular problem in the farming of pigs, where swine are susceptible to a number of *Salmonella* serovars including: Typhimurium, Typhisuis, Dublin, Gallinarum and Choleraesuis (Kim and Isaacson, 2017). Salmonellosis in swine can present as less serious mild diarrhoea and fever, to more severe dehydration, lesion formation and with regards to the Kunzendorf variant of Choleraesuis, septicaemia and fatality (Uzzau et al., 2000; Evangelopoulou et al., 2015; Kim and Isaacson, 2017). Swine farming is both intense and stressful on pigs (Martínez-Miró et al., 2016). These factors can lead to weakened and altered immune responses which increases susceptibility to pathogens including *Salmonella*. Faecal shedding of *Salmonella* among pigs can cause contamination of slaughterhouses, equipment and other pigs, providing possibly transmission routes to humans through the food chain (Kim and Isaacson, 2017).

In addition to health aspects, *Salmonella* has a high economic burden, with a cost of €3 billion per year and >80,000 Salmonellosis cases per year in the European Union alone (Smith et al., 2018). Alternative control measures to antibiotics against *Salmonella spp.* are therefore needed for both an improvement in animal health and welfare, and also to combat the economic burden of *Salmonella* infection in pigs.

1.1.4 Other key zoonotic pathogens

Campylobacter spp., although most prevalent in poultry, is also an issue in the porcine industry (Vanderwaal and Deen, n.d.). Like chickens, pigs carry *Campylobacter spp.* as a commensal with little pathogenicity to self but, the bacterium has potential for zoonotic transmission to humans due to contaminated pork (Aguilar et al., 2014; Facciolà et al., 2017).

The Enterotoxigenic (ETEC) pathotype of *E. coli* is also responsible for diarrhoea in pigs, most commonly from early age aided by poor digestive enzyme production and more alkaline stomach and small intestine (Dubreuil, Isaacson and Schifferli, 2016). Costs associated with this disease can be high, especially in post-weaning diarrhoea (PWD) cases, where mortality, reduction in weight gain and subsequent vaccination programmes all contribute to high costs (Luppi, 2017).

Gram-negative bacterial infections also pose a threat to ruminants, including cattle and others such as reindeer. Ocular infectious keratoconjuctivitis (IKC), is deemed to be the most important ocular disease worldwide in cattle (Sánchez Romano et al., 2018) Although thought to be multi-factorial in origins with many bacterial and viral agents implicated in the disease, *Moraxella spp.* is the most common causative agent (Sánchez Romano et al., 2018) This disease is characterised by conjunctivitis, lacrimation and ulceration of the cornea and subsequent visual impairment and reduction in weight gain (Tryland et al., 2009). This disease has a significant impact on health & welfare of the animals infected.

1.1.5 Alternatives to conventional antibiotics

Bacteriophage are a promising antimicrobial, even after their early therapeutic usage dwindled following the discovery of antibiotics. Now, with growing levels of antibiotic resistance, the highly ubiquitous nature and narrow host ranges of bacteriophage have made them lucrative antimicrobial alternatives to antibiotics in therapeutic settings (Mulani et al., 2019) However, their narrow host range and (typically) single receptor binding enables the target pathogens to acquire resistance readily, reducing their therapeutic potential.

Charged antimicrobial peptides are another approach, utilising naturally- and artificially- produced oligopeptides with antimicrobial properties (Bahar and Ren, 2013) In another strategy, the utilisation of charged metal particles, including Silver nanoparticles (AgNPs) is a targeted approach to disrupting electron transport chains, leading to reactive oxygen species (ROS) production and fatal damage caused to key structural components of the bacterial cell (Dakal et al., 2016) Bacteria have evolved strategies to overcome antimicrobials, therefore inhibitors of these compounds are important as alternative therapies or strategies which could complement antibiotics. β-lactamases are produced by pathogens and can cause hydrolysis of the key β-lactam ring structure common in many antibiotics rendering them non-functional. β-lactamase inhibitors, such as clavulanic acid, are now commonly combined with β-lactam antibiotics in a combinatory therapy (e.g. Amoxiclav), although β-lactamase inhibitors are only effective against a narrow subset of β-lactamases, limiting their effectiveness (Tooke et al., 2019) An alternative strategy, is to use efflux pump inhibitors to prevent efflux pump mediated removal of xenobiotics from target pathogen cells, increasing the susceptibility of these pathogens to other antimicrobials (Blanco et al., 2018).

Whilst each of these alternative approaches have both their advantages and disadvantages, this study will focus on the potential of predatory bacteria, in particular *Bdellovibrio spp*. as a future therapeutic approach.

1.2 BALOs and Predation

1.2.1 Predation in the bacterial kingdom

Predation is not a phenomenon limited to the animal kingdom, examples can be observed within the bacterial kingdom mediated through various key and distinct strategies: 'Wolf-pack', epibiotic, intraperiplasmic and cytoplasmic. In nutrient scarce environments, *Myxococcus spp.* exhibit a so-called 'wolf-pack' behaviour, where high cell densities attack prey cells and secrete extracellular enzymes to aid prey nutrient subversion in an epibiotic approach (Thiery and Kaimer, 2020). Epibiotic predation is a method adopted by *Micavibrio aeruginosavorus* and *Bdellovibrio exovorus* when attached to prey cells (Pérez et al., 2016). Within the order of Bdellovibrionales,

Bdellovibrio bacteriovorus and *Bacteriovorax spp.* achieve predation within the periplasm of the prey cell, secreting extracellular enzymes that result in the characteristic prey cell rounding and nutrient acquisition (Pasternak et al., 2013) Finally, *Daptobacter* is a bacterium that proliferates endobiotically, although in contrast to other *Bdellovibrio* and like organisms (BALOs), this predation is undertaken within the cytoplasm rather than the periplasm (Guerrero et al., 1986).

1.2.2 *Bdellovibrio* and like organisms (BALOs)

Bdellovibrio are vibrioid, highly motile (swimming at speeds of up to 160 $\mu m.s^{-1}$) and small (0.8-1.2µm length) members of the δ-proteobacteria class and represent a group of unique apex predators in the bacterial world (Lambert et al., 2006b; Sockett, 2009). Discovered in 1962 by Stolp and Petzold, this bacteria preys on a myriad of other Gram-negative bacteria (Stolp and Starr, 1963). *Bdellovibrio* are ubiquitous in a broad range of environments, from aquatic (fresh water and marine) to soil environments and the GI tract (Oyedara et al., 2016). The best studied strain is *Bdellovibrio bacteriovorus* type strain HD100, which was first isolated from soil. *Bdellovibrio spp.* are also members of a collective known as BALOs, which encompasses a polyphyletic group of predators within the distinct families of: *Bdellovibrionaceae, Bacteriovoracaceae, Pseudobacteriovoracaceae, Halobacteriovoraceae and Peredibacteraceae* (Figure 1.01) (Paix, Ezzedine and Jacquet, 2019).

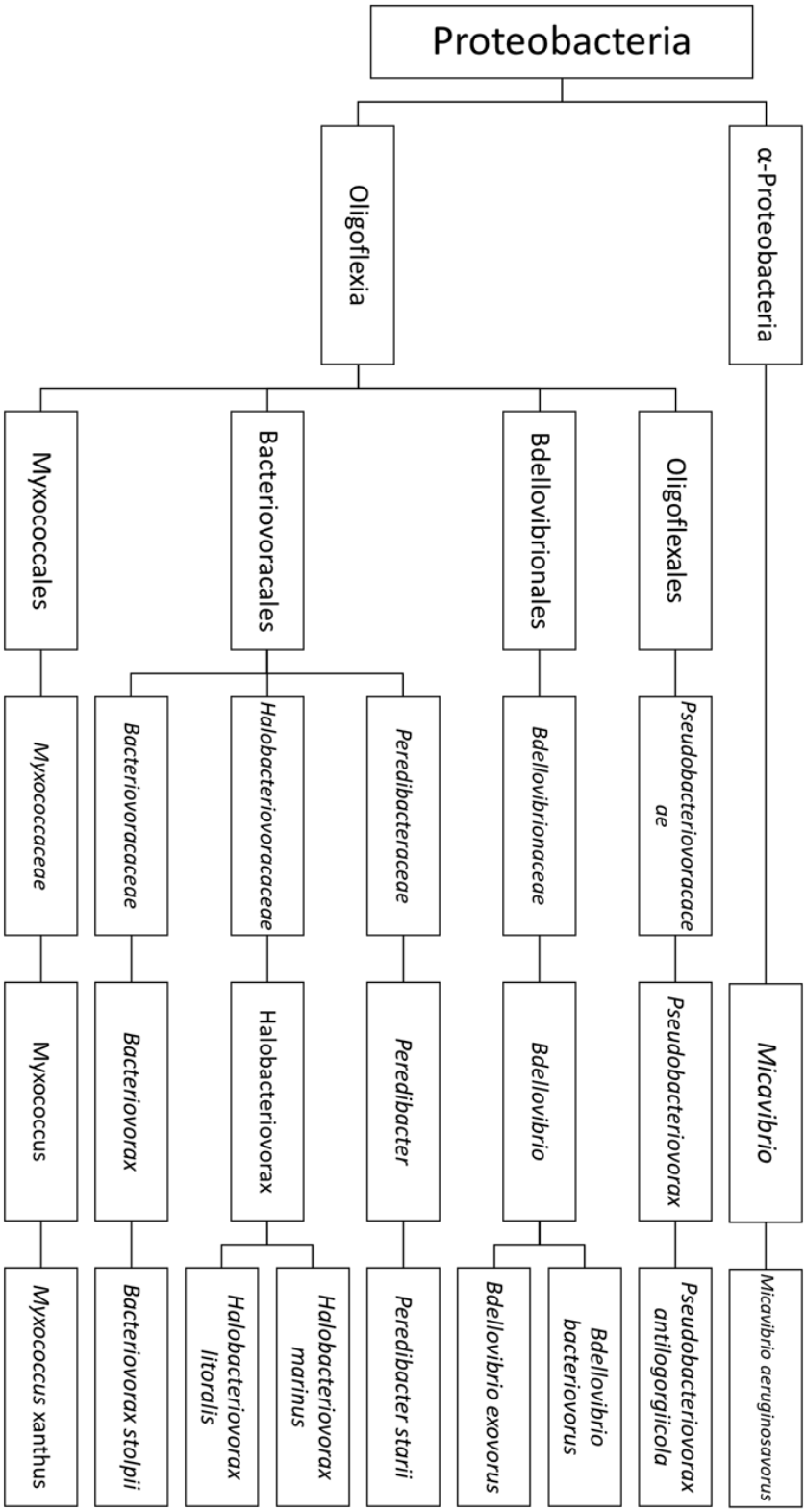


Figure 1.01: Updated phylogenetic tree of *Bdellovibrio* and like organisms (BALOs.

1.2.3 Predatory Life cycle of *Bdellovibrio bacteriovorus*

As described in figure 1.02; Non-replicating and free swimming or gliding attack phase (AP) *Bdellovibrio* cells start the predatory life cycle, where motility plays a role in both encountering and subsequently engaging with prey cells, although motility is not essential for initial entry, only for exit out of the bdelloplast (Hobley et al., 2012b; Negus et al., 2017). Additionally, gliding motility, although slower than free swimming, is also effective at penetrating biofilms of clinically important pathogens (Lambert et al., 2011a).

How *Bdellovibrio* precisely locate prey is still unknown as they do not have a quorum sensing system, but a complex chemotaxis system will allow *Bdellovibrio* to sense areas of higher prey cell density (Lambert, Smith and Sockett, 2003). Many MCPs/chemotaxis related products have been identified in *Bdellovibrio* and are thought to guide it into high densities of prey cells (~10^8 CFU/mL) and amino acids (Lambert, Smith and Sockett, 2003; Medina, Shanks and Kadouri, 2008). MCP-2 is one such chemotaxis mediator, where gene deletion mutants have confirmed its apparent role in directing the predators towards prey but indicated that it has no direct input on the predation process itself (Lambert, Smith and Sockett, 2003). Also important is the novel lipid A structure present in *Bdellovibrio*. The substitution of phosphate groups with α-D-mannopyranose residues in this structure results in the lack of negative charge seen in the *Bdellovibrio* cell envelope (Schwudke et al., 2003). This has been suggested to result in *Bdellovibrio* cells recognising each other as non-prey, while also allowing the predators to directly interact with a prey cell (Negus et al., 2017).

Once the prey cell has been encountered, *Bdellovibrio* use a type IV pilus structure on the opposite pole to the flagellum to enter the prey cell. Deletion mutants of pilus genes have shown that the pilus is essential for prey entry (Evans, Lambert and Sockett, 2007). Squeezed entry into the periplasm is mediated through pilus retraction and the creation of a pore in the prey outer membrane that is later re-sealed after entry (Evans, Lambert and Sockett, 2007; Chanyi and Koval, 2014). Simultaneously, the single sheathed flagella may be shed upon entry, although this event is not always guaranteed with the flagella being occasionally internalised within the prey (Lambert et al., 2006a).

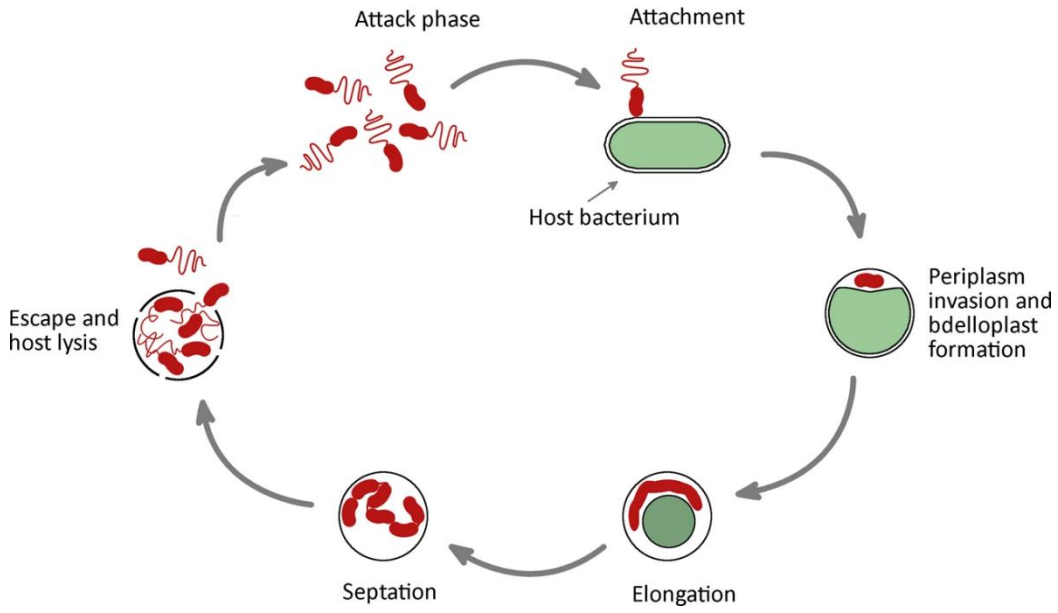


Figure 1.02: *Bdellovibrio* predatory life cycle: Attack phase (AP) and growth phase (GP). Reproduced from (Marine et al., 2020).

Upon entry to the prey cell several endopeptidases are released including Bd0816 and Bd3459 which begin the re-modelling of the host peptidoglycan to create a spherical shaped cell structure known as a 'bdelloplast'. Concurrently, these factors act as an 'occupancy signal', preventing the entry of more than one predator into single prey cell, thus maintaining the 1:1 predator to prey ratio (Lerner et al., 2012; Lambert et al., 2015).

In the 'growth phase' (GP) of the life cycle, *Bdellovibrio* secrete a cocktail of hydrolytic enzymes into the prey cell cytoplasm via several transport systems, these hydrolytic enzymes include proteases, DNAses and RNAses to degrade the prey cell cytoplasmic contents. The degraded cytoplasmic contents are then uptaken as nutrients (Rendulic et al., 2004). Prey cell death occurs quickly after *Bdellovibrio* entry, and the *Bdellovibrio* grows as a filament (Strauch, Schwudke and Linscheid, 2007). The filamentous cell continues to grow until the nutrients are exhausted, at which stage filament septation and bdelloplast lysis occurs, typically releasing 4-6 flagellate progeny (Sockett, 2009; Fenton et al., 2010).

1.2.4 Prey Range of *Bdellovibrio*

Bdellovibrio are known to prey on Gram-negative bacteria, including many clinically relevant pathogens, such as *Acinetobacter, Klebsiella, Escherichia coli, Salmonella enterica* and *Pseudomonas* (Dashiff and Kadouri, 2011). The predation efficiency between different strains of *Bdellovibrio* can also be variable; 109J has been previously shown to cause a greater reduction in *A. baumanii* and *P. aeruginosa* than HD100, which itself caused greater reductions in *P. putida* (Kadouri and Tran, 2013). Different strains of the same prey species can also affect predation efficiency for HD100 and 109J, as shown in *K. pneumoniae* and *E. coli* (Kadouri and Tran, 2013). Another *B. bacteriovorus* strain NC01, was shown to have a limited prey range compared to HD100 (Williams et al., 2019). *Bdellovibrio* do not directly prey upon Gram-positives such as *Staphylococcus aureus*, but have been tentatively shown to acquire nutrients from *S. aureus* indirectly through the secretion of serine proteases (Im, Dwidar and Mitchell, 2018).

Importantly in the context of AMR, *Bdellovibrio* will complete predation on a given prey regardless of the presence of antimicrobial resistance. Previous studies have proved this, where both HD100 and 109J maintained high predation efficiency on prey hosting MDR elements (Kadouri et al., 2013). Further work has shown *Bdellovibrio* to be capable of predation on biofilms of MDR prey, including those expressing resistance to last resort antibiotics such as colistin (Kadouri et al., 2013; Sun et al., 2017; Dharani et al., 2018).

An important aspect of *B. bacteriovorus* in the scope of AMR is its natural resistance to β-lactam antibiotics, thought to be mediated through efflux pumps (Sockett and Lambert, 2004). Although it is difficult to determine the minimum inhibitory concentration (MIC) of *B. bacteriovorus*, a novel liquid-culture based method has been developed which can achieve this (Marine et al., 2020). This is an important concept to consider during future therapeutic applications where a combinatory therapy of *Bdellovibrio* and antibiotics would likely be deployed, as inhibitory concentrations of antibiotics towards *Bdellovibrio* used in therapy would be detrimental for therapeutic success.

1.2.5 Resistance to *Bdellovibrio* predation

During *Bdellovibrio* predation, incomplete clearing and persistence of limited prey cells is observed as these cells display 'plastic resistance'. Importantly, plastic resistance is not genetically encoded and is instead thought to be a stress response to predation (Shemesh and Jurkevitch, 2003; Sockett and Lambert, 2004; Hobley et al., 2020). Interestingly, plastic resistant prey cells regain susceptibility to predation when proliferated and reintroduced to *Bdellovibrio* at a later stage (Shemesh and Jurkevitch, 2003). Prey are capable of resisting predation through the production of a Paracrystalline S layer, as seen in prey such as *Caulobacter crescentus* (Koval and Hynes, 1991; Fagan and Fairweather, 2014). Genetic resistance to *Bdellovibrio* predation is predicted to be infrequent, as there are no single receptor targets for prey binding such as those seen in bacteriophage (Hobley et al., 2020).

Some prey metabolites can protect prey from predation as seen in fluorescent *Pseudomonas* and *Chromobacterium* which produce hydrogen cyanide in normal metabolism. This confers resistance to predation by inducing a tumbling phenotype in otherwise free swimming motile *Bdellovibrio* cells, and causes prevention of *Bdellovibrio* exit from the bdelloplast (Mun et al., 2017). Also important when considering potential prey encountered in the GI tract is Indole. Produced by many bacteria which occupy the GI tract including *E. coli*, indole has inhibitory potency against *Bdellovibrio*, delaying the predation process at lower concentrations, while inhibiting prey cell lysis and reducing *Bdellovibrio* progeny numbers at higher concentrations (Mitchell et al., 2020).

1.2.6 Therapeutic studies using *Bdellovibrio*

Early studies showed the potential for *Bdellovibrio* to be used as a pathogen control agent, reducing numbers of *Shigella.* Studies have also showed the safety of *Bdellovibrio* to mammalian cells, highlighting a lack of predatory behaviour towards them (Westergaard and Kramer, 1977; Lenz and Hespell, 1978).

Beyond the success of using *Bdellovibrio* as a control agent in ocular infections, rodents stand out as a common model to assess the therapeutic potential of *Bdellovibrio in vivo* (Boileau, Clinkenbeard and Iandolo, 2011; Shanks et al., 2013). Mouse models

have demonstrated the ability of *B. bacteriovorus* HD100 and 109J to reduce the intracellular pathogen *Yersinia pestis* in the lungs, in addition to reducing persistence within the phagosome of phagocytes, where *Y. pestis* is localised during infection (Russo et al., 2018; Findlay et al., 2019). Similarly, *Bdellovibrio* was shown to successfully complete predation on *K. pneumoniae* in rat lungs, where both *Bdellovibrio* and *K. pneumoniae* became completely cleared from the lungs after 10 days; importantly, *Bdellovibrio* had no detrimental affect on lung health in this model (Shatzkes et al., 2016). The ability of *Bdellovibrio* to prey intravenously was also assessed using a rat model. However, inoculation of *B. bacteriovorus* and *K. pneumoniae* into rat tail veins did not yield a significant decrease in *K. pneumoniae* in the blood or the organs which it had spread to, this devalued the potential of therapeutic use of *B. bacteriovorus* in blood infections (Shatzkes et al., 2017a).

Zebra fish larvae are a unique *in vivo* model which have been exploited to assess the therapeutic potential of *Bdellovibrio*; *B. bacteriovorus* was shown to have no adverse effects on zebrafish and also caused a >4,000 fold decrease in *Shigella* (Willis et al., 2016). This model in zebrafish and other *in vivo* studies have highlighted the interactions of *Bdellovibrio* with the immune system, where it induces the inflammatory response and the production of inflammatory cytokines (Shanks et al., 2013; Shatzkes et al., 2015a; 2017b; Gupta et al., 2016; Willis et al., 2016) Other studies have demonstrated the ability of *B. bacteriovorus* to persist in phagocytes, where they can potentially predate on intracellular pathogens (Findlay et al., 2019; Raghunathan et al., 2019). *In vivo* study of *Bdellovibrio* has led to the hypothesis that *B. bacteriovorus* works synergistically with the immune system to aid in the clearance of pathogens (Shatzkes et al., 2016; Willis et al., 2016).

The GI tract is an important therapeutic target for *Bdellovibrio*. Assessment of *Bdellovibrio* predation in the GI tract is scarce but has been shown in chickens. In this *in vivo* model, inoculated *Salmonella* were reduced by *B. bacteriovorus* during a period of 3 days in the GI tract, this modest predation was complemented by an apparent reduction in cecal inflammation compared with control groups (Atterbury et al., 2011a). This study showed that despite their adversity to anaerobiosis, *Bdellovibrio* still persisted during

the 3 days and were able to complete predation, however this environment appears to remain a great challenge for long term survival of *Bdellovibrio* in therapeutics. Additionally, indirect effects of *Bdellovibrio* predation in the gut of these chickens lead to an increase in growth of Gram-positive bacteria (Atterbury et al., 2011a).

1.2.7 Challenges to consider when developing *Bdellovibrio* therapeutics

When administered as a therapeutic, *Bdellovibrio* face many challenges in environments that are distinct from the environments from which they were first isolated (Saxon et al., 2014). The gut is a key therapeutic battleground against gut dwelling pathogens (e.g. *Salmonella*). Although *Bdellovibrio* were able to complete a degree of predation in the gut of chickens, this was far from that seen in optimal laboratory conditions (Atterbury et al., 2011). Mg^{2+} and Ca^{2+} have been shown to be essential to *Bdellovibrio* growth beyond one predation cycle, with the lab-type strains requiring ~2mM $CaCl_2$ for predation (Seidler and Starr, 1969; Marbach and Shilo, 1978). In the human gut however, concentrations of 0.15mM and 0.6mM in the gastric fluid and intestinal tract respectively are seen, far lower than optimal, therefore supplements of Ca^{2+} would be required to maximise therapeutic output of *Bdellovibrio* in future *in vivo* studies (van der Zande et al., 2020).

Optimal temperature for *Bdellovibrio* is 29^{o}C and the organism requires an aerobic environment, neither of which are observed in the gut, with the environment exhibiting anaerobic and anoxic qualities as well as a higher ~37 ^{O}C temperature, both of which can be detrimental to *Bdellovibrio* growth (Jackson and Whiting, 1992; Heinken and Thiele, 2015; Shatzkes et al., 2016). *Bdellovibrio* also replicate in a strict environment of pH 7.6, below this predation diminishes; therefore the gut represents an extraordinary challenge for *Bdellovibrio* survival, where the pH ranges from the more acidic 1.5 – 2.0 in the stomach, to the 3.0 – 8.0 in the small intestine (Jackson and Whiting, 1992; Lambert et al., 2008; van der Zande et al., 2020). Despite the presence of a sheathed flagellum and unique LPS makeup, *Bdellovibrio* are still subject to immune recognition and clearance in these therapeutic environments (Koboziev, Karlsson and Grisham, 2010; Shatzkes et al., 2015a; Raghunathan et al., 2019; Chu, Liu and Hoover, 2020).

Although effective during *in vitro* studies, the type strains HD100 and 109J are not as well suited to the physiological challenges faced in the GI tract. It has been proposed however, that the isolation of novel *Bdellovibrio* strains from these challenging environments may exhibit a greater ability for persistence, immune avoidance, survival in wide range of pH and temperatures closer to environment within thehost organism and tolerating anoxic environments, such strains may also exhibit greater predation efficiency compared to current type strains and would therefore be more favourable as a therapeutic option (Atterbury et al., 2011a).

1.3 Molecular understanding of *Bdellovibrio* predation

1.3.1 Current understanding of predatory processes

Attack phase – Translocation to prey

A single polar sheathed flagellum is critical for prey collisions in liquid but not prey entry (Lambert et al., 2011a). Interestingly, the *B. bacteriovorus* HD100 genome contains six copies at different loci of the gene encoding FliC, the main structural component of the flagellum, as well as three copies of genes encoding the integral ATP-driven motor proteins MotA / MotB (Iida et al., 2009). This ensures the flagellum remains functional, should one gene be mutated and has been shown in *fliC5* knockouts where expression of other fliC genes increased to compensate (Iida et al., 2009). Additionally, in the event of a non-functional flagellar phenotype, exit from the bdelloplast can still be achieved by gliding motility (Iida et al., 2009; Lambert et al., 2011b).

Attachment, Entry and Growth

Attachment of *Bdellovibrio* to prey is one of the most integral steps in the predatory lifecycle and is facilitated by a type IV pilus (Evans, Lambert and Sockett, 2007; Chanyi and Koval, 2014). Type IV pili in *B. bacteriovorus* HD100 have been shown to consist of 8 proteins, with *bd1290* encoding the key structural PilA homologue (Prehna, Ramirez and Lovering, 2014). Furthermore, extension of the pilus and

retraction, thus allowing entry into the prey cell, is regulated by Bd0108 and Bd0109, with the former playing a role in the emergence of HI growth; mutations in the gene encoding Bd0108 within the *hit* locus have been shown to be a contributing factor for a shift in growth from host dependence (HD) to host independence (HI) in rich media (Capeness et al., 2013).

Lifestyle switches in bacteria such as those seen in the dimorphic *Bdellovibrio* (attack phase to growth phase) are commonly mediated by the regulatory molecule cyclic-di-GMP. Response to cyclic-di-GMP is achieved through the key protein domains GGDEF, EAL, HD-GYP and PilZ, with the type strain HD100 displaying various examples of enzymes containing these domains. PilZ domains display key cyclic-di-GMP binding motifs: RXXXR and [D/N]XSXXG (Galperin and Chou, 2020). Key events in the predatory lifecycle are mediated by cyclic-di-GMP. It is important to note that gene deletions of key proteins involved in cyclic-di-GMP signalling can substantially change the *Bdellovibrio* phenotype, such as a switch to axenic growth or aberrant motility (Hobley et al., 2012a).

Following attachment, invasion is characterised by the re-modelling of the prey cell peptidoglycan. Cross-link cleavage between D-amino acids is achieved through the peptidases Bd0816 and Bd3459 (Lerner et al., 2012). Present during prey entry, these peptidases also act as occupancy signals, preventing infection by more than one *Bdellovibrio* into the same prey cell (Lambert et al., 2010; Lerner et al., 2012). These proteins show sequence similarity to housekeeping and maintenance peptidases but exhibit specialised functions directed to predation, while their importance is highlighted by slower initial invasion in double gene deletions of both enzymes (Lerner et al., 2012). Importantly, *Bdellovibrio* also encode a protein with ankyrin repeats, Bd3460, which is thought to prevent prey PG remodelling enzymes from acting on self (preventing self-rounding, as seen in gene deletion mutants of *Bd3460*), both Bd0816 and Bd3459 display binding regions for Bd3460 (Lambert et al., 2015).

Chromosomal replication and cell division in *Bdellovibrio* differs from the typical binary fission seen in most Gram-negative bacteria. Cellular replication is instead characterised by the formation of a long singular filament to produce multiple progeny.

Interestingly, the origin of replication (*oriC*) of *Bdellovibrio* has been shown to be located at the invasive pole of the cell, unlike many other well characterised bacteria where this takes place in the middle of the cell; it has also been noted that as of yet, the molecular factors and regulators of replication initiation have yet to be identified (Makowski et al., 2019). Once inside the prey, *Bdellovibrio* use an arsenal of hydrolytic enzymes to digest the prey, allowing nutrient acquisition and subsequent prey cell death.

1.3.2 The hydrolytic enzyme arsenal of *Bdellovibrio*

In light of its predatory lifestyle, it is therefore not surprising that the *Bdellovibrio* genome encodes a high number of hydrolytic enzymes that are involved in the degradation of prey (Bratanis et al., 2020). The work of Rendulic and colleagues showed that the *Bdellovibrio* genome encodes 293 hydrolytic enzymes: 150 proteases, 20 DNases, 9 RNases, 10 glycanases, 15 lipases and 89 unassigned. These enzymes are produced in response to several events in the predatory lifecycle: prey cell entry, degradation of prey components and final egress from the bdelloplast (Rendulic et al., 2004). During initial invasion only 11 out of the 150 proteases are upregulated, with these being thought to be involved in initial penetration of prey membranes (Lambert et al., 2010). 74 of the proteases were expressed during growth within the bdelloplast, some of which will be involved in the degradation of the prey cell contents. These were typically of serine-protease types, with 11 predicted to be extracellular. The remaining types are metal- dependent as well as cysteine and aspartic types (Karunker et al., 2013). Protease production is key for *Bdellovibrio* to scavenge amino acids for in-house protein synthesis, as *Bdellovibrio* are only capable of synthesising 11 amino acids (Rendulic et al., 2004; Barabote et al., 2007).

An example of a serine protease with previously unknown classification is Bd1962, later identified and annotated as BspK, *Bdellovibrio* serine-protease K (Bratanis et al., 2017). This protein was found to have a trypsin-like peptidase domain and to be capable of targeting lysine residues of proteins (Bratanis et al., 2017) Bd1962 was also shown to display high levels of conservation between HD100, Tiberius, and 109J strains,

highlighting its importance within the predatory process. Another protease identified by the same research group in a later study was BspE (Bd2692), *Bdellovibrio* elastase-like protease. The authors commented on the role of BspW role in prey invasion processes and commented on its broad host range, opening the possibility that BspE may regulate other *Bdellovibrio* proteases (Bratanis and Lood, 2019).

Despite *Bdellovibrio* possessing the appropriate biochemical synthetic *de novo* pathways for the production of nucleoside monophosphates, *Bdellovibrio* acquire them primarily through host genome degradation via nucleases to ensure the 4-6 progeny receive a full genomic complement (Fenton et al., 2010; Herencias, Prieto and Nogales, 2020). From the 20 nuclease encoding genes identified in *B. bacteriovorus* HD100, several have been identified through homology as housekeeping genes, e.g. *bd1431*. Comparatively, *bd1244* and *bd1934* were shown to be upregulated and peak in expression levels during the first 30-45 mins of exposure to prey cells (Lambert and Sockett, 2013). Other nucleases (*bd3507, bd0934*) were also implicated in the mid stages of intracellular predatory growth. Intriguingly however, mutants of these nucleases have been shown to have little effect on the predation efficiency of *Bdellovibrio*, this highlights the redundancy exhibited by many *Bdellovibrio* products, whilst also displaying the ability of other nucleases to 'pick up the slack' by increasing expression levels to compensate (Lambert and Sockett, 2013; Bukowska-Faniband, Andersson and Lood, 2020).

Further understanding of the predatory processes in *Bdellovibrio* by identifying high conservation levels of hydrolytic enzymes across multiple *Bdellovibrio* strains, could be key in elucidating the prime and integral players in the predation process, as well as placing them within the predation cycle based on their function. Further work on grouping hydrolytic enzymes by domain (both catalytic and signal domains) and function may clarify this further, both allowing for better understanding of the predation process, but also to enable us to tap into the great reservoir of antimicrobial based therapies that could be developed from the *Bdellovibrio* hydrolytic enzyme arsenal (Rendulic et al., 2004).

1.3.3 Transport Systems in Bdellovibrio

The arsenal of hydrolytic enzymes produced by *Bdellovibrio* must ultimately be secreted for them to act on their targets. In Gram-negative bacteria there are 2 transport systems (figure 1.03) implicated in initial transport of proteins across the inner membrane (IM) into the periplasm: the Sec pathway which transports unfolded proteins possessing a specific hydrophobic sec signal sequence or an SRP protein bound to the secreted protein through the SecYEG channel, and the Tat pathway, which transports folded proteins across the IM, these proteins also contain a specific Tat signal sequence (Green and Mecsas, 2016).

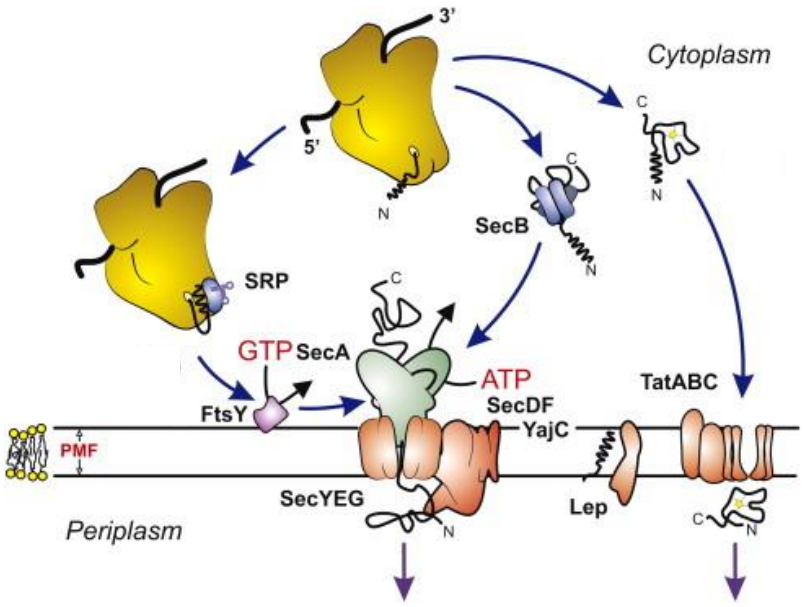


Figure 1.03 – Schematic of Sec and Tat transport in Gram-negative bacteria, showing components of the sec pathway SecB, SecDF and the SecYEG channel, FtsY and SRP protein transported directed to SecYEG and tat channel made up of TatABC. Reproduced from (Natale, Brüser and Driessen, 2008).

Proteins are further transported across the outer membrane with additional secretion systems, in Gram-negative bacteria are there are 6 types: Type 2 secretion systems (T2SS) and type 5 secretion systems (T5SS) both complete the movement of sec or tat transported proteins from the periplasm across the OM (Green and Mecsas, 2016). T3SS are typically secretors of effectors, many of which have roles in virulence, like type 1 secretion systems (T1SS), type 4 secretion systems (T4SS) and type 6

secretion systems (T6SS), proteins translocated by these transporters are done so in a Sec-independent manner, where they possess structures which span both IM and OM for secretion outside of the cell, but also to insert them into target mammalian or bacteria cells (Green and Mecsas, 2016).

Previous studies have sought to review the transport systems in *Bdellovibrio*. In one study, the *Bdellovibrio* genome was analysed and compared with the transport classification database (TCDB) for hits of recognisable and previously characterised transport systems, resulting in the findings of 172 transport systems within *B. bacteriovorus* HD100 (Barabote et al., 2007). *B. bacteriovorus* has been found to encode the key components of both the Sec (SecYEG, SecA, SecDF, FtsY) and Tat (TatA, TatB, TatC) transport systems (Barabote et al., 2007; Tajabadi et al., 2018). The Sec transport system is essential in bacteria, whereas the Tat transport system is dispensible in many bacteria. However, in *Bdellovibrio* it has been shown that the Tat transport system is essential for *Bdellovibrio* survival, in both predatory and host-independent lifecycles (Chang et al., 2011).

1.4 Hypotheses and Main Aims of the Project

Bdellovibrio have been shown to successfully complete predation within biologically relevant environments such as the GI tract of rodents and chickens, although at much lower degrees of predation efficiency compared with laboratory conditions due to the typically anaerobic/microaerophilic, warmer and variable pH environment (Atterbury et al., 2011a; Shatzkes et al., 2015b). *In vivo s*tudies in chickens suggested future research should be focused on the isolation and characterisation of novel *Bdellovibrio* strains from these environments for future use in therapeutics (Atterbury et al., 2011b). The ubiquitous nature of *Bdellovibrio* in a range of known environments creates great optimism for the success of isolating these novel strains from the GI tracts of animals, where if shown to be persistent in the organism from which they originate, would be better adapted to face the non-optimal conditions than the type strains HD100 and 109J.

The main aim of this study:

To isolate novel *Bdellovibrio* spp. from the intestines of farm animals, for future use as antimicrobial therapeutics in animals

Therefore, this project focussed on isolating, purifying and characterising novel *Bdellovibrio* strains from 3 locations within the GI tract of pigs: Stomach acid, Small intestine and Large intestine.

Isolates were also attempted to be purified from faecal samples of pigs, reindeer and sheep. The primary focus on pigs was chosen due to their close similarities with the humans GI tract, in terms of biological relevance (similar pH, temperature at ~37^{o}C, transit times and atmosphere). The other animals were chosen for their agricultural significance (sheep) and as an example of a ruminant (reindeer).

From March 2020 onwards due to the COVID-19 pandemic, the laboratory was closed and all experimental work ceased. As a result, the aim was shifted to investigate *Bdellovibrio* hydrolytic enzymes using a systematic bioinformatics approach. The main aim of this section of the project was:

To further categorise *Bdellovibrio bacteriovorus* HD100 hydrolytic enzymes by predicted domains, transcriptomics and the degree of conservation between *Bdellovibrio* strains.

With the aim being to produce a collated resource of the current data, to use in identifying proteases potentially involved in the predation process both for molecular studies of their role(s) in predation and also for investigations into the potential uses of some of these enzymes as future antimicrobial agents.

2.0 Materials and Methods

2.1 Laboratory methods

2.1.1 Bacterial strains and culturing methods

2.1.1.1 Bacterial strains

The *Bdellovibrio* and prey strains used in this study are listed in Table 2.1.

Table 2.01: Bacterial strains used in this study

Bacterial strains	Genotype/Phenotype	Source/Reference
B. bacteriovorus HD100	Wild-type	(Stolp and Starr, 1963)
B. bacteriovorus 'Rudolphii'	Wild-type	This study
E. coli S17-1	*thi*, *pro*, *hsdR*$^-$. *hsdM*$^+$, *recA*; integrated plasmid RP4-Tc::Mu-Kn::Tn7	(Simon, Priefer and Pühler, 1983)
S. Typhimurium 4/74	Wild-type	
P. putida	Wild-type lab strain	Sockett Lab, University of Nottingham
E. coli XL-1-Blue pGEM-T	*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac*; contained in pGEM-T	

2.1.1.2 – Media used in this study

Table 2.2 below details the media and buffers that were used during this study. All were made with deionised water and autoclaved before use.

For the agar prepared in this study, in addition to YPSC constituents, 10 g/L of agar select per 100 mL of YPSC was used for bottom agar and 6 g/L YPSC for top agar. $CaCl_2$ was added to both YPSC bottom and top agar before pouring (to a final concentration of 0.25 g/L). 10 g/L of agar was also added to YT broth to make YT agar. The pH of all medias and buffers was adjusted using 2M NaOH.

Table 2.02 Growth media used for *Bdellovibrio* and prey

Media and Buffers	Constituents	pH
YPSC Broth	1 g/L BD Bacto Yeast Extract 1 g/L Sigma Aldrich Broadbean Peptone 0.5 g/L Fisher Scientific $C_2H_3NaO_2$ 0.25 g/L Fisher Scientific $MgSO_4 \cdot 8H_2O$	7.6
YT Broth	5 g/L Fisher Scientific NaCl 5 g/L BD Bacto Yeast Extract 8 g/L BD Bacto Tryptone	7.5
Ca-HEPES buffer	5.94 g/L Sigma HEPES 0.284 g/L Fisher Scientific $CaCl_2 \cdot 2H_2O$	7.6
$CaCl_2$ stock solution	25 g/L Fisher Scientific $CaCl_2 \cdot 2H_2O$	

2.1.1.3 Growth of Prey

Escherichia coli S17-1 and *Salmonella* Typhimurium 4/74 were streaked onto YT agar from -80 °C frozen stocks and incubated overnight for 16 hrs at 37 °C. Plates were stored at 4 °C. Overnight liquid cultures were setup by inoculating a single colony into 10 mL of YT broth in a 30 mL universal and incubated at 37 °C with shaking at 200 rpm for 16 hrs.

2.1.1.4 Growth of *B. Bacteriovorus* from frozen stocks

Bdellovibrio were revived from frozen stocks by inoculating 100 µL onto a double-layer agar YPSC plate, where the top layer consisted of 150 µL of the required prey and 5 mL of YPSC top agar Double layer agar plates were incubated at 29 °C. After a zone of clearing was observed in the prey lawn, an agar plug was taken from the cleared zone and added to 2 mL of Ca-HEPES and 150 µL of prey (in a 7 mL bijou tube) creating a predatory liquid culture, which was incubated at 29 °C with shaking at 200 rpm 24 hrs or until prey lysis was observed by microscopy.

2.1.1.5 General maintenance of *B. bacteriovorus* liquid predatory cultures

2 mL predatory cultures were checked for prey cell lysis after 24 hrs of incubation by microscopy using a Nikon 100x A/1.25 lens on a Nikon eclipse E200 microscope, the observation of small vibrioid and highly motile cells was confirmation of the presence of *Bdellovibrio* cells, additionally the lack of prey cells or bdelloplasts (rounded prey cells) was confirmation that the

culture had fully lysed. These cleared cultures were then used to inoculate a subsequent *B. bacterivorous* predatory culture in either 2 mL, 10 mL or 50 mL of Ca-HEPEs with the columns given in Table 2.3 below.

Table 2.03: Volumes of *B. bacteriovorus* and prey added to each size of predatory culture.

2 mL culture (in a 7mL bijou)	**10 mL culture** (in a 30mL universal)	**50 mL culture** (in a 250mL conical flask)
2 mL Ca-HEPES 150 µL Prey 50 µL *B. bacteriovorus*	10 mL Ca-HEPES 600 µL Prey 200 µL *B. bacteriovorus*	50 mL Ca-HEPES 3 mL Prey 1 mL *B. bacteriovorus*

2.1.2 Novel Predator isolation, growth and characterisation

2.1.2.1 Isolation and purification of novel predators from farm animal GI tract and faeces

Initially, pig guts received from an abattoir (R.B Elliot and Son LTD Farm shop, Chesterfield) and faecal samples from sheep, reindeer and pigs (Manor Farm Park, East Leake) were processed by Dakshayeeni Sivasankaran, a previous undergraduate summer student. The work of this student laid the groundwork for this project. . They collected 1 g – 2 g of intestinal material from various locations within the GI tract of the pigs as described in table 2.04.

Table 2.04: Source animals for isolates of novel *Bdellovibrio* and the locations within the GI tract where the sampling took place.

Farm Animal	Location of sampling
Pigs (x6) (Abattoir samples)	Stomach Acid, Small Intestine (2 random locations), Large intestine
Pigs (x2) (Manor Farm Park)	Faeces
Sheep (Manor Farm Park)	Faeces
Reindeer (Manor Farm Park)	Faeces
Goat (Manor Farm Park)	Faeces

The gut material was suspended in either MRD or Ca-HEPES. Resuspended samples were plated using double layer agar plates with either *E. coli* S17-1 or *S.* Typhimurium 4/74 prey and incubated at 29 ^{o}C for 5-7 days. Areas of clearing on overlay plates were picked into 2 mL predatory cultures containing prey which had been washed to remove the YT broth by centrifugation at 13,000 g for 2 mins, removal of the supernatant (to limit nutrient availability to background flora) and the pellet resuspended in Ca-HEPES. After clearing of the predatory cultures was observed (between 2 and 5 days after inoculation) along with the presence of small *Bdellovibrio*-like cells, the predatory cultures were plated on double layer agar plates and incubated at 29 ^{o}C for 5-7 days. Any plaques that formed on these plates were picked into another 2 mL predatory culture. Before plating the subsequent cultures, they were diluted 1 in 5 and filtered through a 0.45 μL filter to remove background flora in these highly mixed cultures (this was an effective method at reducing this background flora, but after several days of growth on YPSC plates, would return to prefiltering levels). This process was repeated in a series of plaque purification rounds until the end point where only pure plaques were seen on a double-layer agar plate, with no other bacterial isolates (except the added prey) growing on the agar. Other methods attempted during the purification process included growing predatory cultures with Carbenicillin and the appropriate antibiotic resistant prey (*E. coli* XL-1-Blue pGEM-T), due to prior knowledge of the penicillin-resistant nature of the *B. bacteriovorus* type-strain HD100. This did not result in a significant difference compared to purification without the antibiotic, possibly due to large numbers of the accompanying "contaminating" bacteria also being penicillin resistant. Growing predatory cultures in YPSC broth rather than Ca-HEPES was also unsuccessful.

2.1.2.2 Confirmation of bacterial vs bacteriophage novel isolates

1 mL of an overnight predatory culture containing a novel predator strain was centrifuged at 13,000 g for 8 mins, with the supernatant being plated on double-layer YPSC plates. The pellet was resuspended in 1 mL Ca-HEPES and plated.

2.1.2.3 Growth of novel predator 'Rudolphii' Isolates

The novel predatory isolate was found to grow preferentially in a microaerophilic environment. Frozen stocks of Rudolphii were spotted onto a double layer agar plate with *E. coli* S17-1 as prey and incubated at 29 ^{o}C for up to 5 days as for other *B. bacteriovorus* strains. To transfer the predatory cells into a liquid culture, the area of clearing was cut out with a scalpel and added to 20 mL of Ca-HEPES, then broken up (without introducing air bubbles) to resuspend

the predatory cells into the Ca-HEPES. 1 or 2 mL of the resuspension was added to 150 µL of prey in a 7 mL bijou tube, these tubes were then incubated statically at either 29 °C or 37 °C. After 3-5 days the cultures were checked for prey lysis via microscopy and subsequently subcultured into a 10 mL predatory culture and again incubated at either 29 °C or 37 °C statically.

2.1.2.4 Quantification of predation by novel strain 'Rudolphii' at differing temperatures

Several 1 mL and 2 mL lysates were combined and filtered through a 0.45 µm filter (Sartorius) before use to remove any remaining prey cells and combined into a 10 mL culture. Cultures were incubated at 20 °C, 29 °C and 37 °C with *E. coli* S17-1 prey, again these cultures were incubated statically. To enumerate both prey and predator, dilutions of each culture were plated onto YT to determine prey numbers and YPSC to determine predator numbers.

2.2 Bioinformatics

2.2.1 Analysis of domain composition

The initial list of hydrolytic enzymes was taken from the study by Rendulic et al., and the protein domains reanalysed using SMART (Anon., 2021h) to determine domains, whilst COG grouping analysed using HHpred (Anon., 2021c). The hydrolytic enzymes were then grouped together based on their primary catalytic domain, which had been identified in SMART. Verification of the presence of signal peptides (both Sec and Tat) was done using SignalP (Anon., 2021g), and transmembrane domains using TMHMM(Anon., 2021j). Genomic localisation was visualised using the genome browser in xbase (Anon., 2021l).

2.2.2 Collation of transcriptional, proteomics and experimental protein localisation data from published sources

Transcriptional data from previously published studies (Lambert et al., 2010; Karunker et al., 2013) was collated from the provided supplemental data from each study; this allowed for comparison of the gene expression profiles of the hydrolytic enzymes under focus during this study. Proteomics data from the publicly available thesis of Dr Rene Becker (Avidan et al., 2017) was also searched for information relating to the proteins of interest . Experimental data which described genes implicated in different stages during the *Bdellovibrio* lifecycle were obtained from the study of Duncan and colleagues which used Tn-FACseq screening.

2.2.3 Conservation of genes between *B. bacteriovorus* HD100, Tiberius and *B. exovorus* JSS

Lists of non-conserved genes between *B. bacteriovorus* HD100 and *B. bacteriovorus* Tiberius were available from the supplemental data from (Hobley et al., 2012b) Each of the genes found in Tiberius and not in HD100 were analysed using SMART, to determine those containing domains found in hydrolytic enzymes. SMART was then used to find proteins in HD100 with the same combination of protein domains as found in these unique Tiberius genes. Enzymes in *B. exovorus* containing each of the enzymatic domains found in HD100 proteins were also identified using SMART.

2.2.4 Alignments of protein sequences

Alignments of protein sequences were produced using Clustal Omega (Anon., 2021b) with the default settings.

3.0 Results

3.1 Isolation and preliminary characterisation of a novel microaerophilic predatory bacterium

3.1.1 Optimisation of isolation techniques for predatory bacteria from faecal and GI-tract samples

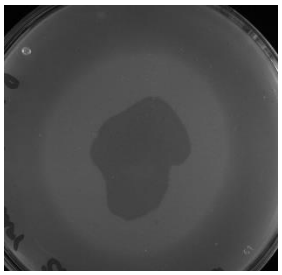
From 24 selected samples, only one predatory bacterial isolate from reindeer faeces was successfully purified following eight successive rounds of plaque purification with the filtering and washed prey techniques. Purity was determined after two rounds of plating on double-layer agar plates with plaques forming on *E. coli* S17-1 as prey, with no observable contaminating colonies within the *E. coli* lawn. A notable finding at this stage was the inability for this isolate to clear prey in liquid culture, and that this isolate did not efficiently clear the *E. coli* prey population in the strict aerobic environment of a shaking incubator. However, when this isolate was grown in liquid cultures statically to create a pseudo-microaerophilic environment mirroring that of the reindeer GI tract from which it was isolated from, increased prey clearing was observed in addition to an increased number predators as determined by plaque formation on serial-dilution plates, suggesting a greater survival rate of the predator.

3.1.2 Characterisation of *Bdellovibrio spp.* Rudolphii: Growth and predation

Following confirmation of bacterial rather than bacteriophage make-up, this isolate from reindeer, which we putatively named *Bdellovibrio spp.* Rudolphii, was observed to be a small rod-shaped bacterium under phase-contrast microscopy. The isolate also displayed very uncoordinated and dysfunctional motility; very few cells were observed to exhibit swimming motility and those that were swimming were observed to swim much slower than the *B. bacteriovorus* type strain HD100. The plaque forming and therefore predatory ability of this isolate was initially confirmed through spot tests on overlays containing *E. coli* (Figure 3.01a), *S.* Typhimurium and *Pseudomonas putida*. As well as displaying plaque forming ability, the plaques produced by *Bdellovibrio spp.* Rudolphii were formed within 24 hrs, much faster than the *B. bacteriovorus* HD100 type strain, which typically requires 3-5 days. *Bdellovibrio spp.* Rudolphii plaques also presented as much smaller than HD100, appearing as only 2-5 mm across. Unlike phage,

where the plaques also appear within 24 hrs but do not then typically grow in size, *Bdellovibrio spp.* Rudolphii plaques continued to grow over time (Figure 3.01b) over a period of 7 days when grown on *E. coli* S17-1 on YPSC agar, the plaques also began to turn cloudy between 2-7 days, suggestive of re-growth of the *E. coli* prey.

a)



b)

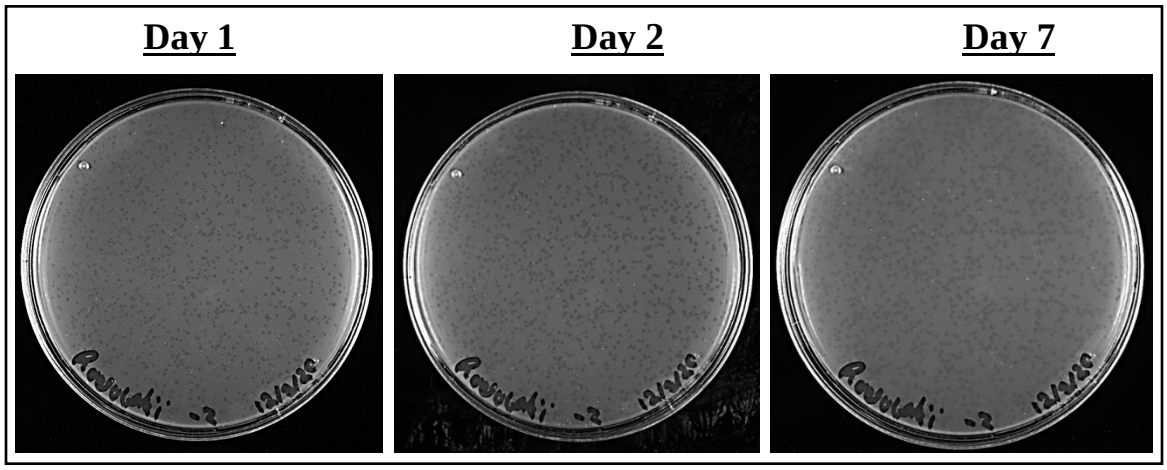


Figure 3.01: Predation of *Bdellovibrio spp*. Rudolphii on lawns of *E. coli* S17-1. a) *Bdellovibrio spp.* Rudolphii spotted onto the top of a double-layer agar plate containing a lawn of *E. coli* S17-1,image taken after 24 hrs b) Plaques of *Bdellovibrio spp.* Rudolphii become visible after 1 day of incubation at 29 °C, continue to grow over the subsequent 6 days, whilst slowly becoming cloudy by day 7 of incubation.

3.1.3 Characterisation of *Bdellovibrio spp.* Rudolphii: Temperature

After the enhanced predation efficiency under microaerophilic conditions by *Bdellovibrio spp.* Rudolphii was identified, it was further hypothesised that this isolate may also display other adaptations for growth optimised in the GI tract environment of the organism from which it was isolated. Growth temperature was therefore investigated, with the inclusion of the following temperatures: 20 ^{o}C, 29 ^{o}C (the preferred growth temperature of *B. bacteriovorus* type strain HD100), and 37 ^{o}C which is closer to the reindeer core body temperature of 38 ^{o}C. Growth at these temperatures was quantified in 10 mL liquid cultures containing *E. coli* S17-1 as prey. The experimental data set included two repeats, a final repeat was not possible due to the COVID-19 outbreak.

The *E. coli* viable counts and numbers of predators was measured every 2-3 days from the start of the experiment for a total of 18 days, by which stage the lysates were observed by microscopy to be fully cleared of prey. Figure 3.02 shows the graph of *E. coli* viable counts, data points for 20 ^{o}C and 29 ^{o}C began at day 7 due to days 2 and 4 being too many to count on the plates as the dilutions of prey used did not provide colonies within the countable range due to a slower reduction in prey than expected compared to laboratory *B. bacteriovorus* strains. Despite this, there is a trend of 2× lower viable *E. coli* cells at 37^{o}C compared to the other two temperatures, with the reduction in viable prey beginning 3 days earlier at 37 ^{o}C compared to the other two temperatures.

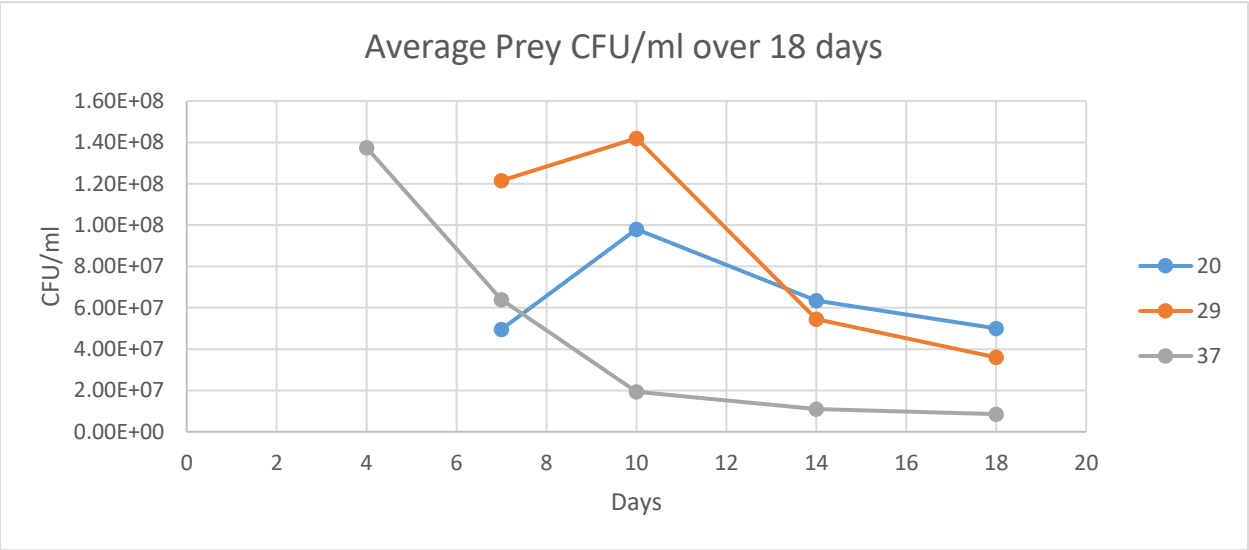


Figure 3.02 – Reduction in *E. coli* prey numbers during predation by *Bdellovibrio spp*. Rudolphii at different temperatures. The graph shows the average *E. coli* prey numbers over a period of 18 days at the three temperatures 20 °C (blue curve), 29 °C (orange curve) and 37 °C (grey curve). Results are from two replicate, day 2 and day 4 *E. coli* counts were not countable on the plated dilutions and are therefore absent from the dataset.

Figure 3.03 shows the *Bdellovibrio spp.* Rudolphii numbers during the same 18 day experiments. Figure 3.03 shows that at 20 °C, the predator numbers slowly declined over 14 days, with a more pronounced decline during the first 10 days, but a slight increase in numbers between days 14 and 18. At this temperature, the predator number remained below 2.00×10^3 pfu/mL. Similarly, at 29 °C the predator number did not significantly exceed that seen at 20 °C but did show an initial increase between days 4 and 10, before the numbers of predators oscillated between increasing and decreasing for the remainder of the 18 days. Conversely, the predator numbers at 37 °C increased more significantly in the first 10 days to 1.20×10^4 viable cells per mL. by a one order of magnitude increase compared to when incubated at 29 °C. However, the predator numbers began to decrease between days 10 and 18 but remained at a higher level than when incubated at the lower temperatures. This decrease of the predator numbers between days 10 and 18 as seen across all growth temperatures is concurrent with the slowing of prey death in the same time period as seen in figure 3.02.

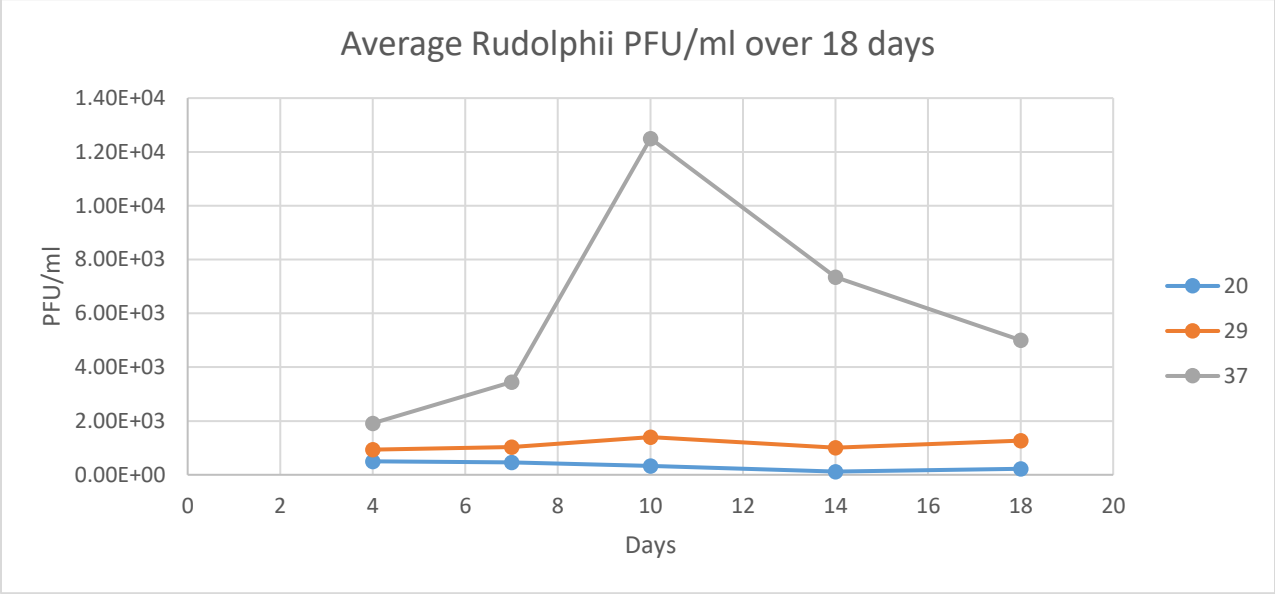


Figure 3.03 – Increase in *Bdellovibrio spp*. Rudolphii population size occurs at higher temperatures. Graph showing the *Bdellovibrio spp.* Rudolphii average viable counts over a period of 18 days during incubation with *E. coli* prey at 20 °C (blue curve), 29 °C (orange curve) and 37°C (grey curve). Due to experimental limitations the data begins at day 4 after the start of incubation.

3.1.4 Summary of experimental findings regarding *Bdellovibrio spp*. Rudolphii.

This study has shown that the novel predatory bacterial isolate, which we have putatively named *Bdellovibrio spp.* Rudolphii, is a predator of the Gram-negative bacterial species *E. coli*, and has shown that *Bdellovibrio spp*. Rudolphii exhibits a potential preference for both a microaerophilic environment and a 37 °C growth temperature, a notable difference to the previously characterised *Bdellovibrio bacteriovorus* strains which thrive in oxygen rich environments and at a lower temperature optimum of 29 °C. At this stage of the study, further experiments to continue the characterisation of *Bdellovibrio spp.* Rudolphii were planned (discussed in Chapter 4) but the COVID-19 pandemic meant that the university and laboratories shut for more than 6 months, beyond the end of the initial MRes timeframe, as a result no further results concerning *Bdellovibrio spp.* Rudolphii were able to be collected, leaving this study with limited experimental data for this isolate.

3.2 Bioinformatics of Hydrolytic enzyme arsenal

3.2.1 Classification into Proteases, Nucleases, Glycanases & Lipases, and Other hydrolytic enzymes

Analysis conducted at the time of the original sequencing of the *Bdellovibrio bacteriovorus* HD100 genome identified 293 hydrolytic enzymes in the *Bdellovibrio* protein arsenal, this original analysis grouped these enzymes into 6 distinct categories: Proteases, DNases, RNases, Glycanases, Lipases and other hydrolases (Rendulic et al., 2004). This study also sub-grouped these enzymes by catalytic type. However, since this study was published in 2004, conserved domain databases such as SMART (Anon., 2021h) have been updated such that some of the categorisation of these proteins performed in this study is no longer up to date, plus greater information about each can be obtained (for example the metal-dependent proteases can be further classified using the system developed as part of the MEROPS database (Anon., 2021d). Transcriptomic studies comparing gene expression at different points within the predatory cycle have been published (Lambert et al., 2010; Karunker et al., 2013) and the publicly available data from these studies can be used to determine the transcriptional profile of genes of interest in the *Bdellovibrio bacteriovorus* HD100 genome.

This study into the hydrolytic enzymes of *B. bacteriovorus* HD100 has allowed prediction of the potential functions of these proteins through the identification of conserved domains, and also to predict the roles of these enzymes within the lifestyle of *Bdellovibrio*; where we can hypothesise as to which proteins are simply housekeeping (ie involved in general growth of *Bdellovibrio*, likely expressed both during predatory growth and Host-Independent growth) and which are likely to have integral roles in the attachment, prey cell entry and intracellular growth stages of predation.

In this study, the domains of all the hydrolytic enzymes were obtained using SMART (Anon., 2021h), this identified catalytic domains and potential signal peptides, in addition to a COG group placing. SMART was also used to determine the conservation of enzymes containing the same domain combinations in *B. exovorus* JSS compared to *B. bacteriovorus* HD100. Conservation of genes between *B. bacteriovorus* Tiberius and HD100 was achieved by searching the list of unique genes in each genome previously

given in (Hobley et al., 2012b) to identify genes encoding potential hydrolytic enzymes, where again SMART was used to compare the domain combinations with their potential HD100 counterparts. Transcriptomic data for each gene of interest was collated from two previously published studies (Lambert et al., 2010; Karunker et al., 2013). Lambert and co-workers (Lambert et al., 2010) used microarray analysis of *B. bacteriovorus* RNA for both predatory *Bdellovibrio* during free-swimming attack-phase and 30 minutes into the predatory cycle and Host-Independently growing *B. bacteriovorus*, comparing each stage to attack-phase (AP) levels to determine up or downregulation of expression. These data were grouped into 6 categories: genes upregulated in both HI and after 30 mins, upregulated at 30 mins and not HI, downregulated in HI and not at 30 mins, upregulated in HI and not at 30mins, down in both HI and at 30 minutes and genes which were downregulated at 30 mins during the transition out of AP. The study by Karunker and co-workers (2013) analysed the expression of each gene using an RNA-seq approach in two conditions, AP and GP, where AP cells were attack-phase (pre-infection) free-swimming *B. bacteriovorus* in the absence of prey, and GP samples were collected 3 hours after *E. coli* infection with *B. bacteriovorus*. Together these two studies give an insight into the gene expression at two stages of predation, 30 minutes representing the establishment of the bdelloplast structure and 3 hours representing a late-stage of predation during which the prey cell is being degraded and the subsequent products this degradation being up taken by the *Bdellovibrio* and used in cell elongation and replication. Combined together the analysis described in this thesis has sought to group these enzymes further by catalytic domain in sub-groups, which exist within the original 6 groupings of proteases, DNases & RNases, Lipases & Glycanases and other hydrolases, investigate the conservation of the genes across the *Bdellovibrio* genus, and identify those proteins likely to be involved in intra-periplasmic predation.

3.2.1.1 Hydrolytic arsenal: Proteases

Proteases are enzymes which show degradative activity against proteins, where they can break the peptide bond found between adjacent amino acids in a polypeptide chain (Razzaq et al., 2019). Following the analysis in this study, the total number of identified proteases in *B. bacteriovorus* HD100 now stands at 164. The full list of proteases and their classifications are shown in Appendix A, table A1 and summary in figure 3.04 below:

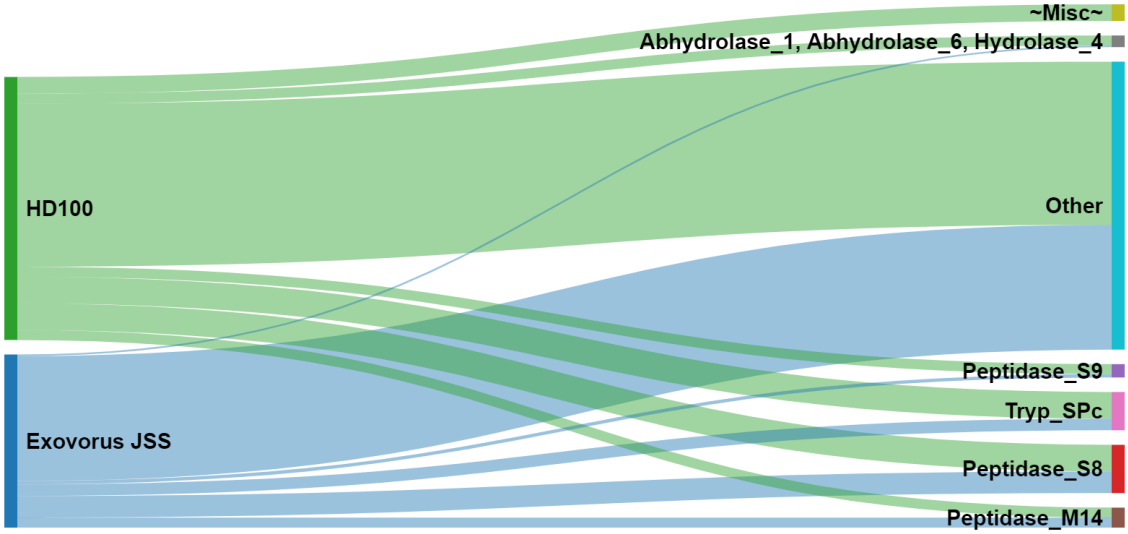


Figure 3.04: Summary diagram showing the most abundant categories found within the proteases of *B. bacteriovorus* HD100 and comparisons with near relative *B. exovorus* JSS.

This study found five enzymes which were not previously identified in the original genome analysis (Rendulic et al., 2004) by cross-referencing the functional domain with the HD100 genome in SMART (Anon., 2021h), analysis of the functional domains of these enzymes placed them within the proteases grouping. Nine enzymes were re-categorised into the protease grouping from the other grouping categories (as they had been previously categorised in the original HD100 genome analysis paper (Rendulic et al., 2004) based on the protein domain analysis using the SMART tool. The 164 proteases were further grouped by their functional domain into 57 sub-groupings, where 30 of these belong to individual peptidase families which have serine (S), metal

dependent (M), cysteine (C), aspartic acid (A) or unclassified catalytic types (U) based on the MEROPS classification system (Anon., 2021d). Trypsins with the domain Tryp_SPc and the peptidase S8 family of enzymes were the most abundantly found domains in the protease grouping, being found in 17 and 16 proteins respectively. Due to the high number of enzymes within the proteases grouping and limited constraints for time in this project, only a few of the domain types were analysed in depth, this included the abundant S8 peptidase type, M14 peptidase and S13 peptidase types, covering peptidases with 3 different catalytic types.

3.2.1.2 Hydrolytic arsenal: Nucleases

DNases and RNases are known collectively as nucleases and have phosphodiesterase activity, cleaving the bond between the phosphate group and organic base in a DNA or RNA polymer (Yang, 2011). This study found the nucleases grouping to contain 32 enzymes in *B. bacteriovorus* HD100 (Appendix A, table A2 and summarised in figure 3.05), this includes the addition of 3 enzymes which were not included in the original genome study (Rendulic et al., 2004) and were placed into the nucleases grouping based on functional domains.

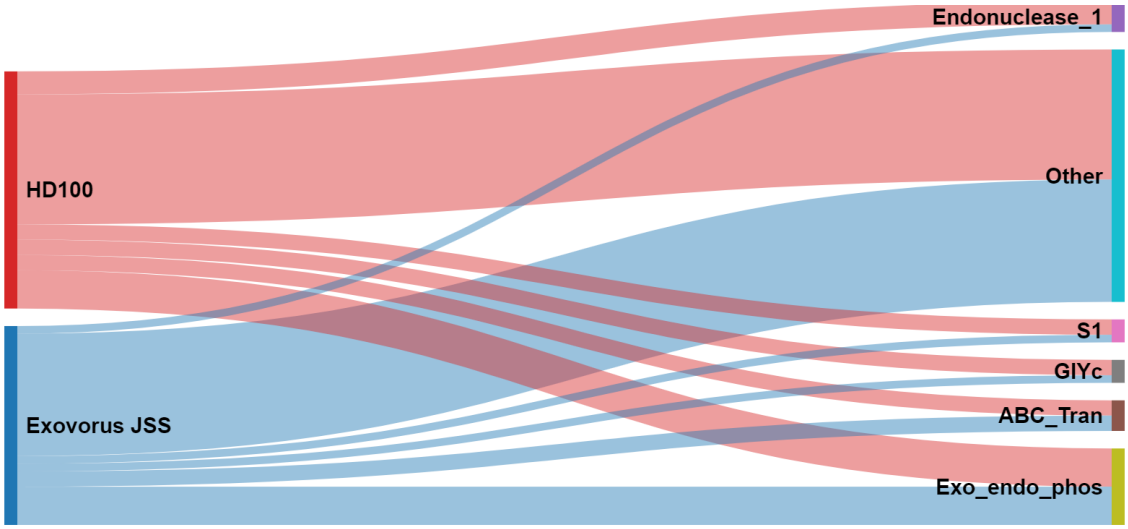


Figure 3.05: Summary diagram showing the most abundant categories found within the nucleases of *B. bacteriovorus* HD100 and comparisons with near relative *B. exovorus* JSS.

The nuclease grouping has been further classified into 19 groups based on the functional domains of the enzymes. Endo_exo_phos is the most abundantly found domain type of the sub-grouping, being found in 5 enzymes. Endonuclease_1 is the second most abundant, with 3 enzymes. The remaining sub-groupings had domains which were only found in one or two enzymes. Three sub-groupings within the nucleases grouping were subjected to further analysis, Exo_endo_phos, endonuclease_1 and SNases. The remaining nucleases are summarised in table 3.14.

3.2.1.3 Hydrolytic arsenal: Glycanases and Lipases

Lipases are enzymes capable of hydrolysing the carboxyl ester bonds present in triacylglycerols and phospholipids, releasing the organic acids associated with them (Jaeger et al., 1994; Borrelli and Trono, 2015). Glycanases are enzymes responsible for the degradation of glycoproteins, examples of glycoproteins in bacteria include those found in the S-layer of surface-layer producing bacteria (Upreti et al., 2003). This study has identified 25 enzymes which belong in the collective grouping of glycanases and lipases (Appendix A, table A3 and summarised in figure 3.06).

Figure 3.06: Summary diagram showing the most abundant categories found within the glycanases and lipases of *B. bacteriovorus* HD100 and comparisons with near relative *B. exovorus* JSS.

Five of these enzymes were not previously identified (Rendulic et al., 2004) and belonged to this grouping based on the presence of functional domains. Sub-grouping based on functional domains revealed 11 sub-categories, with the SLT domain being the most abundantly found in 11 enzymes, SLT enzymes are transglycosylases capable of cleaving the β-1,4-glycosidic bond between NAG and NAM units in peptidoglycan (Thunnissen et al., 1994). Some of these SLT enzymes are likely needed to modify the peptidoglycan of the *Bdellovibrio* cell during growth, 5 of the genes encoding proteins with SLT domains are highly expressed 3 hours into the predatory cycle (Appendix B, table B4, (Karunker et al., 2013) suggesting that this may be their function in the *Bdellovibrio* lifecycle. Whereas only one, Bd3575, of the genes encoding an SLT-domain containing protein was upregulated 30 minutes after the onset of predation (Appendix B, Table B4, (Lambert et al., 2010), whilst *Bdellovibrio* is establishing the bdelloplast structure, which may indicate that this particular SLT may be required in the modification of the prey peptidoglycan. Further molecular studies would allow for the testing of this hypothesis.

3.2.1.4 Hydrolytic arsenal: Other hydrolytic enzymes

This grouping does not have an overarching domain or enzymatic function, but instead contains a very diverse group of 89 identified enzymes from this study that do not fall into one of the other three groupings (Appendix A, table A4 and summarised in figure 3.07). One of these enzymes was re-categorised into other hydrolases from the nucleases grouping from the initial genome analysis (Rendulic et al., 2004). A further 5 enzymes were found to belong to this grouping. Sub-grouping based on functional domains revealed 40 sub-categories, with HDc, metallophos and lactamase_B being the most abundantly found domains in the new sub groupings. 10 genes encoding proteins with HDc domains were identified, HDc domains have phosphodiesterase activity and potential roles in signal transduction. The HD-GYP phosphodiesterases have an HDc domain and are involved in secondary messenger cyclic-di-GMP signal transduction. Cyclic-di-GMP has been shown to regulate both predation and host-independent growth in *Bdellovibrio* (Hobley et al., 2012a). The crystal structure of one of the HD-GYP

proteins, Bd1817, of *Bdellovibrio* has been solved (Lovering et al., 2011) however the precise function of the HD-GYP proteins has not yet been reported.

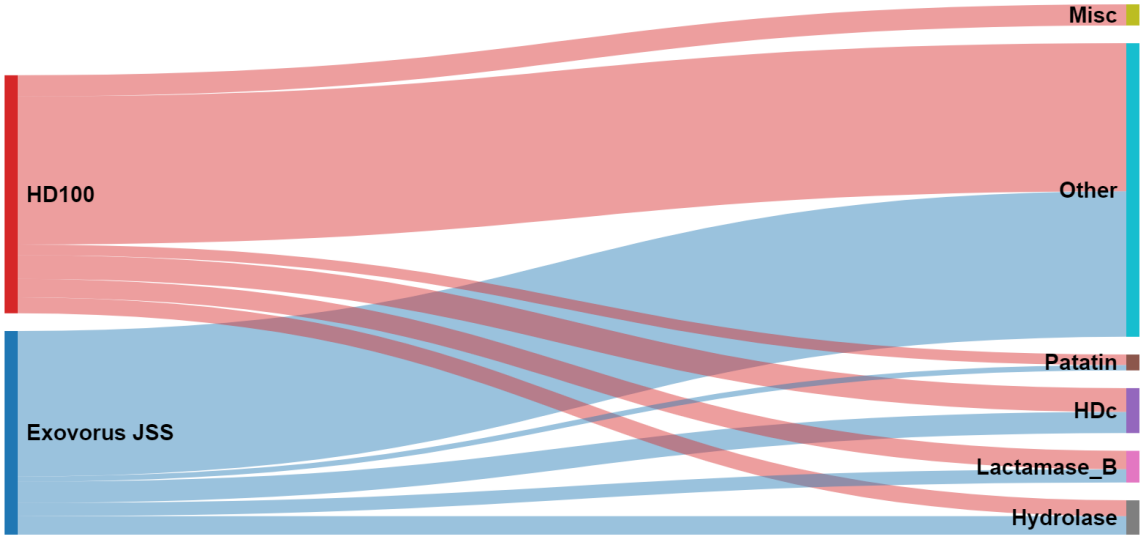


Figure 3.07: Summary diagram showing the most abundant categories found within the other hydrolytic enzymes of *B. bacteriovorus* HD100 and comparisons with near relative *B. exovorus* JSS.

3.2.2 Conservation of hydrolytic enzymes in other *Bdellovibrio* strains and species

The potential role(s) within the predatory lifecycle and the predatory approach these enzymes are involved in, might be further elucidated by comparing different strains and even species of *Bdellovibrio* to the type strain HD100, which exhibits a periplasmic-growing predatory lifecycle. Other species such as *B. exovorus* (in particular the type strain JSS) which is an epibiotic predator and other strains of periplasmically-growing *B. bacteriovorus* such as the strain Tiberius can provide insights into what has been conserved between these predators and what differs between them, catering to their different lifestyles.

3.2.2.1 Genes found in *Bdellovibrio bacteriovorus* HD100 but not Tiberius

Data used to identify genes which are found in the HD100 genome, but not in Tiberius, were acquired from (Hobley et al., 2012b), this original study used manual BLAST and Reciprocal Smallest Distance analysis (RSD) to determine if the genes were unique or not unique to HD100 compared with Tiberius. In this study, 13 proteins containing hydrolytic enzyme domains were identified to be unique in HD100, with no homologue found in Tiberius (Table 3.02).

Table 3.02 – Enzymes which were found in this study to be unique in *B bacteriovorus* HD100 compared to *B. bacteriovorus* Tiberius, with their general grouping, subgrouping and functional domain and the function.

Bd number	Grouping	Functional domain for sub-grouping	Function
Bd0922	Proteases	Trypsin	Diverse function, acts as a trypsin-serine like protease (Patel, 2017)
Bd1898	Proteases	Tryp_SPc	Diverse function, acts as a trypsin-serine like protease (Patel, 2017)
Bd2667	Proteases	Peptidase_M41	Metalloprotease that degrades N or C termini of proteins and is annotated as FtsH (Anon., 2021d)
Bd1084	Proteases	Peptidase_M4	Endopeptidase activity (Anon., 2021d)
Bd1171	Proteases	Misc / Unknown	
Bd2674	Proteases	Misc / Unknown	
Bd2328	Proteases	Peptidase_U32	Collagenase activity (Anon., 2021d)
Bd2675	Proteases	PHB	Function unknown
Bd3695	DNases & RNases	Methylase_S	Endonuclease activity
Bd0664	Glycanases & Lipases	Coesterase	Acts upon carboxylic esters
Bd2297	Other hydrolases	Hydrolase	Alpha/beta hydrolase with diverse function
Bd3503	Other hydrolases	Metallophos	Phosphodiesterase activity (Anon., 2021e)
Bd2755	Other hydrolases	NUDIX	Hydrolyses nucleotide diphosphates linked to some other moiety

	(Bessman, Frick and O'Handley, 1996)

Interestingly, 8 of the 13 unique HD100 genes were found in the protease grouping, these had diverse domain makeup and 3 of these were different classes of peptidases. The presence of these genes which are unique to HD100 compared with Tiberius may be explained by the difference in lifestyles between the two strains: HD100 is purely host dependent and so rely on the acquistion of macromolecules from prey to feed the growing *Bdellovibrio* filament, whereas Tiberius is capable of exhibiting a HI lifestyle (without the acquisition of genetic mutations). HD100 may have a critical use for these enzymes for intraperiplasmic growth, whereas Tiberius may have lost these during evolution as they are potentially not critical for HI lifestyles. The transcriptional data in table 3.03 further hints to a redundant role for 5 of these enzymes in HI growth, as they were shown to be downregulated in HI grown HD100 (Lambert et al., 2010).

Table 3.03 – Transcriptional data for enzymes unique to HD100 (Lambert et al., 2010; Karunker et al., 2013).

Enzymes	Upregulated During Attack Phase (AP), Growth Phase (GP) or Silent?	Expression change in *B. bacteriovorus* Enzymes at 30 mins and in Host independent (HI) vs Attack Phase (AP)
Bd0922	Silent	Down in HI
Bd0664	AP	Down in HI
Bd1084	GP	No Change
Bd1171	Silent	Up in HI
Bd1898	Silent	Up at 30
Bd2297	Silent	Down in HI

Bd2328	Silent	Down in HI
Bd2667	Silent	Down in HI
Bd2674	AP	Up in HI
Bd2675	AP	No Change
Bd2755	Silent	No Change
Bd3503	Silent	No Change
Bd3695	Silent	No Change

3.2.2.2 Genes found in *Bdellovibrio bacteriovorus* Tiberius and not in HD100

A full list of the genes which were unique to Tiberius (and not found in HD100) was obtained from supplementary data that accompanied the original Tiberius genome paper (Hobley et al., 2012b), each gene was then analysed with SMART to determine those with any domains found in hydrolytic enzymes. The same combination of protein domains was cross-referenced with HD100 using SMART to identify any conserved proteins. 43 hydrolytic enzymes were identified from this data in Tiberius (Appendix C), 2 of which contained similar domain structures with equivalent HD100 enzymes: Bdt0871, Bdt1340. Bdt0871 possesses a signal peptide sequence and DJ-1_Pfpl domains, this domain family have peptidase activity, this combination of domains was also found in Bd1521 and Bd3678 of HD100, alignments of these 3 proteins can be found in figure 3.08.

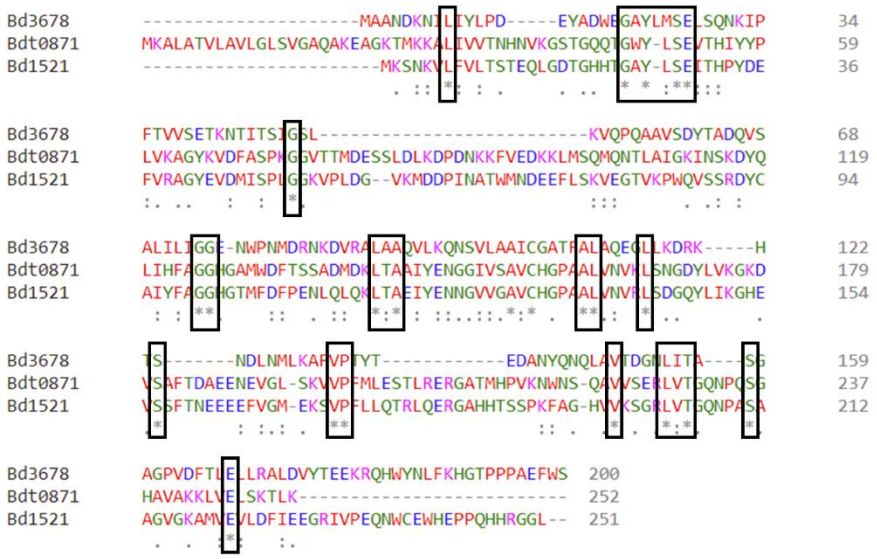


Figure 3.08 – Multiple sequence alignment of HD100 enzymes Bd3678 and Bd1521 with Tiberius enzyme Bdt0871, black boxes indicate regions of high conservation.

These enzymes all show 21 completely conserved amino acid residues, there is clear high similarity and conservation between Bd1521 and Bdt0871 level conservation between two of the enzymes but not all three. The conserved region GXYXSE is a highly conserved motif among all three enzymes and could be an important motif for function, possibly serving as an active site pocket in these enzymes.

Another Tiberius enzymes: Bdt1340 was shown to both contain both an S8 peptidase and PA domain, this domain combination is also present in Bd2269, Bd3238 and Bd1444 in HD100. Figure 3.09 is an alignment of all 4 enzymes. The 3 active site motifs for peptidase S8A as described in 3.2.3.2, are present in all 4 enzymes, Asp-Thr/Ser-Gly, His-Gly-Thr-His and the full motif for Gly-Thr-Ser-Met-Ala-Xaa-Pro.

Figure 3.09 – Multiple sequence aligment of the S8 peptidase domain from the HD100 enzymes Bd1444, Bd2269 and Bd3238 and the Tiberius enzyme Bdt1340, black boxes indicate conserved S8A peptidase active site motifs.

3.2.2.3 Comparisons of the hydrolytic enzymes in *Bdellovibrio exovorus* JSS with those found in *B. bacteriovorus* HD100

Once the HD100 enzymes had be categorised into the new sub-groupings by functional domains, SMART was used to identify enzymes in B. exovorus JSS matching the domains and combinations seen in the HD100 subgroupings. Although many subgroupings had B. exovorus equivalent enzymes, there were 65 HD100 enzymes which did not have any corresponding enzyme in B. exovorus. 36 of the enzyme subgroupings missing in B. exovorus can be found in the protease grouping, where B. exovorus does not have enzymes with peptidase types M15, M13, M48, M16, U32 and S11 (Table 3.04).

Table 3.04 – HD100 Peptidase containing enzymes from the proteases grouping missing from *B. exovorus*, including sub-grouping, function and transcriptional data (Lambert et al., 2010; Karunker et al., 2013).

Bd number	Functional domain for sub-grouping	Function	Upregulated During Attack Phase (AP), Growth Phase (GP) or Silent?	Expression change in *B. bacteriovorus* Enzymes at 30 mins and in Host independent (HI) vs Attack Phase (AP)

Bd0923	Peptidase U32	Collagenases	Silent	Up at 30 and down in HI
Bd1077	Peptidase M16	Diverse function	AP	Up in HI
Bd1078	Peptidase M16	Diverse function	Silent	Up in HI
Bd1287	Peptidase M48	Endopeptidase activity	AP and GP	Up in HI
Bd1552	Peptidase M16	Diverse function	GP	Up in HI
Bd2044	Peptidase S11	DD-carboxypeptidase activity	Silent	Down in HI
Bd2068	Peptidase M48	Endopeptidase activity	GP	Up in HI
Bd2328	Peptidase U32	Collagenases	Silent	Down in HI
Bd2654	Peptidase M15	DD-Carboxypeptidase activity	GP	Down in HI
Bd2798	Peptidase M48	Endopeptidase activity	GP	No Change
Bd3547	Peptidase M13	Endopeptidase activity	Silent	Up in HI
Bd3869	Peptidase M16	Diverse function	Silent	Up in HI

As these enzymes are not found in *B. exovorus,* it can be hypothesised that they may have potential function(s) within the predatory lifecycle attributed to intraperiplasmic growth. Two of these HD100 enzymes (Bd1077 and Bd1287) are highly expressed during AP (Karunker et al., 2013), and due to their endopeptidase activity, it can be hypothesised that they could be involved in breaking down the prey cell peptidoglycan crosslinking during prey cell entry. Bd1287 however does not possess a signal peptide for Sec or Tat-transport, meaning this may be localised to the cytoplasm of the *Bdellovibrio* cell as this enzyme is also highly expressed during GP and may play a role in

the biogenesis of the *Bdellovibrio* cell wall. Alternatively, a transport system which spans the entire cell envelope may deliver this enzyme to the prey. A further 5 of these enzymes are upregulated during GP, either at 3 hrs or at 30 mins in the early stages of predatory growth. It is possible that these enzymes are required for the growth of a *Bdellovibrio* filament as seen in *B. bacteriovorus* during growth in the bdelloplast, and are not required for the binary fission model of division and growth seen in *B. exovorus*.

Within the nucleases, glycanases and other hydrolases groupings there were also missing *B. exovorus* enzymes for the equivalent HD100 enzymes, including SNases and LrgAB, analysis of these enzymes can be found in sections 3.2.4.3 and 3.2.3.1.2 respectively.

3.2.3 Proteases: S13, S8 and M14 Peptidases

3.2.3.1 Peptidase S13

Serine-type proteases have been found to have exo- and endopeptidase activity in both eukaryotes and bacteria where they target cell wall components of peptidoglycan. The peptidoglycan polymer is made up of repeating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) monomers linked by 1,4-glycosidic bonds (Vollmer, Blanot and de Pedro, 2008). NAG-NAM monomers are cross linked with other chains via a tetrapeptide sequence and short peptide bridge: L-Ala-D-Glu-Mesdiaminopimelic acid-D-Ala-D-Ala, with the final D-Ala molecule being lost during crosslinking to form the tetrapeptide (Figure 3.10).

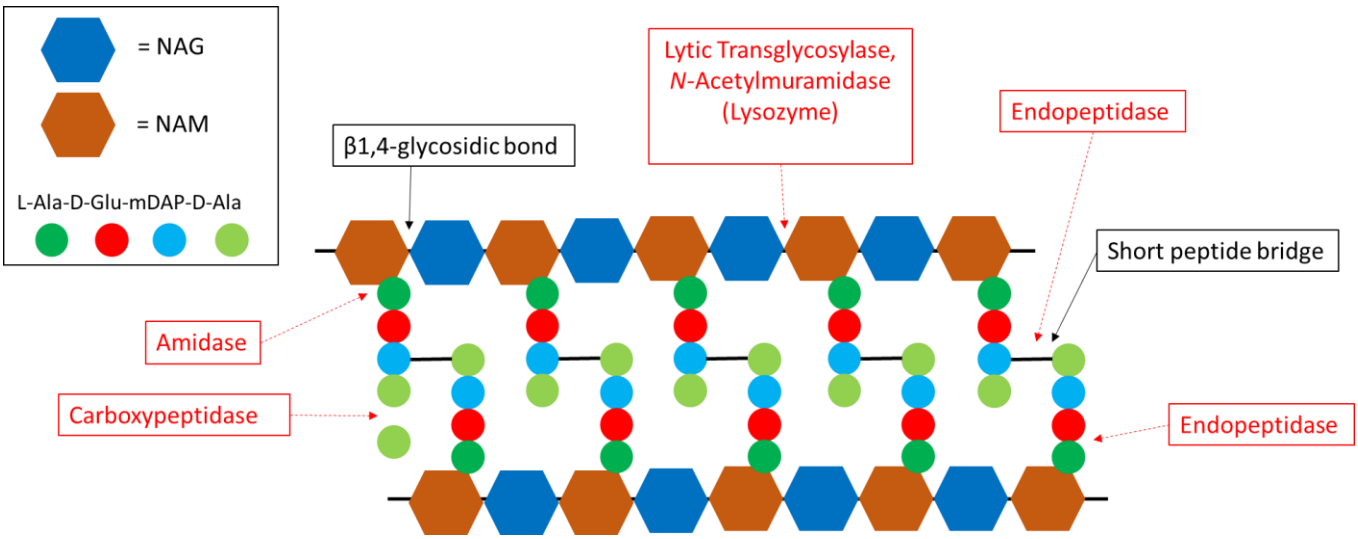


Figure 3.10: Structure of peptidoglycan, with *N*-acetylglucosamine and *N*-acetylmuramic acid repeating units joined by β1,4-glycosidic bonds and the tetrapeptide crosslinks between the glycan strands, lytic enzyme targets are also highlighted.

Penicillin binding proteins (PBPs) catalyse the reactions of NAG-NAM polymerization and transpeptidation events which create the cross-linking. These PBPs can be categorised into 3 classes: A, B and C. Class A PBPs are responsible for transpeptidase and transglycosylase activities, while class B are responsible for transpeptidation (Sauvage et al., 2008). The so called 'low weight' Class C PBPs are however incapable of carrying out transpeptidase duties. They are strict DD-carboxypeptidases capable of cleaving the second D-Ala residue from the initial pentapeptide to produce the final tetrapeptide seen in mature peptidoglycan (Sauvage et al., 2008). Peptidases of the type S13 fall under the class C classification of PBPs. S13 peptidases share the known active site motif: Ala-Ser-Xaa-Xaa-Lys-Xbb, where a serine and lysine dyad form the basis for catalysis to occur via nucleophilic attack (Sauvage et al., 2008, Anon., 2021d).

3.2.3.1.1 Peptidase_S13 domain containing proteins in *B. bacteriovorus* HD100

The genome of *Bdellovibrio bacteriovorus* HD100 encodes 3 enzymes containing S13 type peptidases domains: Bd0816, Bd3244 and Bd3459, the domain structures of

each can be seen in figure 3.11, Bd3244 contains only one copy of the domain while Bd0816 and Bd3459 (not shown on diagram due to overlap / mistake by SMART) contain two S13 domains.

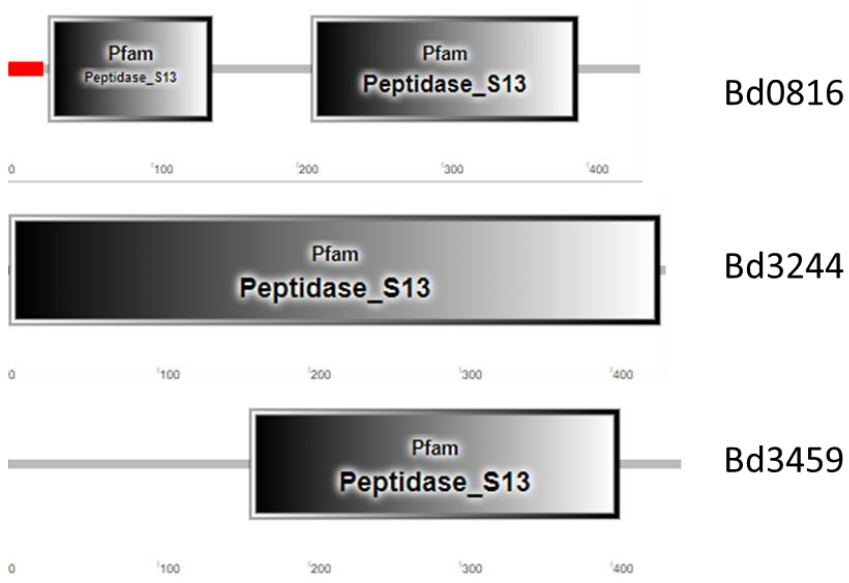


Figure 3.11: Domain structures for Peptidase S13 domain-containing enzymes from *B. bacteriovorus* HD100 determined using SMART.

Through analysis in SMART (Anon., 2021h) only Bd0816 had a predicted signal peptide domain, upon further investigation with SignalP-5.0 (Anon., 2021g) both Bd0816 and Bd3459 were shown to possess signal peptides directed to the Sec-transport system, whilst Bd3244 was predicted to have no signal peptide by both SMART and SignalP-5.0.

As part of their published study on the functions of these proteins, Lerner and colleagues (, 2012) produced a sequence alignment of the 3 *Bdellovibrio bacteriovorus* S13 peptidases and the *E. coli* housekeeping PBP DacB, a shortened version showing the active site of the protein is reproduced below in figure 3.12, notable is the presence of the Lysine/Serine dyad motif in all three of the *Bdellovibrio* S13 peptidases.

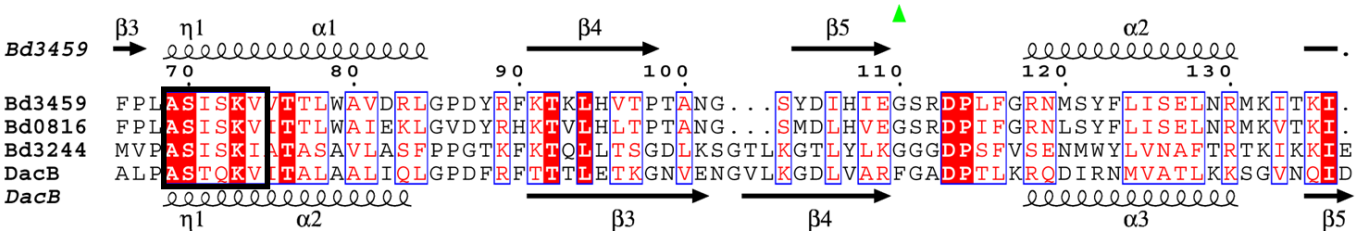


Figure 3.12: Multiple alignment of part of the Bd3459, Bd0816, Bd3244 and *E. coli* DacB enzymes including the active site residues taken from (Lerner et al., 2012), the serine/lysine dyad active site residues are highlighted in the black box, other conserved motifs are also highlighted in blue.

Collated transcriptional analysis from the studies by Lambert and co-workers , 2010) and Karunker and colleagues , 2013) shown in table 3.05 shows that Bd3244 is neither upregulated in either attack phase (AP) and 3 hours into predatory growth (GP) or at 30 mins of predation or in host-independent (HI) growth, therefore eluding to a possible function as an enzyme required for normal growth and peptidoglycan maintenance of *Bdellovibrio*. This was further evidenced in the experimental study by Lerner and co-workers (Lerner et al., 2012), who showed the constitutive expression of this protein throughout the predatory cycle suggesting its role as a 'housekeeping protein'. Conversely both Bd0816 and Bd3459 are upregulated at 30 mins (table 3.05), which suggested a role in initial invasion and formation of the bdelloplast. Lerner and co-workers (Lerner et al., 2012) also showed this, with RT-PCR revealing upregulation of both genes during the first 15-30 mins post exposure to prey. They also found roles for both proteins in prey cell rounding prior to entry by *Bdellovibrio* cells and prevention of the entry of more than one *Bdellovibrio* cell into the prey.

Table 3.05: Transcriptomic data of Bd0816, Bd3244 and Bd3459 collated from studies by Karunker and colleagues (Karunker et al., 2013) and Lambert and co-workers (Lambert et al., 2010), showing that both Bd0816 and Bd3459 are both increased in expression 30 minutes after the onset of predation.

Enzymes	Upregulated During Attack Phase (AP), Growth Phase (GP) or Silent?	Expression change in B. bacteriovorus Enzymes at 30 mins and in Host independent (HI) vs Attack Phase (AP)
Bd0816	Silent	Up at 30
Bd3244	Silent	No Change
Bd3459	Silent	Up at 30

3.2.3.1.2 Insights gained from the genomic location of the S13 peptidase genes

The genomic co-localisation of genes can sometimes be indicative of common functions and/or pathways, whilst co-transcription of genes is often used to ensure that genes with related functions are both expressed at the same time and at similar levels within the cell. In this section a 20 kb region around each of the three genes encoding the S13 peptidases was analysed, looking for other hydrolytic enzymes, and/or transporter systems that might be involved in either the export of the enzymes, or import of the products of their enzymatic activity, as well as any other genes that may be involved in similar processes.

Bd3244

Within the *Bdellovibrio* genome, *bd3244* is located next to *bd3243* which corresponds to Bd3243, as seen in figure 3.13, *Bd3244* and *bd3243* are both transcribed in the same direction suggesting possible co-transcription, however experimental evidence is needed to confirm this hypothesis.

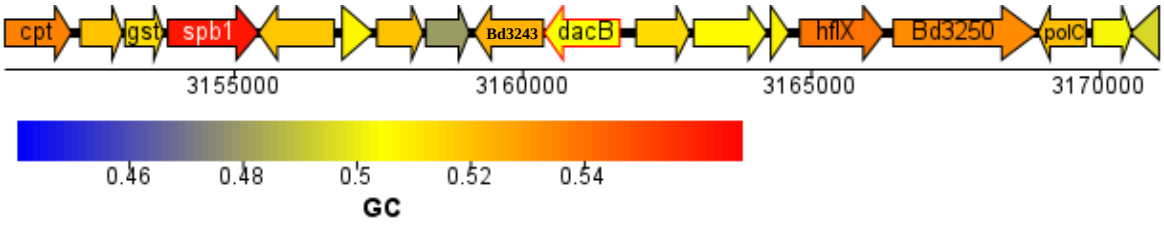


Figure 3.13: Genomic region around Bd3244 (*dacB*). A 20 kb region around Bd3244 (*dacB*) is shown, the diagram is reproduced from xbase (Anon., 2021l).

Interestingly, Bd3243 is also predicted to interact with peptidoglycan, due to the presence of an SLT domain in its structure, and has been annotated as a putative membrane bound murein transglycolase. SLT domains represent a group of enzymes which act as transglycosylases, with a biological role of cleaving the β-1,4-glycosidic bond which exists between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan, while also contributing to the formation of a 1,6-anhydro bond in these structures (Anon., 2021k). The domain structure of Bd3243 is shown in figure 3.14a, where a signal peptide domain was predicted by signalP and SMART, upon further investigation with SignalP-5.0 this was confirmed to be a sec transport pathway signal peptide. As Bd3243 was originally annotated as a membrane bound transglycosylase, the presence of transmembrane domains were assessed using TMHMM (figure 3.14c) which did not predict the presence of any transmembrane domains.

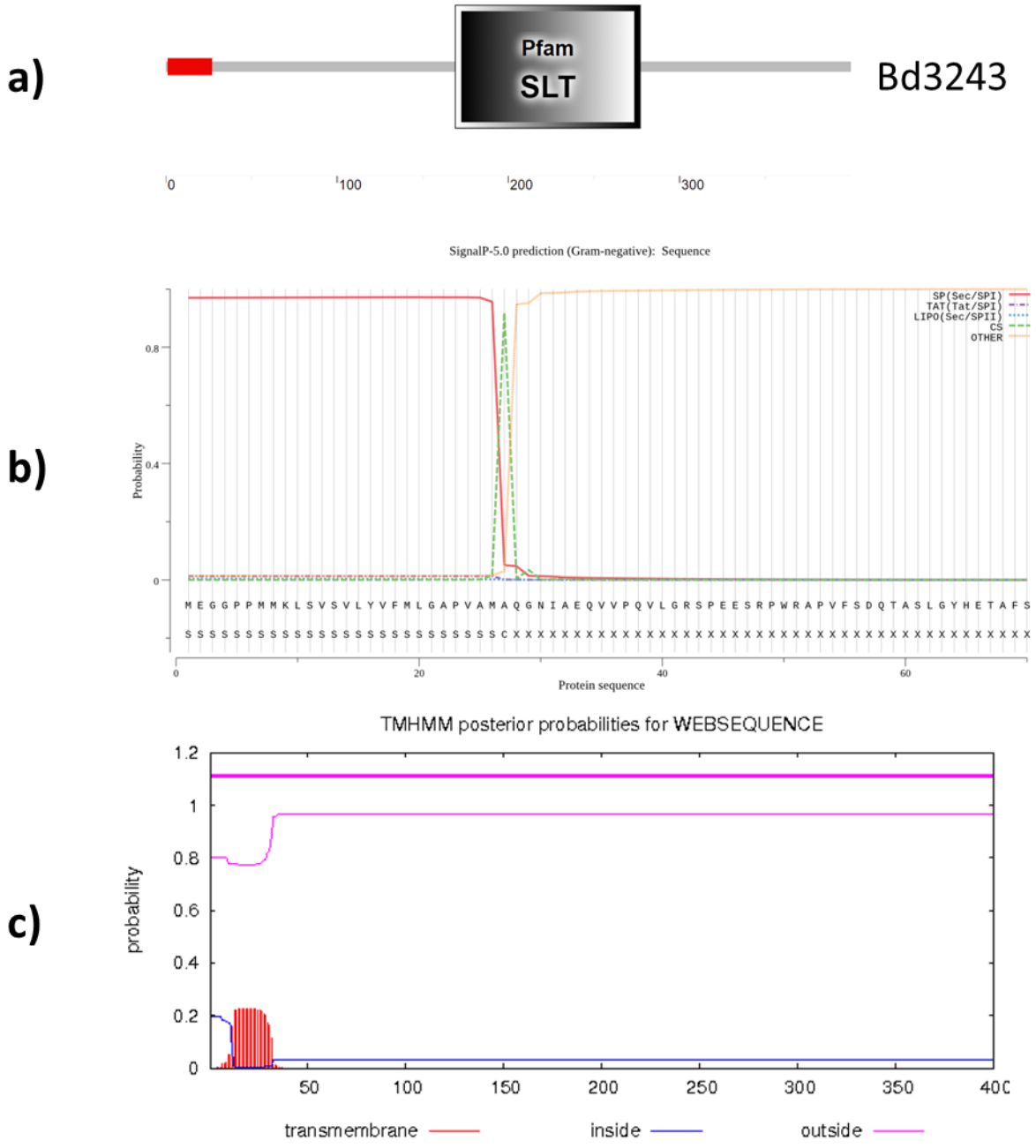


Figure 3.14: a) Domain structure of Bd3243 and a) and b) signal peptide confirmation through SMART and SignalP-5.0 for sec pathway transport. c) Transmembrane domain prediction using TMHMM did not show any evidence of possible TM domains, despite the original annotation suggesting this is a membrane-located enzyme.

Interestingly, this is the only SLT domain containing protein in *B. bacteriovorus* HD100 which contains a signal peptide domain The signal peptide in Bd3243 suggests a

role for this protein within the periplasm of the *Bdellovibrio* cell, modifying the *Bdellovibrio* peptidoglycan rather than acting upon prey in a predatory manner.

Transcriptomic studies: showed that Bd3243 was significantly upregulated during AP but not during GP (3 hours after the onset of predation) (Karunker et al., 2013) and a downregulation of expression of Bd3243 both 30 minutes after the onset of predation and during HI growth (Lambert et al., 2010) suggesting that this gene is primarily expressed in free-swimming, non-replicating attack phase cells, which again supports the hypothesis that this is a peptidoglycan maintenance protein that is not involved in the predatory process.

Bd3459

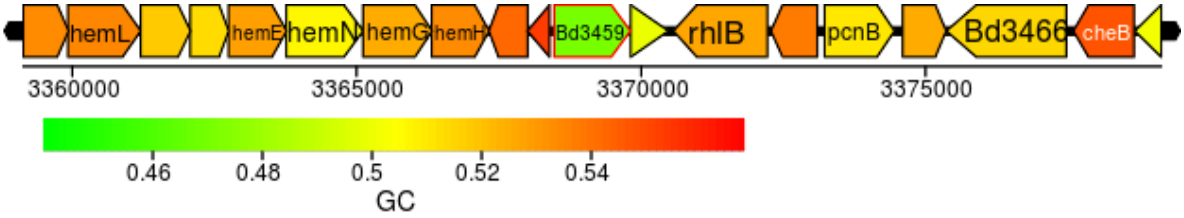


Figure 3.15 – Genomic region around Bd3459. A 20 kb region around Bd3459 is shown, the diagram is reproduced from xbase (Anon., 2021l).

Several genes of potential interest are located near Bd3459, the first of these is Bd3457 which has been previously annotated as a putative murein hydrolase. In this study Bd3457 has been classified in the grouping of glycanases and lipases (section 3.2.1.3 and Appendix A, table A3). Analysis of the domain structure of Bd3457 has revealed the presence of an LrgB domain as well as 6 transmembrane domains, whilst no signal peptides were identified through SMART or SignalP (figure 3.16). Bd3458 was shown by smart analysis to contain an LrgA domain as part of its domain structure, 4 transmembrane domains and no signal peptide domains. BLAST analysis also confirms the likely designation of LrgA of Bd3458 (100% identity, 100% cover) (Figure 3.11b) and LrgB of Bd3457 (100% identity, 100% cover) (Figure 3.11a).

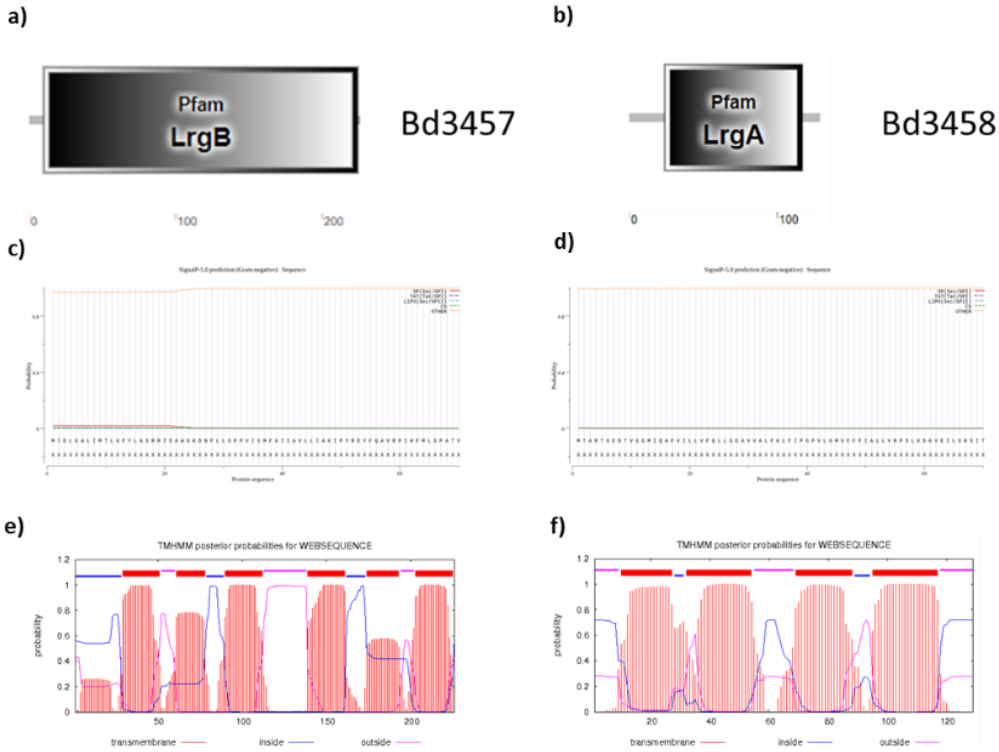


Figure 3.16 – Analysis of the protein domains of Bd3457 and Bd3458 showed that they are homologues of LrgB and LrgA respectively. a) and b) domain structures of LrgB (Bd3457) and LrgA (Bd3458), c) and d) SignalP diagrams for Bd3457 and Bd3458 showing no signal peptide for either Sec or Tat-transport, e) and f) TMHMM diagrams showing 6 transmembrane regions in Bd3457 and 4 transmembrane regions in Bd3458.

Bd3457 and Bd3458 also have different transcriptional profiles to Bd3459. Bd3457 was shown to be upregulated during GP (Karunker et al., 2013) and down regulated in HI (Lambert et al., 2010) when compared with AP. Bd3458 was deemed to be silent based on the work of Karunker and co-workers (Karunker et al., 2013) whilst Lambert and colleagues showed that Bd3458 was also downregulated in HI compared to attack-phase cells (Lambert et al., 2010) Both of these sharply contrast with the upregulation of Bd3459 at 30 minutes into predation.

The evidence suggests that both these proteins have roles closely linked with being membrane bound for function, with LrgA and LrgB units forming part of the LrgAB operon. LrgA has similarity to the bacteriophage murein hydrolase like transporter protein family known as holins, lytic enzymes capable of degrading the cell wall of infected cells in preparation for the final stages of lysis and are controlled by anti-holin proteins to regulate the process (Brunskill and Bayles, 1996; Wang, Smith and Young, 2000). It has been previously shown in *Staphylococcus aureus* that due to high levels of hydrophobicity in the LrgA and LrgB proteins that neither are hydrolases themselves but instead act to regulate murein hydrolases; increased hydrolase activity resulted from ΔLrgAB cells, while these levels returned to WT levels when complemented with complete copies of LrgAB (Groicher et al., 2000). Groicher and colleagues hypothesised that this indicated a role for LrgAB in *S. aureus* as antiholins. Other studies have implicated the Cid operon with regulating the *lrgAB* operon, with CidR acting as a positive regulator and CidA acting as a potential holin protein (van den Esker, Kovács and Kuipers, 2017; Claunch et al., 2018).

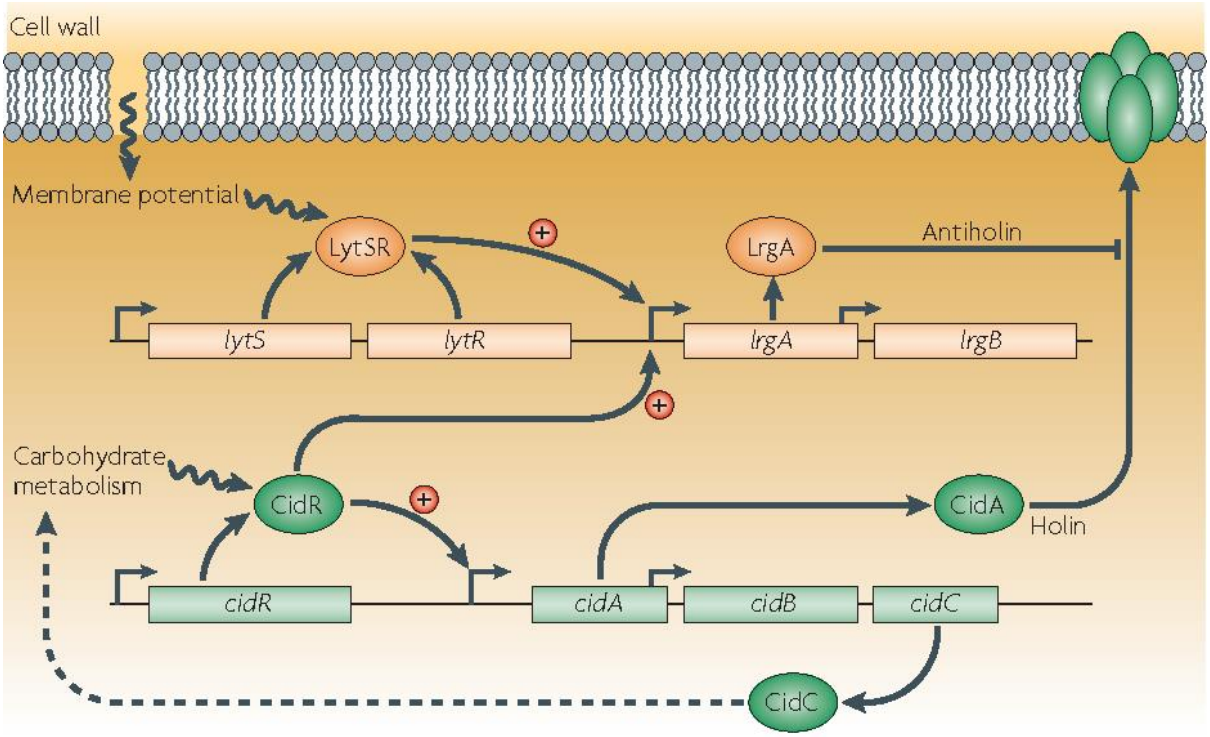


Figure 3.17 – Schematic for the regulatory action of LrgA and LrgB as antiholin proteins, including the role of CidR, which acts like an LysR-type regulator which

regulates the expression of the Cid and Lrg operons in response to metabolism of carbohydrates (Bayles, 2007).

Lrg/Cid operons may also have a role in pyruvate uptake, possibly overlapping with the lytic functions (van den Esker, Kovács and Kuipers, 2017; Ahn et al., 2019). Although much of what is currently know about the LrgAB system has been determined in Gram-positive bacteria, it is possible *Bdellovibrio* may have similar or even more specialised functions for LrgAB directed to the predation process, by having possible involvement in the final lysis stages of the bdelloplast to release new progeny. It is unlikely however, that Bd3457 and Bd3458 activities are directly related to the activity of Bd3459 as a murein hydrolase, but all three proteins may all play a combinatory role in a holin/anti-holin system. To determine whether LrgAB has a role in late stage predation, experimental studies of a Bd3457 and/or Bd3458 deletion strain of *Bdellovibrio* would be required.

Bd0816

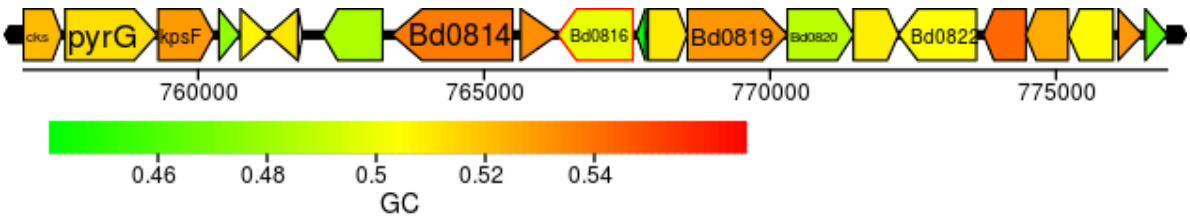


Figure 3.18 - Genomic region around Bd0816. A 20 kb region around Bd0816 is shown, the diagram is reproduced from xbase (Anon., 2021l).

Located on the chromosome near Bd0816 are very few enzymes of interest in regard to hydrolytic enzymes and transport systems. Three genes of note are Bd0823-0825, these appear to be part of an ABC transporter system, capable of glutamine amino acid transport and are annotated as *glnQPH*. The placement and orientation of Bd0816 means it will not be co-transcribed with any other proteins near it other than the small hypothetical protein encoded by the open reding frame Bd0817.

3.2.3.2 Peptidase_S8

Peptidase S8 domain containing proteins belong to the subtilisin-like serine proteases, having diverse functions in prokaryotes and eukaryotes, such as pathogenesis, nutrient acquisition, interruption of complement immune pathways and have peptidase activity, the majority are endopeptidases, but a few have been shown to have exopeptidase activity (Rawlings and Barrett, 1993). Proteins within this family typically possess active site residues of Asp-Ser-His working as a catalytic triad to hydrolyse peptide bonds (Anon., 2021f) Within S8 peptidases there are two types S8A and S8B: S8A typically have active site residues of Asp-Thr/Ser-Gly. Other active site motifs seen in S8A include: His-Gly-Thr-His and Gly-Thr-Ser-Met-Ala-Xaa-Pro (Anon., 2021d).

3.2.3.2.1 Peptidase_S8 domain containing proteins in *B. bacteriovorus* HD100

In *B. bacteriovorus* HD100 there are 16 enzymes containing the Peptidase S8 catalytic domain as seen in figure 3.19 below, with 10 containing the S8 domain alone.

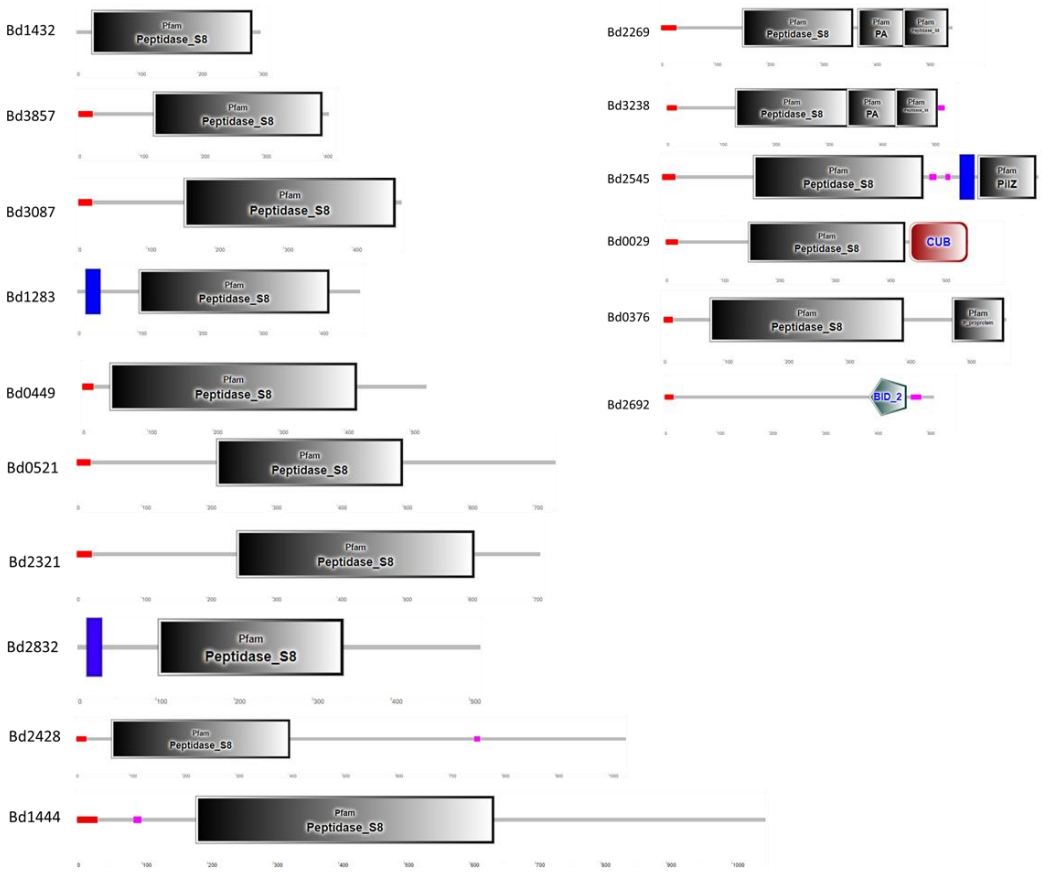


Figure 3.19: Domain organization of the 16 enzymes from *B. bacteriovorus* HD100 with Peptidase S8 protease domains. 13 of the proteins also contain signal peptides, 2 contain transmembrane domains and 6 contain other potential catalytic domains.

Other domains seen combined with S8 include the important cyclic-di-GMP receptor PilZ, PA and CUB.

Based on data from SMART (Anon., 2021h), 13 of the peptidase S8 containing enzymes also contained a signal peptide sequence and 3 contained transmembrane domains (table 3.06 below). Further analysis with SignalP (Anon., 2021g) revealed all peptidase S8 enzymes with signal peptides fell into the sec transport related signal

sequences of type I and II. Analysis with TMHMM (Anon., 2021j) confirmed that 3 of the peptidase S8 enzymes contained transmembrane domains.

Table 3.06 – Signal peptides of sec pathway grouping and transmembrane domain frequency for S8 peptidases in *B. bacteriovorus* HD100. Signal peptides were predicted using SignalP, and transmembrane domains using TMHMM.

Enzyme	Signal Peptide of Sec Transport Pathway	Number of Transmembrane Domains
Bd0029	SPI	-
Bd0376	SPII	-
Bd0449	SPI	-
Bd0521	SPI	-
Bd1283	-	1
Bd1432	SPI	-
Bd1444	SPII	-
Bd2269	SPI	-
Bd2321	SPI	-
Bd2428	SPI	-
Bd2545	SPI	1
Bd2692	SPI	-
Bd2832	-	1

Bd3087	SPI	-
Bd3238	SPI	-
Bd3857	SPII	-

SPI and SPII refers to the enzyme which targets these signal sequences for cleavage following secretion. Signal peptidase I (SPI) is the general peptidase with no clear residue targets, whereas SPII typically acts on lipoproteins, cleaving near a conserved region known as a 'lipobox' (Schneewind and Missiakas, 2014). The large proportion of peptidase S8 proteins containing a sec signal peptide highlights that these enzymes either act in the periplasm of the *Bdellovibrio* cell, or are exported out of the cell entirely (via unknown transport systems to cross the *Bdellovibrio* outer membrane), likely then entering the prey cell, being involved in prey degradation. By comparison, the three proteins containing the identified transmembrane domains will be incorporated into the inner membrane of the *Bdellovibrio* cell and remain within the predator.

Another domain which is found combined with Peptidase S8 in *Bdellovibrio* proteins include: PA, which is found in 3 of the peptidase S8 proteins. These protease associated domains (PA), are found commonly combined with S8 and M28 proteases with a role in potentially acting as a lid to cover the active site of the protease, limiting substrate access to regulate enzymic activity (Luo and Hofmann, 2001). The CUB domain is found in Bd0029, CUB domains are also typically found in peptidases, these domains have diverse functions including involvement in complement in eukaryotes, where they are thought to aid oligomerization of substrates by driving protein-protein interactions (Briggs and Day, 2008). Bd0029 has an SPI signal sequence (table 3.06), so it could be hypothesised that if this protein is secreted into the prey cell then the CUB domain may be involved in the oligomerisation of prey cell proteins that are then degraded by the peptidase activity from the S8 domain. Of particular interestis that a PilZ domain is found in Bd2545, this is a cyclic-di-GMP receptor domain, that responds to the presence of cyclic-di-GMP in the cell often acting as a regulator of the accompanying protein

domain. Cyclic-di-GMP has been shown to regulate both predatory and host-independent growth in B. bacteriovorus HD100, but the role(s) of each of the cyclic-di-GMP receiver proteins has not yet been reported (see section 1.3.1**)**. Bd2545 contains 4 domains, a signal peptide, peptidase_S8, transmembrane, and PilZ domain. The organisation of these domains suggests that the protein is likely to be integrated into the cytoplasmic membrane of the *Bdellovibrio* cell, with the n-terminal peptidase_S8 domain in the periplasm, and the PilZ domain in the cytoplasm. This localisation suggests that Bd2545 is unlikely to be involved directly in the degradation of prey cell proteins, instead likely acting on proteins within the *Bdellovibrio* periplasm.

The S8 domains from each of the 16 *Bdellovibrio* enzymes were aligned with a canonical S8 peptidase domain from *Bacillus licheniformis* (subC) (Figure 3.20). Motifs implicated in the S8A type of S8 peptidases were found in all 16 of the *B. bacteriovorus* S8 enzymes: the Asp-Thr/Ser-Gly active site triad were found to be conserved in 12 of the 16 enzymes, with Bd0521 and Bd3238 containing the Asp-Ser-Gly residue variant of the triad. Also highlighted are the other active site motifs associated with S8A type peptidases: His-Gly-Thr-His and Gly-Thr-Ser- Met-Xaa-Pro. The former motif was seen to be closely conserved in 13 of the enzymes, with Bd0449, Bd0376 and Bd2832 lacking the fully conserved motif. The latter motif was not conserved in all the enzymes, with only the partial G-T-S being found in some, suggesting reduced or differing function compared to canonical S8 enzymes which contain the full motif.

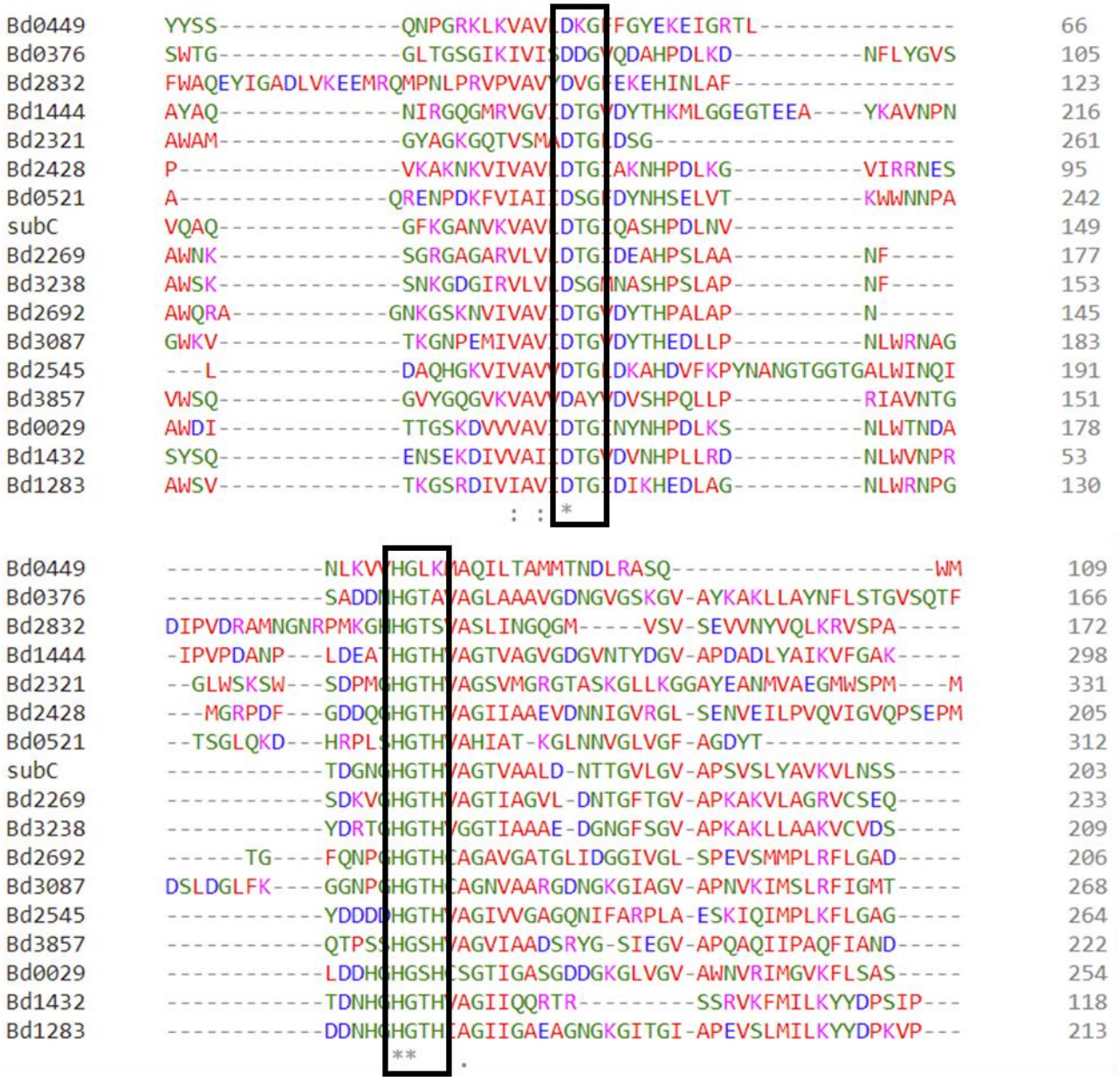


Figure 3.20: Alignment of the Peptidase_S8 domain from the 16 identified enzymes in *B. bacteriovorus* HD100 and a canonical Peptidase_S8 from the SubC protein of *Bacillus licheniformis* (UniProt P00780). The conserved catalytic residue motifs are highlighted, including the well-conserved catalytic triad.

The transcriptional profiles of the 16 S8 domain containing enzymes is seen in table 3.07. Of interest are the 3 genes that are downregulated both 30 minutes into predation and in host-independent growth when compared to attack phase cells, Bd1283, Bd1432 and Bd2545 table 3.07 and (Lambert et al., 2010). Of these, Bd1283 and

Bd2545 were both shown as being highly expressed in attack phase (table 3.07 and (Karunker et al., 2013). Thus, the combined transcriptional data suggest that these three proteins are all likely to be involved in *Bdellovibrio* cell maintenance during the free-swimming attack phase. An additional gene, Bd3857, is also down regulated after 30 mins of predation, but its expression in host-independent growth is not significantly different to attack phase, suggesting that this protein is not likely to be involved in predation, but again in cell maintenance of free-swimming *Bdellovibrio* cells (not all host-independent cells are actively engaged in growth and replication at the same time, many appear to be in a state similar to the non-replicating attack-phase cells). Five genes are upregulated in their expression at 30 minutes into the predatory cycle, but not at either 3 hours of predation or in host-independent growth: Bd0521, Bd1444, Bd2428, Bd2692 and Bd3238 (Table 3.07). These peptidases are thus likely to be involved in the early stages of predation, either entry into the prey cell or establishment of the bdelloplast. Finally, four genes were shown to be increased in expression both in host-independent growth and 3 hours into the predatory cycle: Bd0449, Bd2269, Bd2321 and Bd3087. These enzymes are likely involved in general growth of the *Bdellovibrio*, and are unlikely to be specifically used in the degradation of the prey cell. Together the transcriptional data regarding these enzymes highlights the potential ways in which *Bdellovibrio* may use members of a single class of peptidases at different stages in its lifecycle.

Table 3.07 – Transcriptional data for each gene encoding an S8 Peptidases in *B. bacteriovorus* HD100. Data was collated from the previously published data sets from the studies by Karunker and colleagues (Karunker et al., 2013) and Lambert and co-workers (Lambert et al., 2010).

Enzyme	Upregulated During Attack Phase (AP), Growth Phase (GP) or Silent?	Expression change in *B. bacteriovorus* Enzymes at 30 mins and in Host independent (HI) vs Attack Phase (AP)
Bd0029	AP and GP	No Change
Bd0376	AP	Down in HI

Bd0449	GP	Up in HI
Bd0521	Silent	Up in both 30 and HI
Bd1283	AP	Down at 30 and HI
Bd1432	Silent	Down at 30 and HI
Bd1444	Silent	Up at 30
Bd2269	GP	Up in HI
Bd2321	GP	Up in HI
Bd2428	Silent	Up in both 30 and HI
Bd2545	AP	Down at Both 30 and in HI
Bd2692	AP	Up at 30 and down in HI
Bd2832	Silent	No Change
Bd3087	GP	Up in HI
Bd3238	Silent	Up at 30 and down in HI
Bd3857	Silent	Down at 30

3.2.3.2.2 Insights gained from the genomic location of the S8 peptidase genes

Co-localisation of genes in the genome of bacteria sometimes correlates with roles within related processes or pathways. Genes located in operons are co-transcribed, allowing for precise co-ordination of expression of genes whose products form parts of a pathway. In this section the genes around some of the S8 peptidase genes of interest have been investigated, with those involved in other enzymatic

pathways, transporters or related regulatory systems (such as cyclic-di-GMP related genes) highlighted as being of interest.

Bd1432

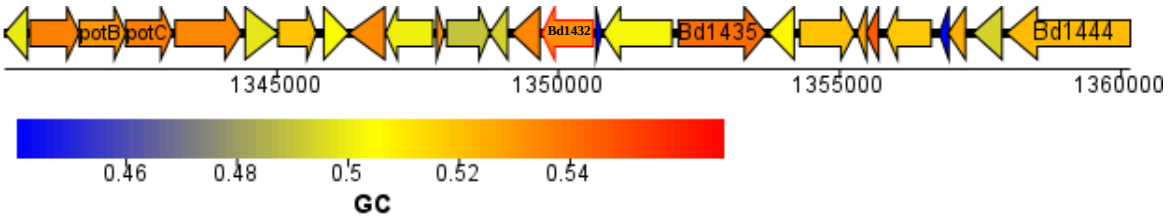


Figure 3.21 – Genomic region around Bd1432. A 20 kb region around Bd1432 is shown, the diagram is reproduced from xbase (Anon., 2021l).

Several genes of interest are located close to Bd1432 (Figure 3.21). Firstly, Bd1431 is annotated downstream as a nuclease, containing an SNase domain (see section 3.2.4.3 for a discussion of the SNases in *B. bacteriovorus*) and Bd1430 as a hypothetical protein, these are both transcribed in the same direction as Bd1432. Upstream, Bd1434 is another gene of interest near to Bd1432, it encodes a GGDEF domain and has been shown to produce the secondary messenger cyclic-di-GMP (Hobley et al., 2012a) where it was shown to be required for a switch to host-independent growth. Bd1434 is also transcribed in the same direction as Bd1432 but is separated by a small open reading frame potentially preventing possible co-transcription, further experimental transcriptomic studies would be needed to confirm this as this could be a simple mistake from the gene prediction algorithm. Lastly, Bd1429 has been previously annotated as the periplasmic solute-binding component of an ABC-transporter, further analysis by Barabote and co-workers (Barabote et al., 2007) showed this protein to be part of a predicted polar amino acid uptake transporter (PAAT). This could have a possible role in the uptake of released amino acids produced by the peptidase activity of Bd1432. This is however, transcribed in the opposite direction, ruling out co-transcription with Bd1432.

Bd2545

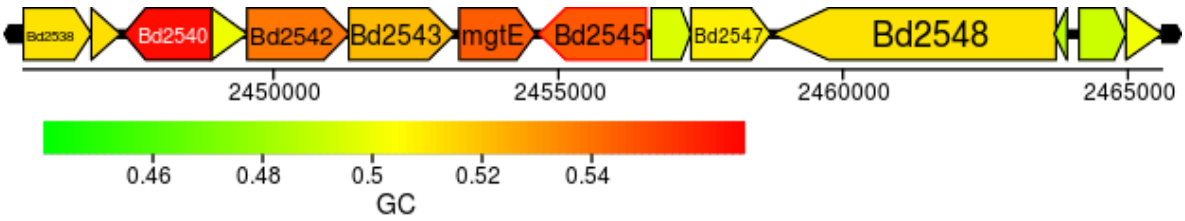


Figure 3.22 - Genomic region around Bd2545. A 20 kb region around Bd2545 is shown, the diagram is reproduced from xbase (Anon., 2021l).

Bd2545 is the predicted membrane localised S8 peptidase that also contains a cyclic-di-GMP receiving PilZ domain. Genes of interest close to Bd2545 include Bd2544 which has been annotated as *mgtE*, a Mg^{2+} transporter, this is however transcribed in the opposite direction to Bd2545 ruling out co-transcription. In addition, both Bd2542 and Bd2543 have been predicted to be multidrug ABC transporter proteins, with both having permease/ATPase activity. Both share the similar domain structure seen in figure 3.23, TMHMM also predicts Bd2542 to have 5 transmembrane regions and Bd2543 to have 6. Both proteins are now in the Transporter Classification Database (TCBD link) as members of the TC family 3.A.1.208, which are the drug conjugate transporter family. Also located here is Bd2541, which contains a P4Hc domain (Prolyl-4-hydroxylase), responsible in prokaryotes for the hydroxylation of antibiotic peptides. Together, Bd2541, Bd2542 and Bd2543 may all be involved in the transport and processing of antibiotics, potentially making this ABC transporter setup more drug targeted than for the secretion of hydrolytic enzymes.

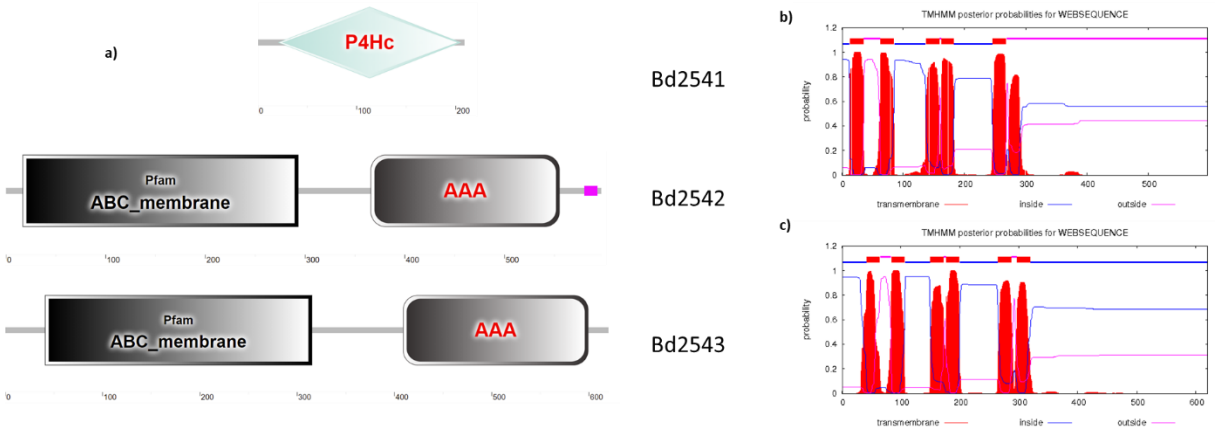


Figure 3.23 – Domain structures of Bd2541, Bd2542 and Bd2543. a) Domain structures of Bd2541, Bd2542 and Bd2543 as determined by SMART, b) TMHMM transmembrane predictions for Bd2542 and c) transmembrane prediction for Bd2543.

Bd3857

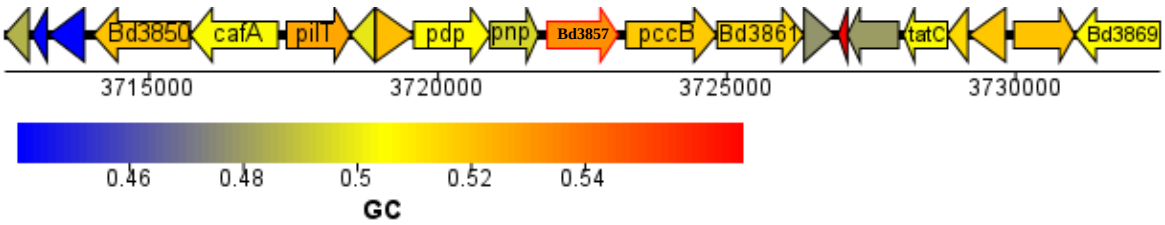


Figure 3.24 - Genomic region around Bd3857. A 20 kb region around Bd3857 is shown, the diagram is reproduced from xbase (Anon., 2021l).

The only genes of interest localised near to Bd3857 are Bd3865 and Bd3866 which encode two components of the Tat transport system (TatC and TatB), these will not be co-transcribed with Bd3857 as they are 8 genes away and are transcribed in the opposite direction. Whilst the Tat transport system is responsible for the transport of folded proteins across the inner membrane of the cell, and it has been shown experimentally that the Tat system is essential for *B. bacteriovorus* HD100 (Chang et al., 2011), none of the enzymes analysed using SignalP in this study had a predicted Tat signal peptide, so it is unlikely that the Tat transporter system plays a role in the secretion of any of these enzymes.

Bd2269

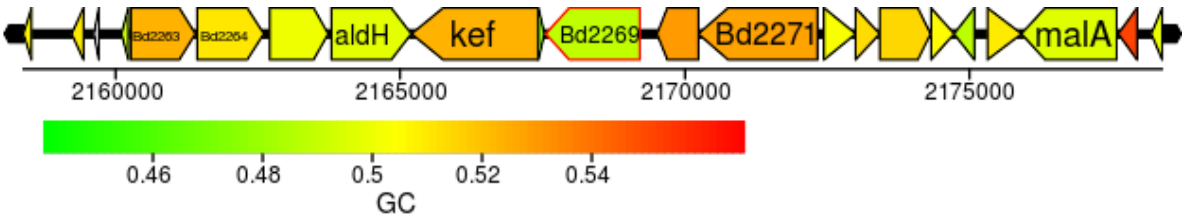


Figure 3.25 - Genomic region around Bd2269. A 20 kb region around Bd2269 is shown, the diagram is reproduced from xbase (Anon., 2021l).

Of initial interest were five genes around Bd2269 (Bd2268, Bd2272, Bd2273, Bd2274 and Bd2275) which are all annotated as spb1 for serine related proteins, but SMART did not identify any functional domains (other than signal peptides in Bd2274 and Bd2275) suggesting incorrect annotation of these predicted proteins. Analysis using the String database (Anon., 2021i) does not predict any interaction of Bd2269 with any of these 5 proteins. Of greater interest is Bd2267 which is also located near to Bd2269 and is annotated as kef (putative potassium/proton antiporter). SMART predicts a Na/H exchanger domain and two TrkA_C domains (figure 3.26) and TMHMM predicts 15 transmembrane regions. The work of Barabote and co-workers (Barabote et al., 2007) confirms this protein belongs to the monovalent cation:proton antiporter-2 CPA2 family, responsible for Na^+ or K^+ transport and are therefore unlikely to be involved in either protein or amino acid transport.

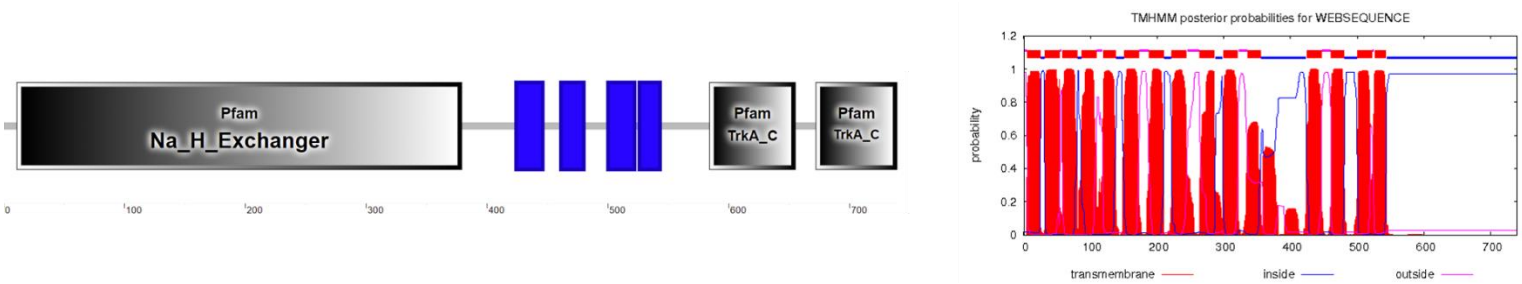


Figure 3.26 – Domain structure of Bd2267 and transmembrane region prediction.

Bd0449

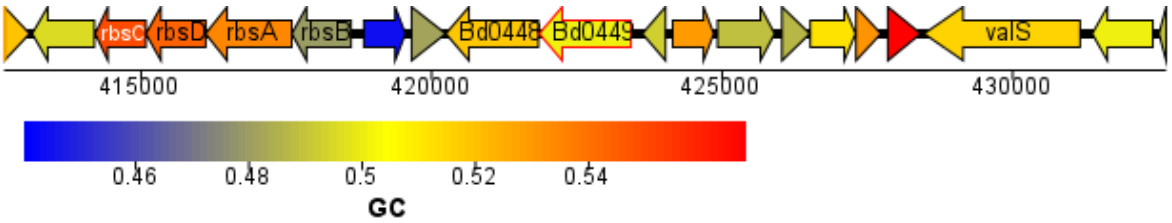


Figure 3.27 - Genomic region around Bd0449. A 20 kb region around Bd0449 is shown, the diagram is reproduced from xbase (Anon., 2021l).

Bd0448 is annotated as a phospholipase D, and is immediately next to Bd0449 and may be co-transcribed. Bd0448 has been classified in this study to belong to the group of Glycanases and Lipases (section 3.2.1.3). Genes encoding transporters were also found localised near Bd0449: Bd0442-Bd0445 are annotated as components of an ABC transporter, Barabote and co-workers (Barabote et al., 2007) categorised this to be a carbohydrate uptake transporter-2 type of ABC transporter, meaning this system is unlikely to transport amino acids or proteins.

Bd2321

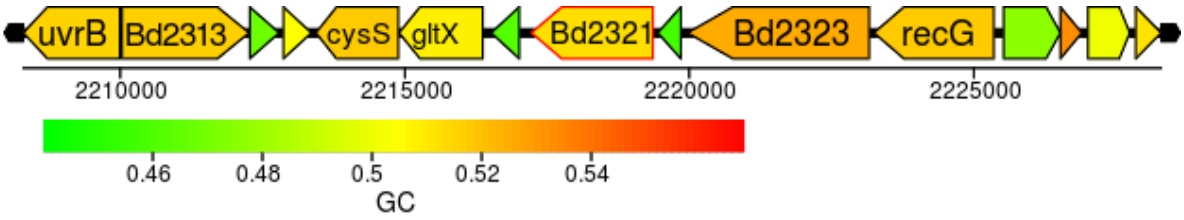


Figure 3.28 - Genomic region around Bd2321. A 20 kb region around Bd2321 is shown, the diagram is reproduced from xbase (Anon., 2021l).

Genes of interest in the region around Bd2321 include two other enzymes classified in this study. The first of these, Bd2325, is an HD-GYP protein (figure 3.28), which is a phosphodiesterase involved in cyclic-di-GMP degradation. In this study Bd2325 has been placed in the category of 'other hydrolases' (section 3.2.1.4). As previously described above, synthesis of cyclic-di-GMP has been shown to regulate both predatory and host-independent growth of *Bdellovibrio* (Hobley et al., 2012a), so it is likely that Bd2325 will also have a role in regulation of the lifecycle of *Bdellovibrio*. Also in the region shown in fig 3.28 is Bd2328, which is annotated as a protease and found in

this study to be a peptidase U32 (fig 3.29 and appendix A, table A1), which are a type of collagenase.

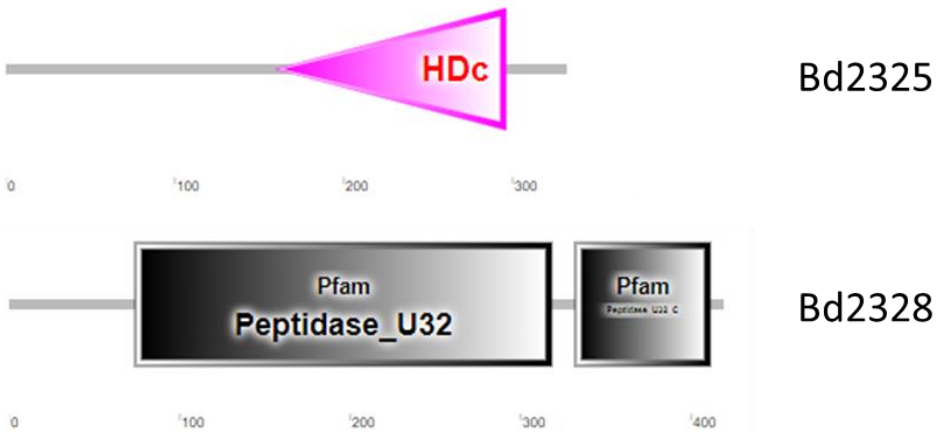


Figure 3.29 – Domain structures of Bd2325 and Bd2328.

Bd1283

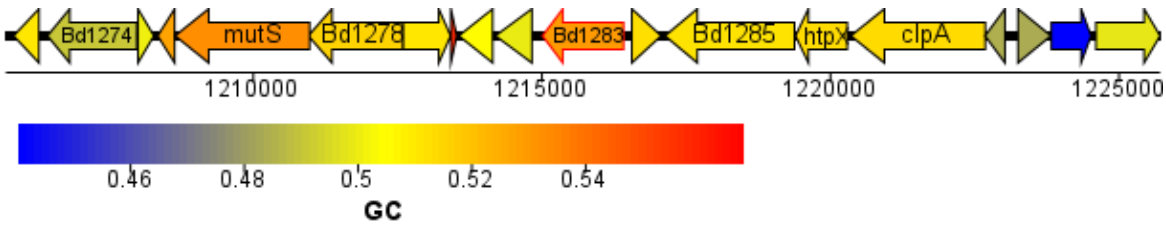


Figure 3.30 - Genomic region around Bd1283. A 20 kb region around Bd1283 is shown, the diagram is reproduced from xbase (Anon., 2021l).

Within the 20 kb region of the genome surrounding Bd1283 (figure 3.30) are a number of enzymes that have been identified as part of this study. Located near Bd1283 is Bd1285, this is annotated as a lytic murein transglycosylase and contains an SLT domain, a SPII signal peptide which overlaps with the predicted transmembrane region (figure 3.31). Bd1285 has been classified in this study as belonging to the grouping of glycanases and lipases (section 3.2.1.3 and appendix A, table A3). Despite Bd1283 and Bd1285 both being transcribed in the same direction they are separated by a small gene

encoding a hypothetical protein which does not contain any detectable domains other than a signal peptide, thus it is unlikely for Bd1283 and Bd1285 to be co-transcribed. Further upstream, Bd1288 is annotated as *clpA*, which encodes the ATP-dependent specificity component of the ClpAP protease, and has been included in the protease category in this study (section 3.2.1.1 and appendix A, table A1). The ClpAP protease has a biological role in degrading misfolded proteins. Interestingly, the work of Duncan and co-workers, 2019) showed that *Bdellovibrio* with a gene deletion of Bd1288 displayed poor attachment and poor prey rounding, however survival was unaffected. Bd1288 and Bd1285 are separated by a protein that is transcribed in the same direction as Bd1283 and Bd1285, this is Bd1287 and is annotated as heat shock protein X (*hspX*). The domain structure, as analysed with SMART, reveals this to be a peptidase of the M48 B- type, as well as containing 4 transmembrane regions with no sec or tat pathway directed signal peptide, indicating that this peptidase is localised in the *Bdellovibrio* membrane. M48 peptidases (*hspX*) are activated by heat shock and are thought to have involvement in the degradation of abnormal proteins, this is a similar function to that of Bd1288, transcription of these two proteins in the same direction could mean these two are co-transcribed. Bd1283, Bd1285, Bd1287 and Bd1288 although all sharing similar predicted peptidase activity, are unlikely to all be transcribed together due to the gene encoding the hypothetical protein Bd1284 which is transcribed in the opposite direction, however further transcriptional data would be required to fully determine this.

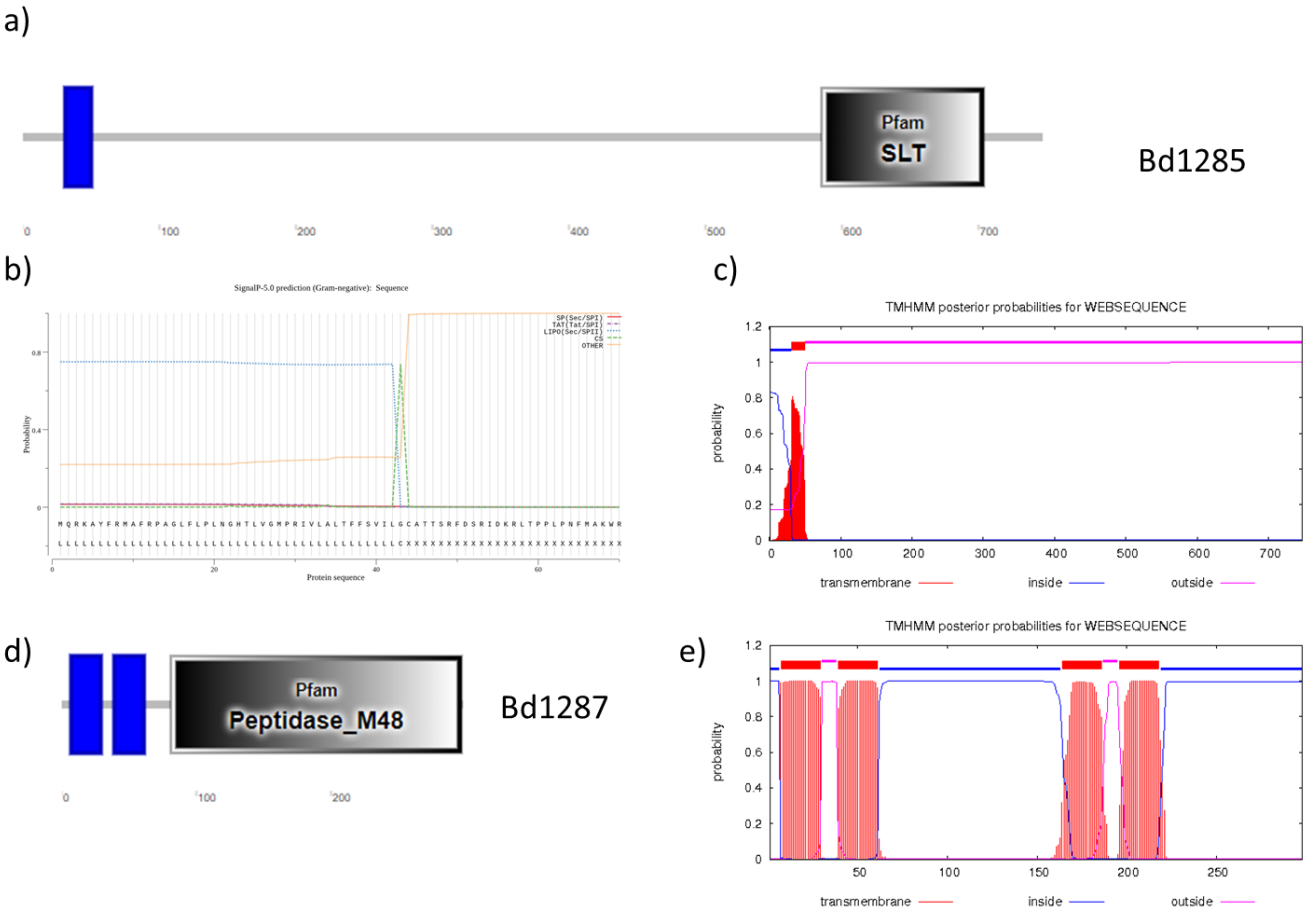


Figure 3.31 – Domain structure of the SLT-containing protein Bd1285 and the peptidase_M48 protein Bd1287. a) Domain structure of Bd1285 as determined by smart b) Signal peptide sequence prediction for Bd1285 c) Transmembrane prediction for Bd1285 d) Domain structure of Bd1287 e) Transmembrane prediction for Bd1287.

Miscellaneous S8 Peptidases

The remaining S8 peptidase-containing genes either did not have many co-localised genes which encoded proteins with either transport and hydrolytic activities, or had hydrolytic enzymes near the gene of interest e.g. Bd0521 which has Bd0519, a murein transglycosylase A, which were transcribed in the opposite direction, ruling out the potential for co-transcription. Additionally, other remaining S8 peptidases had

proteins of interest which were 10 or more genes along the chromosome from the S8 peptidase gene, again limiting the potential for co-transcription e.g. Bd1444. Some were also located near genes of interest for other cellular activities such as chemotaxis, e.g. Bd2832 is located near *mcpA*, *cheR*, *cheD*, *cheW2* with *mcpA* and *cheR* being transcribed in the same direction, which, although interesting to the lifecycle of *Bdellovibrio*, chemotaxis and other processes such as this fall outside of the remit of this project.

3.2.3.3 Peptidase_M14

M14 peptidases are metalloproteases, with carboxypeptidase activity, with the primary target of single C-terminal amino acids in peptides. Type A M14 peptidases target aromatic residues while type B target simple amino acids (Anon., 2021d). Interestingly, M14 type peptidases are typically synthesised without signal peptide sequences, with only a pro-protein sequence needing post-translational processing. These types of peptidases are dependent on interaction with metal ions, including zinc, cobalt, copper and manganese. M14 peptidases bind a single catalytic zinc atom which is important for catalysis, which is bound by two histidine and glutamic acid residues within the peptidase as well as a water molecule. This creates an active site motif of His-Xaa-Xaa-Glu for one of the histidine residues, while the other can be found elsewhere in the enzyme (Anon., 2021d).

3.2.3.3.1 Peptidase_M14 domain containing proteins in *B. bacteriovorus* HD100

The genome of *B. bacteriovorus* HD100 encodes 6 M14 peptidase-like enzymes, 4 of these fall under the M14B type, and the remaining two fall into M14C or M14 carboxypeptidase T. 3 of the *B. bacteriovorus* M14 peptidases also contain zinc peptidase (Zn_pept) domains: Bd3426, Bd0306 and Bd3234 as seen in figure 3.27. Bd3426, Bd1776 and Bd3508 also possess AstE_AspA domains, these domains are the functional domains in succinylglutamate desuccinylases, which are involved in arginine metabolism. Of note are the presence of sec pathway SPI signal peptides in Bd3234 and Bd3508 confirmed by SignalP, this is very unusual for M14 peptidases and can also be seen in Appendix D.

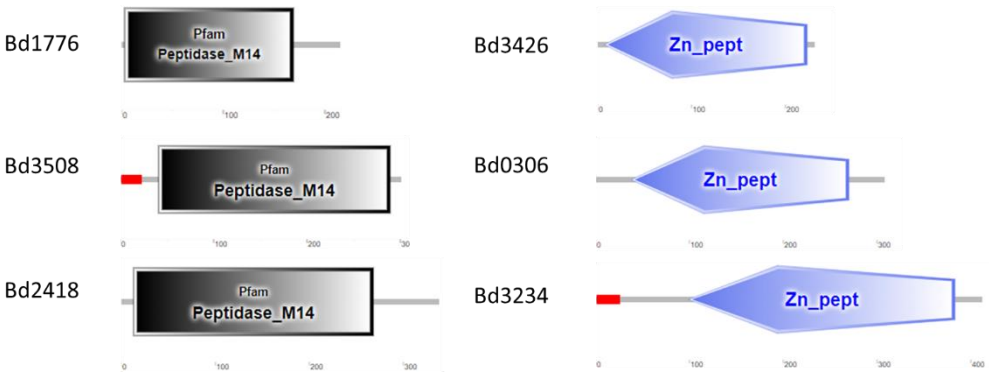


Figure 3.32 – M14 peptidases in *B. bacteriovorus* HD100 Domain structure of M14 peptidases in *B. bacteriovorus* HD100.

All 6 of the M14-like peptidase domains from the *B. bacteriovorus* HD100 proteins were aligned with that of a M14 peptidase, MpaA from *E. coli* B7A. In figure 3.33, the His-Xaa-Xaa-Glu motif can be seen to be fully conserved in all *B. bacteriovorus* HD100 M14-like peptidases, in addition to the other histidine residue that forms part of the zinc complex. The high degree of conservation among these proteins highlights the importance of these motifs in the catalytic ability of M14 peptidases.

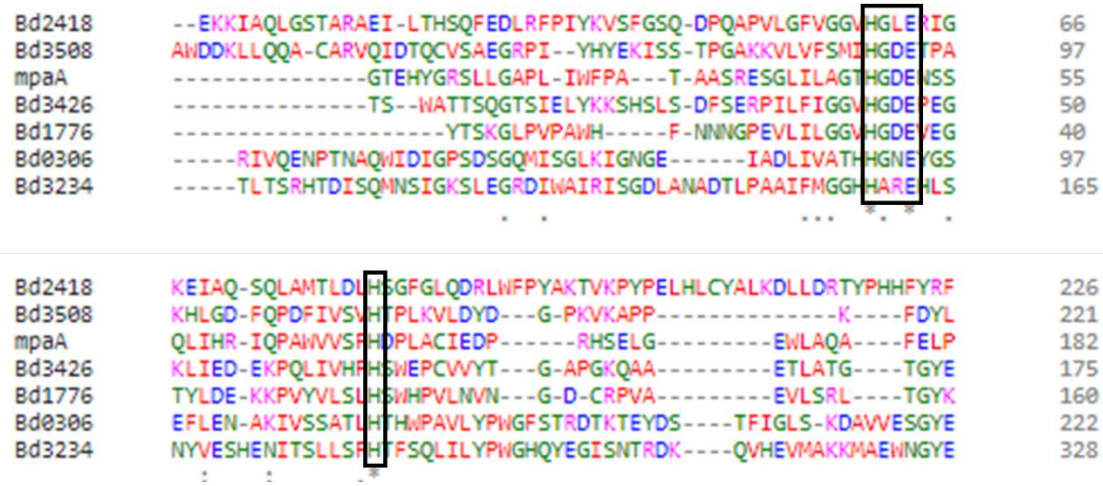


Figure 3.33 – Sequence alignment of the M14 peptidase domains in genes from *B. bacteriovorus* HD100 with the domain from the MpaA canonical M14 peptidase from *E. coli*.

Collation of the transcriptomic data (Lambert et al., 2010; Karunker et al., 2013) has shown that Bd3234 is not significant upregulated during predatory activities (GP or AP) or in HI, this could be attributed to non-predatory growth of *Bdellovibrio* and act as a

'housekeeping protein'. Bd0306 may also be housekeeping for 'normal *Bdellovibrio* growth', as no significant upregulation is observed in AP or GP. The additional upregulation of this protein in HI could highlight its importance for growth in the absence of prey. Both Bd1776 and Bd2418 are GP expressed genes, but the upregulation of expression in HI may also attribute these proteins to a role in general growth and replication. Bd3508 is upregulated during both AP and GP yet is down regulated both in HI and after 30 mins of predation. One possible explanation for this discrepancy could be that this protein plays a role in the maintenance of AP cells prior to contact and invasion of prey, but is not required during the onset of *Bdellovibrio* growth within the bdelloplast and is then expressed 3 hours into the predatory cycle as the *Bdellovibrio* cells are preparing to septate and become attack phase cells.

Table 3.08 – Transcriptomic profile for peptidase M14 proteases in *B. bacteriovorus* collated from previous studies (Lambert et al., 2010; Karunker et al., 2013).

Enzymes	Upregulated During Attack Phase (AP), Growth Phase (GP) or Silent?	Expression change in B. bacteriovorus Enzymes at 30 mins and in Host independent (HI) vs Attack Phase (AP)
Bd0306	Silent	Up in HI
Bd1776	GP	Up in HI
Bd2418	GP	Up in HI
Bd3234	Silent	No Change
Bd3426	GP	No Change
Bd3508	AP and GP	Down in Both 30 and HI

3.2.3.3.2 Insights gained from the genomic location of the S8 peptidase genes

As many of the genes encoding S8 and S13 peptidases in *B. bacteriovorus* HD100 have been shown to be co-localised with other hydrolytic enzymes and transporters (sections 3.2.3.1.2 and 3.2.3.2.2) the chromosomal regions around each of the M13 peptidase genes was also investigated.

Bd1776

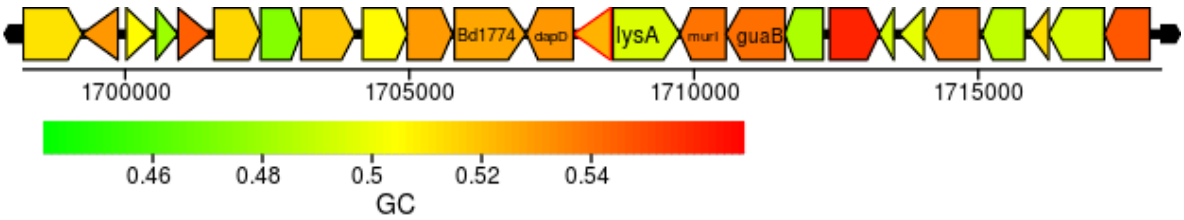


Figure 3.34 – Genomic region around Bd1776. A 20 kb region around Bd1776 is shown, the diagram is reproduced from xbase (Anon., 2021l).

Several genes of interest encoding transporter-related proteins were identified in the genome around Bd1776. Bd1768-1774 are all annotated as ABC-transporter related genes. Further analysis by Barabote and co-workers (Barabote et al., 2007) categorised each of these genes as encoding orphan members of the ABC superfamily, as such their substrates were not predicted.

Bd3508

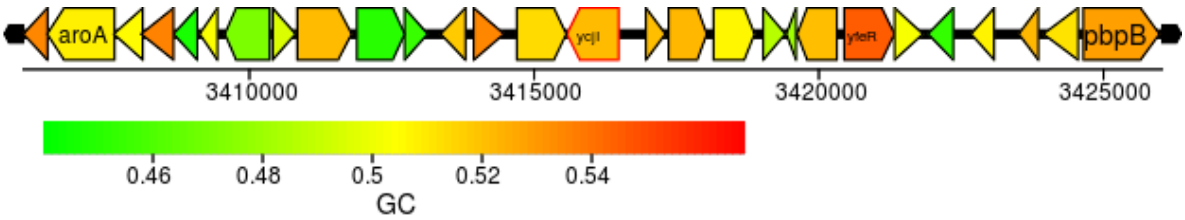


Figure 3.35 - Genomic region around Bd3508. A 20 kb region around Bd3508 is shown, the diagram is reproduced from xbase (Anon., 2021l).

Two genes near to Bd3508 are of interest, the first is the potential nuclease Bd3507, this is convergent with Bd3508 ruling out possible co-transcription, this enzyme belongs to the endonuclease 1 family and further analysis of this protein can be found in section 3.2.4.2. Bd3510 is annotated as encoding the GspD component of the general secretory pathway. The work of Barabote and co-workers (Barabote et al., 2007) showed

this protein to be an outer membrane bacterial secretin, which have the potential to secrete macromolecules including proteins. It could be hypothesised that Bd3509 could have a potential role in allowing both Bd3508 and Bd3507 enzymes to leave the outer membrane, the presence of the SPI signal peptide in both Bd3508 and Bd3507 also adds weight to this hypothesis as these enzymes will already be localised to the periplasm, experimental evidence will be needed to test this hypothesis, first identifying the cellular localisations of each enzyme, and then determining any change in localisation in the absence of GspD.

Bd0306

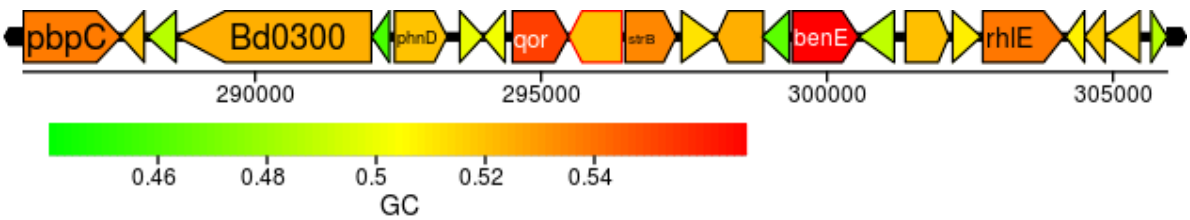


Figure 3.36 - Genomic region around Bd0306. A 20 kb region around Bd0306 is shown, the diagram is reproduced from xbase (Anon., 2021l).

Whilst Bd0306 is not co-transcribed with any neighbouring genes, some nearby genes are of interest as they are annotated as transporters or as having similar functions to Bd0306. Upstream of Bd0306, Bd0311 annotated as a benzoate membrane transport protein, which was confirmed by the work of Barabote and co-workers (Barabote et al., 2007) who showed this to be a Benzoate H+ symporter. Downstream is Bd0304, annotated as a cell wall hydrolase, but SMART analysis did not reveal any predicted functional domains other than a signal peptide, therefore it is likely that Bd0304 does not act as a hydrolytic enzyme and was misannotated.

Bd3426

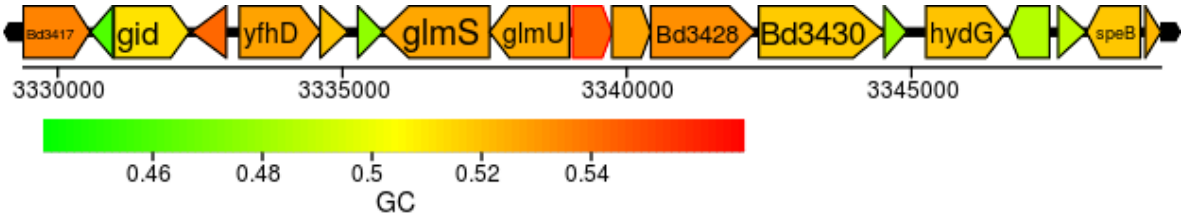


Figure 3.37 - Genomic region around Bd3426. A 20 kb region around Bd3426 is shown, the diagram is reproduced from xbase (Anon., 2021l).

One gene in the region around Bd3426 is of note, upstream and transcribed in the same direction as Bd3426 is Bd3427, SMART analysis revealed a HAD_2 domain present in this hydrolase (classified in this study as a 'other hydrolase' (section 3.2.1.4 and appendix A, table A4). The transcriptional profiles of the two genes differ, suggesting they are not likely to be co-transcribed, with Bd3427 not showing any significant upregulation in GP or AP based on the work by Karunker and co-workers (Karunker et al., 2013), while Bd3246 was shown to be upregulated during HI growth. It is also possible that Bd3427 could play a role in GP in a time period earlier or later than 3 hrs during predatory growth. Bd3426, on the other hand, was shown to be upregulated during GP and showed no change at 30 mins or in HI.

Bd2418

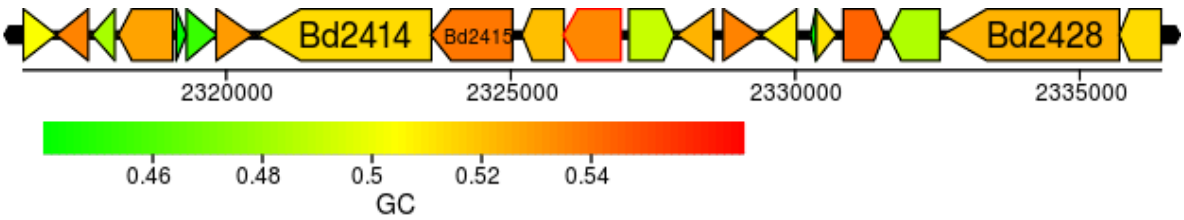


Figure 3.38 - Genomic region around Bd2418. A 20 kb region around Bd2418 is shown, the diagram is reproduced from xbase (Anon., 2021l).

The genome structure around Bd2418 is suggestive that Bd2418 may be the first gene in a 2 or 4 gene operon. Immediately down stream of Bd2418 and transcribed in the same direction is Bd2417, this is annotated as an alpha/beta hydrolase but was shown to have a low e-value probability toAbhydrolase_1 with blast. Analysis with

SignalP and TMHMM revealed no signal peptide or transmembrane domain sequences. Further downstream from Bd2417 are the genes Bd2415 and B2414, these are also transcribed in the same direction as Bd2418 and Bd2417, but no functional domains were found encoded by either of these genes, just signal peptides and a coiled coil domain in Bd2414. Analysis of the available transcriptional data (Lambert et al., 2010; Karunker et al., 2013) is shown in table 3.09 reveals that whilst both Bd2417 and Bd2418 are upregulated in HI, they are not expressed in the same pattern during predation. Bd2415 and Bd2414 are both upregulated at 3 hours into predation (GP) but do not have similar expression patterns earlier in the predation process. Together this data is suggestive that the four genes are not co-transcribed, however experimental validation of this is needed to confirm.

Table 3.09 – Transcriptional profiles for Bd2418 and neighbouring genes Bd2417, Bd2415 and Bd2414.

Enzymes	Upregulated During Attack Phase (AP), Growth Phase (GP) or Silent?	Expression change in B. bacteriovorus Enzymes at 30 mins and in Host independent (HI) vs Attack Phase (AP)
Bd2414	GP	Up at 30 mins
Bd2415	GP	No Change
Bd2417	Silent	Up in HI
Bd2418	GP	Up in HI

Two further genes are of interest in this region: Bd2421, which encodes an HD-GYP protein, responsible for degrading secondary messenger cyclic-di-GMP (as discussed for Bd2325 in section 3.2.3.2.2). Also in this region of the genome is Bd2428, shown in section 3.2.3.2.1 to encode an S8 peptidase.

Bd3234

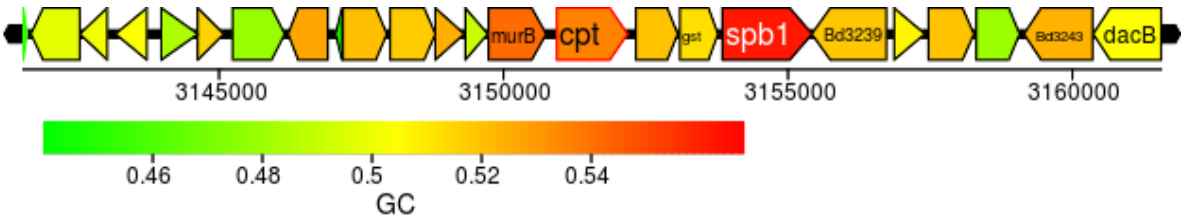


Figure 3.39 - Genomic region around Bd3234 (cpt). A 20 kb region around Bd3234 is shown, the diagram is reproduced from xbase (Anon., 2021l).

The genome around Bd3234 includes several genes encoding for hydrolytic enzymes. Bd3238 is annotated as an S8 family protease, confirmed in this study in section 3.2.3.2.1; Bd3244 is annotated as an S13 peptidase, again confirmed in this study in section 3.2.3.1.1; and Bd3243 a membrane bound lytic transglycosylase, confirmed and described in section 3.2.3.1.2. In addition, Bd3233 is annotated as a UDP-N-acetylpyruvoylglucosamine reductase, with SMART analysis confirming this (figure 3.40), This enzyme is part of the pathway involved in the biosynthesis of peptidoglycan. Further analysis of this protein did not reveal any transmembrane or signal peptide sequences of the tat or sec transport pathway, suggesting more of a housekeeping role in peptidoglycan maintenance in the *Bdellovibro* cell rather than acting on prey peptidoglycan. Although Bd3233-Bd3238 are all transcribed in the same direction, analysis of the available transcriptional data (table 3.10) shows a different transcriptional profile for each gene, indicating that they are unlikely to be co-transcribed.

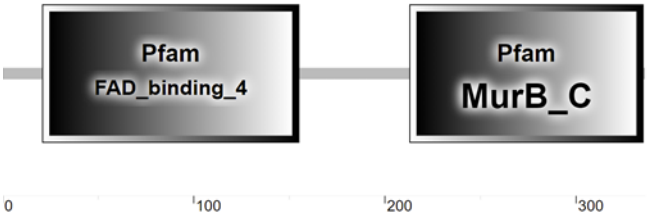


Figure 3.40 – The domain structure of Bd3233 as determined using smart.

Table 3.10 - Transcriptional data of Bd3233-Bd3238 (Lambert et al., 2010; Karunker et al., 2013).

Enzymes	Upregulated During Attack Phase (AP), Growth Phase (GP) or Silent?	Expression change in B. bacteriovorus Enzymes at 30 mins and in Host independent (HI) vs Attack Phase (AP)
Bd3233	GP	Up in HI
Bd3234	Silent	No Change
Bd3236	Silent	Down in HI
Bd3237	GP	Up in HI
Bd3238	Silent	Up at 30 and Down in HI

3.2.4 Nucleases: Exo-endo-phos, endonuclease 1, SNases

3.2.4.1 Exo-endo-phos

The exonuclease, endonuclease and phosphatase (exo_endo_phos) family of enzymatic domains are found in a wide range of proteins including Ca^{2+} dependent nucleases involved in DNA repair and cell signalling. Essential active site residues are thought to be histidine and aspartic acid, which potentially bind Mg^{2+} for catalytic function of the enzyme (Mol et al., 1995; Dlakić, 2000).

3.2.4.1.1 Exo_endo_phos domain containing proteins in *B. bacteriovorus* HD100

In *B. bacteriovorus* HD100 there are 5 exo_endo_phos containing enzymes: Bd3670, Bd3524, Bd1711, Bd3586, and Bd2451, there are also an equal number of these enzymes in *B. exovorus* JSS. Figure 3.41 below describes the domain structures of the 5 enzymes from HD100. Whilst smart predicts a signal peptide in both Bd3586 and Bd3524, only the signal peptide in Bd3586 is predicted using SignalP (Anon., 2021g), using SignalP to analyse Bd3524 a signal peptide was not predicted (Appendix D).

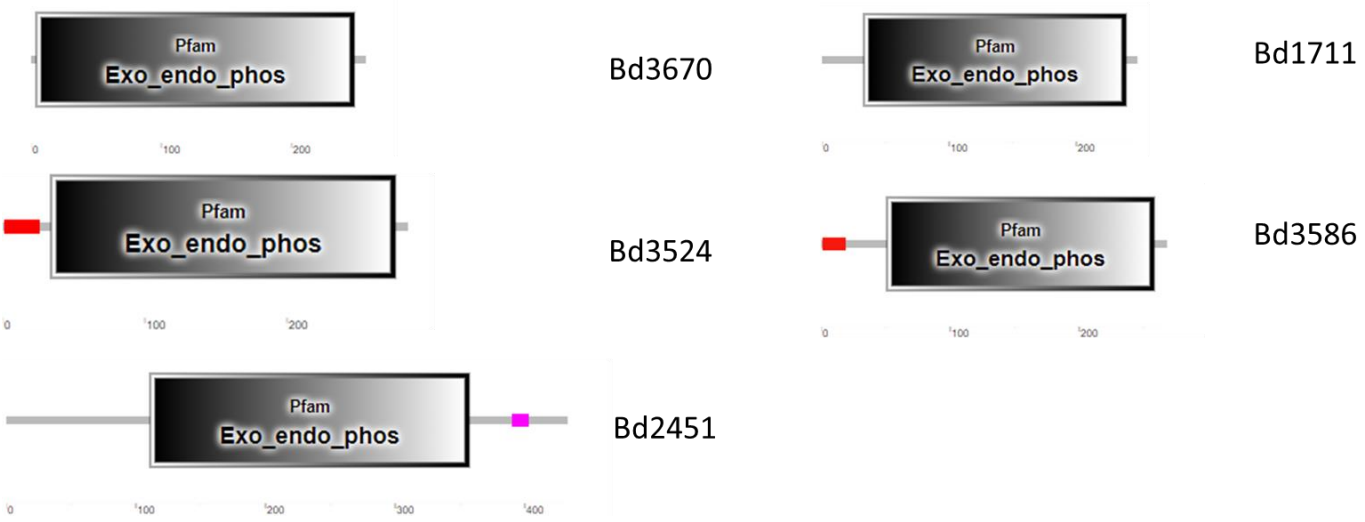


Figure 3.41 – Domain structures for Exo_endo_phos domain containing enzymes in *B. bacteriovorus* as determined using smart. Note that the signal peptide shown in Bd3524 is not predicted using the latest release of SignalP.

The Exo_endo_phos domains from the 5 *B. bacteriovorus* proteins were aligned with the domain from the *E. coli* K-12 enzyme XthA in figure 3.42, and the amino acids that potentially form the Ca^{2+} binding sites are highlighted.

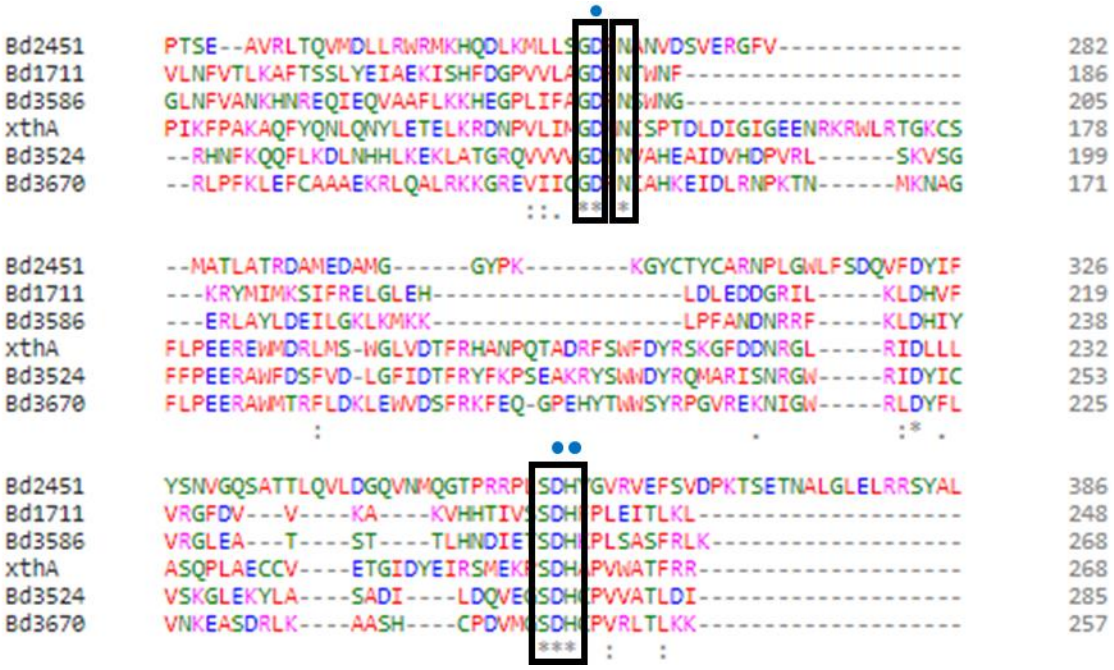


Figure 3.42 - Alignment of *the* Exo_endo_phos domains from the enzymes encoded by *B. bacteriovorus* HD100, with the corresponding domain from XthA from *E. coli* K-12.

Transcriptional data (Karunker et al., 2013) showed that whilst there was no change of expression detected for any of the genes in the data produced by Lambert and colleagues (Lambert et al., 2010), discouting a role for any of these enzymes in the first 30 minutes post infection, or in Host-independent growth, although Bd1711 was detected in the study by Karunker and coworkers (Karunker et al., 2013) as being significantly upregulated during AP (Table 3.11). Three of the remaining enzymes were determined to be upregulated during GP (at 3hrs) suggesting a role of these three enzyme during the later stages of the predation cycle with their nuclease activity directed towards prey cell DNA.

Table 3.11 – Gene expression of the Exo_endo_phos containing enzymes in *B. bacteriovorus* HD100.

Enzymes	Upregulated During Attack Phase (AP), Growth Phase (GP) or Silent?	Expression change in *B. bacteriovorus* Enzymes at 30 mins and in Host independent (HI) vs Attack Phase (AP)
Bd1711	AP	No Change
Bd2451	Silent	No Change
Bd3524	GP	No Change
Bd3586	GP	No Change
Bd3670	GP	No Change

3.2.4.1.2 Insights gained from the genomic location of the Exo_endo_phos nuclease domain containing genes

Bd3524

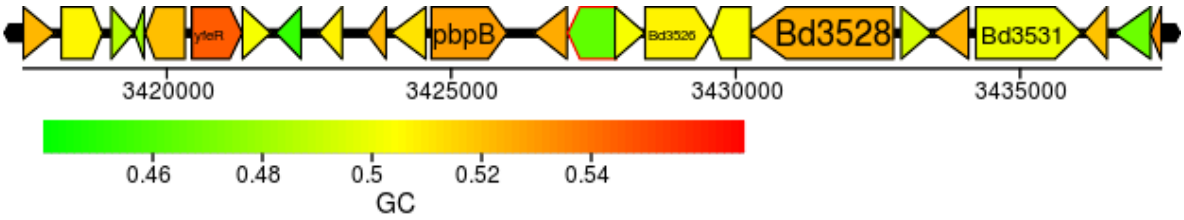


Figure 3.43 - Genomic region around Bd3524. A 20 kb region around Bd3524 is shown, the diagram is reproduced from xbase (Anon., 2021l).

There are four genes in the 20 kb region around Bd3524 that are of potential interest. Bd3522 is annotated as *pbpB*, which this study has confirmed it encode a transpeptidase (section 3.2.1.1 and appendix A, table A1). *pbpB* is transcribed in the opposite direction to Bd3524 meaning the two genes will not be co-transcribed. Further away from Bd3524 is Bd3533, a serine esterase containing an Alpha/Beta hydrolase 2 domain (section 3.2.1.3 and appendix A, table A3). Bd3534 is also an enzyme of the peptidase S24 family (section 3.2.1.1 and appendix A, table A1) and Bd3510 is annotated as *gspD,* general secretory protein D, and is further discussed later in section 3.2.3.3.2.

Bd2451

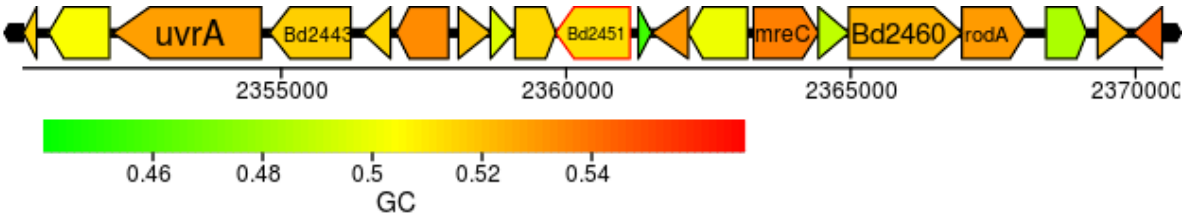


Figure 3.44 – Genomic region around Bd2451. A 20 kb region around Bd2451 is shown, the diagram is reproduced from xbase (Anon., 2021l).

Due to divergent and convergent transcription of neighbouring genes Bd2451 is not transcribed with any other genes. Identified close to Bd2451 is an ABC transporter annotated by Barabote and colleagues (Barabote et al., 2007) as an orphan member of the ABC superfamily with an associated periplasmic solute-binding receptor component encoded by Bd2450. The work by Barabote and co-workers (Barabote et al., 2007) did

not identify what substrate is transported in this system. Upstream is Bd2448, this is a protein containing a Maf domain, this has a potential role in cell division arrest and is typically responsible for preventing the insertion of non-canonical nucleotides into DNA. Also in this region is Bd2442, previously annotated as a *uvrA* excinuclease but domain analysis in this study has now revealed that this actually encodes a protein that forms part of an ABC transporter. Finally, two further enzymes have been identified: Bd2460 which encodes a transpeptidase (section 3.2.1.1 and appendix A, table A1) and Bd2462, which is a transglycosylase with an SLT domain (section 3.2.1.3 and appendix A, table A3).

Bd3670, Bd1711 and Bd3586

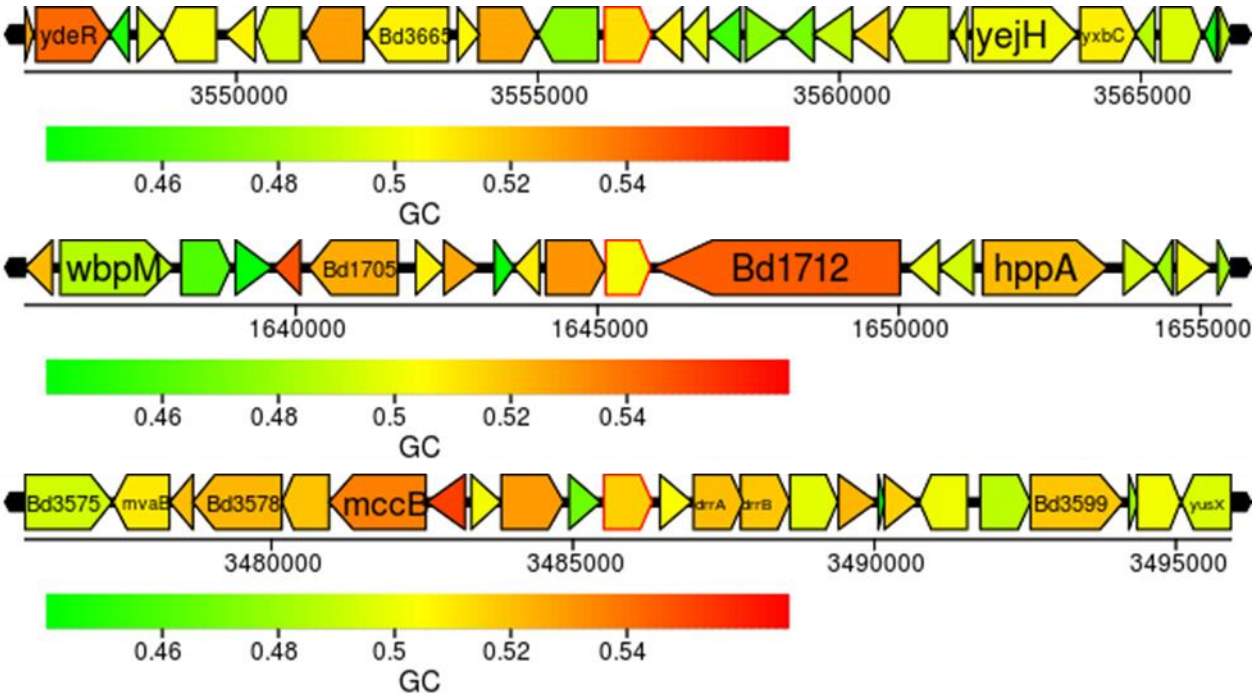


Figure 3.45 - Genomic region around each of Bd3670, Bd1711 and Bd3586. A 20 kb region around each gene is shown, the diagrams were reproduced from xbase (Anon., 2021l).

The genomic regions around the remaining three Exo_endo_phos domain containing genes do not contain many genes of interest and/or relevance for this study. The only gene of interest in the region around Bd3670 is Bd3662 which encodes a metallophosphatase (section 3.2.1.4 and appendix table A4).

However, the gene adjacent to Bd1711, Bd1712, is of interest. Whilst this is annotated as a cell wall surface anchor family protein, analysis in SMART has revealed it to encode a peptidase of the S74 family (section 3.2.1.1). Interestingly, these S74 peptidase domains are found in phage tail enzymes, implicated in breaking through polysaccharide capsules in bacteria, Bd1712 could potentially have been acquired by *Bdellovibrio* from a bacteriophage, incorporated into the *Bdellovibrio* genome and possibly adapted for use in *Bdellovibrio* predation.

In the genome surrounding Bd3586 there are a few genes of interest: Bd3590 – Bd3592 are all annotated as ABC transporter superfamily proteins, with Barabote and co-workers (Barabote et al., 2007) categorising Bd3590 as a cytoplasmic ATP-hydrolysing protein and Bd3591/Bd3592 as integral membrane proteins, but the substrates that this system transports has not been predicted. Also in this region is Bd3575, this is annotated as a lytic murein transglycosylase containing an SLT domain (section 3.2.1.3 and appendix A, table A3).

3.2.4.2 Endonuclease_1

Endonuclease 1 type enzymes create double or single stranded DNA breaks to elicit biological function, although the exact function is unknown. In *E. coli*, these enzymes are thought to have highly conserved cysteine residues, as well as metal binding sites (Glu and Asn) which coordinate with a magnesium ion and water (Li et al., 2003)

3.2.4.2.1 Endonuclease_1 domain containing proteins in *B. bacteriovorus* HD100

There are 3 enzymes with endonuclease_1 domain in *B. bacteriovorus*, but only one in *B. exovorus*, with all containing signal peptides of SPI Sec-transport type directed to the periplasm. Structures of the 3 *B. bacteriovorus* endonuclease_1 domain containing enzymes can be seen in figure 3.46 below.

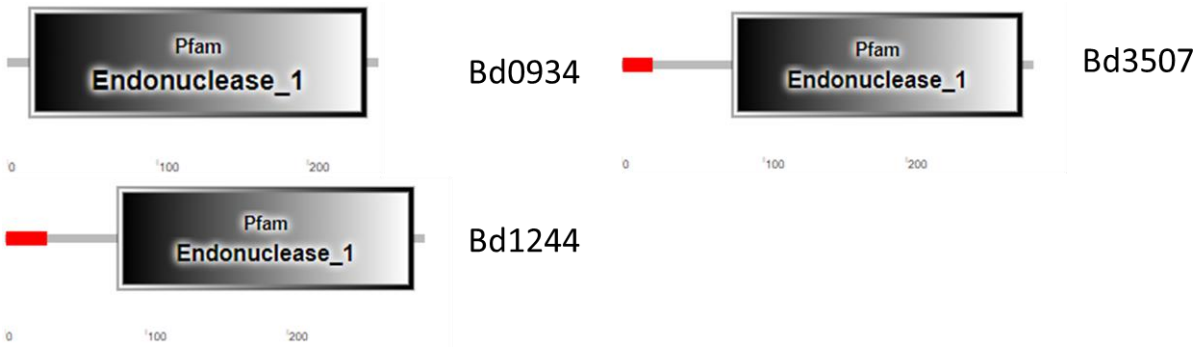


Figure 3.46 – Domain structures for Endonucelase_1 domain-containing enzymes from *B. bacteriovorus* HD100 determined using SMART.

The Endonuclease 1 domains from the enzymes identified in *B. bacteriovorus* were aligned with Endonuclease domain from the nuclease SbcB from *E. coli* K-12 (figure 3.47). There are 14 amino acid residues which are conserved across both the *E. coli* and all 3 *B. bacteriovorus* endonucleases. There was no conservation of the glutamic acid residue belonging to the predicted metal binding site identified in an Endonuclease_1 domain from a *V. vulnificus* nuclease, although the asparagine residues of the other predicted metal binding sites were conserved between all *B. bacteriovorus* endonucleases, but not in *E. coli*. There was also high levels of conservation between the three *B. bacteriovorus* proteins (figure 3.47).

Figure 3.47 – Sequence alignment of the endonuclease 1 domains from the enzymes in *B. bacteriovorus* along with the canonical SbcC endonuclease 1 domain from *E. coli*.

Both Bd3507 and Bd0934 were previously shown (Lambert et al., 2010) to be upregulated during HI growth with no detectable change in expression after 30 mins of predation compared to AP (Table 3.12). Additionally, both of these genes were shown to be significantly expressed during GP (after 3hrs) (Karunker et al., 2013), in addition increased expression 1 hr after infection occurred was detected as part of an experimental study of nucleases in *Bdellovibrio* (Bukowska-Faniband, Andersson and Lood, 2020), placing the expression of these nucleases within the middle of predatory growth phase, leading to the hypothesis that they may be involved in the degradation of prey cell DNA. This study also found contradicting results to the original transcriptomic study by Lambert and co-workers (Lambert et al., 2010), as they describe upregulation of expression of both Bd0934 and Bd3507 at 30 mins, whilst the data from Karunker and colleagues showed significant expression of Bd3507 during AP. Interestingly, gene deletion of both Bd3507 and Bd0934 affect predation, highlighting their redundancy (Bukowska-Faniband, Andersson and Lood, 2020). Bd1244 has been shown to be highly expressed during both AP and GP, and upregulated in both HI growth and 30 minutes into predation. This increased expression early in predation was again shown (Lambert

and Sockett, 2013) using RT-PCR analysis, the expression levels of Bd1244 were shown to increase upon exposure to prey and peaked around 30-45mins (Lambert and Sockett, 2013).

Table 3.12 – Transcriptional data of the genes encoding endonuclease 1 enzymes in *B. bacteriovorus* collated from (Lambert et al., 2010; Karunker et al., 2013).

Enzymes	Upregulated During Attack Phase (AP), Growth Phase (GP) or Silent?	Expression change in B. bacteriovorus Enzymes at 30 mins and in Host independent (HI) vs Attack Phase (AP)
Bd0934	GP	Up in HI
Bd1244	AP and GP	Up at 30 and HI
Bd3507	AP and GP	Up in HI

3.2.4.2.2 Insights gained from the genomic location of the endonuclease_1 genes

Analysis of the genome region around each of the genes encoding endonuclease_1 domains revealed a few co-localised genes of interest related to DNA/RNA synthesis, other hydrolytic enzymes and transporters endonuclease_1 domains revealed a few co-localised genes of interest related to DNA/RNA synthesis, other hydrolytic enzymes and transporters

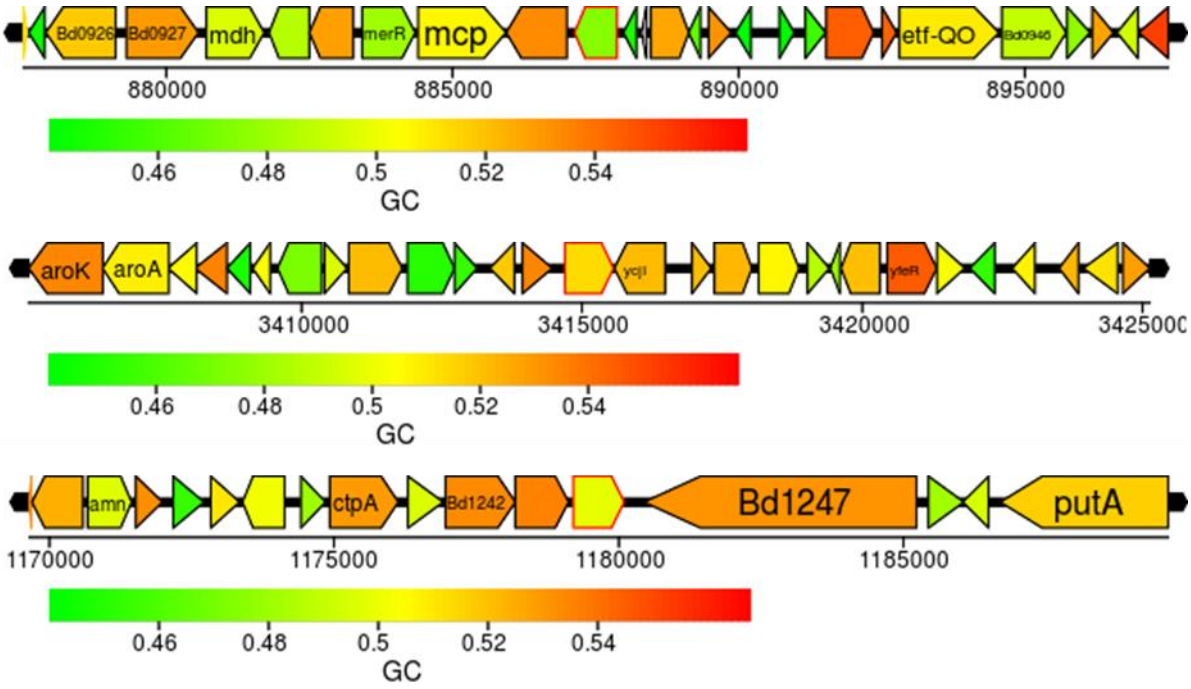


Figure 3.48 – Genomic region around each of Bd0934, Bd3507 and Bd1244. A 20 kb region around each gene is shown, the diagrams were reproduced from xbase (Anon., 2021l).

In the region of the genome around Bd0934 is an RNA methylase (Bd0927) and a putative methyltransferase (Bd0926). Other genes of note are copies of the ribosomal subunits 30S (Bd0940) and 50S (Bd0941).

The region around Bd3507 contains genes encoding for both some hydrolytic enzymes and some transport system components. Bd3508 encodes an M14 peptidase (described in section 3.2.3.3), Bd3507 encodes a phosphoesterase (section 3.2.1.4 and appendix A, table A3), whilst Bd3510 is annotated as *gspD* – part of the general secretory pathway and involved as part of T2SS and T3SS in other prokaryotes. Barabote and co-workers (Barabote et al., 2007)escribe this protein as an outer bacterial membrane secretin.

Finally, the region around Bd1244 contains three genes of interest for this study. Bd1243 is annotated as an integral membrane protein and smart analysis revealed that it has both transmembrane and Rhomboid domains, with the latter being found in some

S54 peptidase proteins. Bd1242 has an RDD domain and a single transmembrane domain; in *Halobacillus andaensis* this RDD domain acts as an Na^+/H^+ antiporter.

The last gene of interest in this region is Bd1237, annotated as an amino acid ABC transporter, Barabote and co-workers (Barabote et al., 2007) have characterised this as a member of the ABC superfamily and as the periplasmic solute-binding receptor, but did not identify the substrate being transported.

3.2.4.3 SNases

Staphylococcal-like nuclease (SNase) type enzymes are Ca^{2+} mediated nucleases capable of degrading both single and double stranded DNA and RNA (Wang et al., 2011). These enzymes have been known to have the following catalytic or Ca^{2+} binding residues: Asp-21, Arg-35, Asp-40, Glu-43 and Arg-87 (Ponting, 1997).

3.2.4.3.1 SNase domain containing proteins in *B. bacteriovorus* HD100

Within *B. bacteriovorus* HD100 there are two known SNase domain containing proteins: Bd1934 and Bd1431 (Figure 3.49). Interestingly, there are no proteins containing this domain in *B. exovorus* JSS. In addition to SNase domains in these enzymes, both have signal peptides of SPI or SPII sec pathways type.

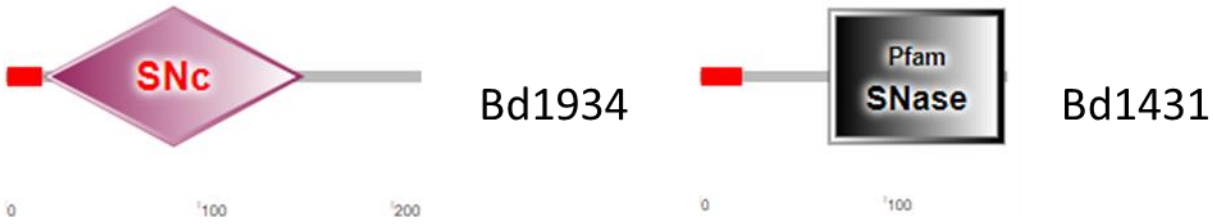


Figure 3.49 - Domain structures for SNase domain containing enzymes in *B. bacteriovorus* as determined using smart.

A multiple alignment of Bd1934 and Bd1431 with the *E. coli* 99.0741 SNase: EC970259_A0031, showed the conservation of the Asp and Arg residues in the potential Ca^{2+} binding sites, but the Glu residue was not conserved in the *B. bacteriovorus* and *E. coli* proteins.

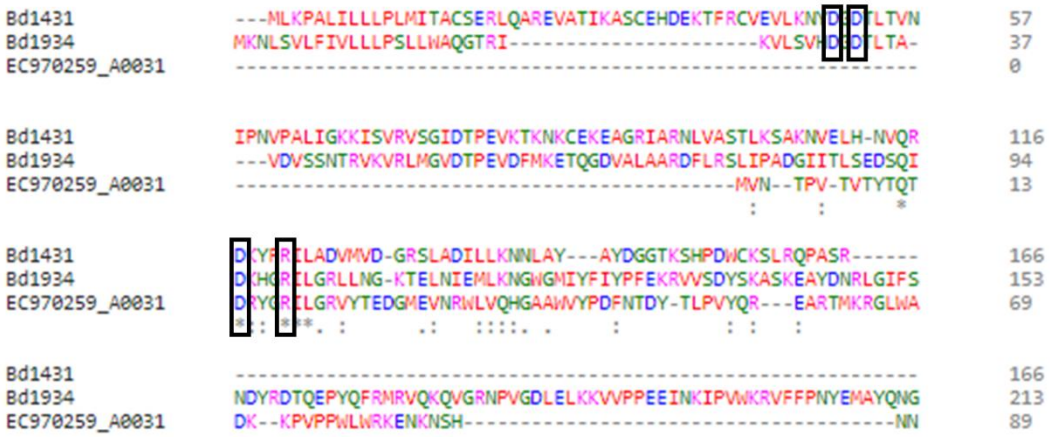


Figure 3.50 - Multiple sequence alignment of Bd1934, Bd1431 and EC0970259_A0031, potential Ca^{2+} binding sites are highlighted in the black boxes.

Transcriptional data (Table 1.13) suggests that Bd1934 has a role in the initial stages of predation, due to its upregulation at 30 mins(Lambert et al., 2010) however Karunker et al, (Karunker et al., 2013) noted this gene was not significantly expressed in either GP or AP. The work of Lambert et al, gives further weight to a potential role for this nuclease as an AP located protein, due to its upregulation in this study shortly after exposure to prey and peaking between 30 and 45 mins. In comparison, Bd1431 was highly expressed 3 hours into the predatory cycle, and was downregulated in HI growth, suggesting that this enzyme might be specifically involve in intraperiplasmic growth rather than general growth.

Table 1.13 - Transcriptional data for the SNase domain containing proteins Bd1431 and Bd1934, (Lambert et al., 2010; Karunker et al., 2013).

Enzymes	Upregulated During Attack Phase (AP), Growth Phase (GP) or Silent?	Expression change in *B. bacteriovorus* Enzymes at 30 mins and in Host independent (HI) vs Attack Phase (AP)
Bd1431	GP	Down in HI
Bd1934	Silent	Up at 30 mins

3.2.4.3.2 Insights gained from the genomic location of the SNase nuclease domain containing genes

Bd1934

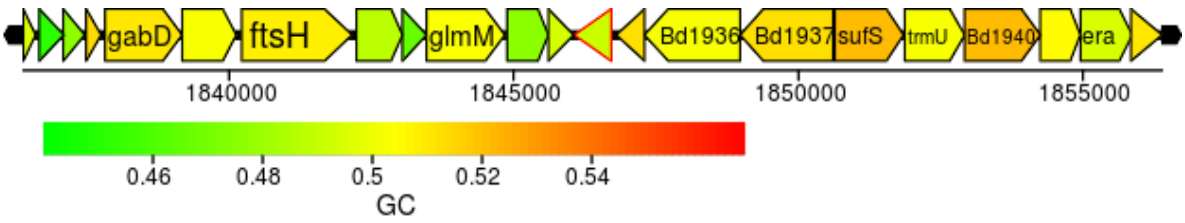


Figure 3.51 - Genomic region around Bd1934. A 20 kb region around Bd1934 is shown, the diagram is reproduced from xbase (Anon., 2021l).

The region of the genome around Bd1934 (Figure 3.51) only contains one gene of interest to this study: Bd1941 encodes a Ribonuclease_3 domain which is described further in section 3.2.4.4.

Bd1431

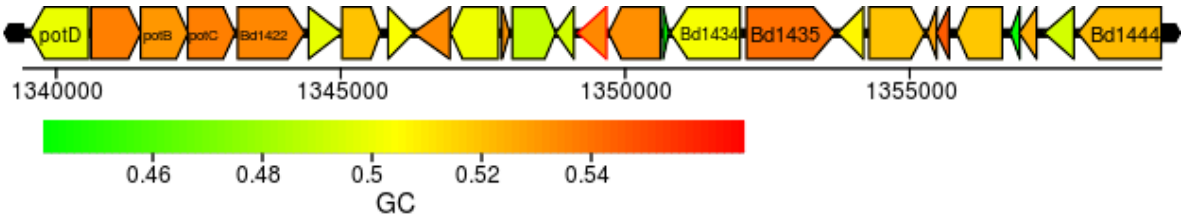


Figure 3.52 - Genomic region around Bd1431. A 20 kb region around Bd1431 is shown, the diagram is reproduced from xbase (Anon., 2021l).

By comparison, the genomic region around Bd1431 contains multiple genes of interest. Immediately upstream of Bd1431 is Bd1432, this is annotated as a serine protease of the S8 type (section 3.2.3.2), both are transcribed in the same direction, suggesting possible co-transcription, however the published transcriptional data shows that the two genes have different transcriptional profiles in the conditions tested thus far, indicating that the two genes are unlikely to be co-transcribed. Bd1431 has a SPII Sec transport system signal sequence, Bd1432 also has a Sec transport signal sequence but the SPI type. Another gene of interest in this region is Bd1434 which encodes a GGDEF protein involved in cyclic-di-GMP signalling (Hobley et al., 2012a). Another S8 peptidase protein can be found further upstream: Bd1444.

The region also contains genes encoding more hydrolytic enzymes: Bd1444 encodes another S8 peptidase (section 3.2.3.2); Bd1427 encodes a C1 peptidase (section 3.2.1.1) and Bd1426 encodes a putative hydrolase with an HDc domain (section 3.2.1.4). One transporter system associated gene is found in this region: Bd1429 is annotated as a component of an ABC transporter, Barabote and co-workers (Barabote et al., 2007) categorised this as an orphan member of the ABC superfamily and as a periplasmic solute-binding receptor, but did not identify the substrate this protein would bind.

3.2.4.4 Other nucleases

Remaining nuclease enzymes can be found in table 3.14 below, for these enzymes there are either one or two enzymes per domain type, suggesting these

enzymes may have low levels of redundancy compared to previous enzymes covered in this grouping which contained >two enzymes per domain type.

Table 3.14 – Functions, annotations and descriptions of neighboring genes of interest for remaining nucleases based on the new domain grouping from this study.

Domain	Enzymes	Function and Annotations	Nearby Genes of Interest
ABC_Tran	Bd2442, Bd0159	Both are uvrA subunits of the ATP-binding domain found in ABC Transporters annotated as excinucleases	
S1	Bd3851, Bd0423	Bd3851 annotated as cafA, a ribonuclease G with S1 being the RNA-binding domain. Bd0423 annotated as vacB, which is a ribonuclease R	Bd3855 – pdp ispyrimidine-nucleoside phosphorylase and Bd3856 – pnp purine nucleoside phosphorylase
GIYc	Bd0254, Bd2311	Bd2311 is a uvrC involved in nucleotide excision repair (*E. coli*), possibly a *Bdellovibrio* 'housekeeping' gene	Bd0255 – ATP dependent helicase and Bd0253 is annotated as a DNA methylation and regulatory protein Bd2312 is the uvrB subunit linked with Bd2311 for nucleotide excision repair
Exonuclease VII	Bd0197, Bd0198	Bd0197 is the L subunit and Bd0198 is the S subunit and catalyzes exonucleolytic cleavage producing nucleoside 5'-phosphates.	Two proteases downstream: Bd0201 (Patatin glycoprotein) and Bd0202 (peptidase of M24 family and aminopeptidase)
TatD_Dnase	Bd1042	Member of the deoxyribonuclease family	Bd1041 – holB annotated as a DNA polymerase III δ-subunit

DHH	Bd2232	Annotated as recJ – in *E. coli* this is involved in DNA repair and recombination	Two components of Sec transport: Bd2233 – secF and Bd2234 – secD
Methylase_S	Bd3695	Annotated as hsdS, type I restriction modification system S subunit	Bd3694 – annotated as hsdR, Bd3696 – annotated hsdM – all three subunits would complete a type I restriction system
Ribonuclease_3	Bd1941		Bd1934 – SNase but not co-transcribed due to opposite direction
ExoIII	Bd1346		Bd1342 – annotated as a DNA alkylation repair enzyme
Rnase_H	Bd2138, Bd3131	Hydrolyses RNA in RNA:DNA hybrids	Bd2140 – polC which is a DNA polymerase subunit
Rnase_HII	Bd2116	Hydrolyses RNA in RNA:DNA hybrids	
Rnase_PH	Bd2700		
RDDEXK_1	Bd3140, Bd3139	Likely involved in DNA recombination repair	
ENDO3C	Bd0591		
DUF3108	Bd1501	Has no known function, no predicted domains to elicit function, possibly misannotated as a nuclease	

3.2.5 Summary of bioinformatics analysis of *Bdellovibrio* hydrolytic enzymes

The hydrolytic enzyme catalogue of *Bdellovibrio spp.* is vast and diverse: having started with the original hydrolytic enzyme list set out in a previous study (Rendulic et al., 2004) and having added some more recently identified genes encoding these enzymes, the 6 hydrolytic enzymes groupings of proteases, DNases, RNases, Glycanases, Lipases and other hydrolases were expanded further into 127 sub-groupings based on functional domains. Combining this analysis with that of previous transcriptomic work allowed for the putative placement of these enzymes within the predatory lifecycle (Lambert et al., 2010; Karunker et al., 2013). Within the proteases, S13 peptidases have been shown experimentally to have roles in initial invasion and formation of the bdelloplast (Lerner et al., 2012) while also being genomically located adjacent to the potential LrgAB 'anti-holin' prey cell lysis regulator. Many of the S8 peptidases and M14 peptidases were predicted to have 'housekeeping' roles during GP or roles in the initial prey invasion stages. Notable among the proteases analyzed in this study was both the abundance of signal peptide sequences directed at Sec transport to the periplasm, and their genome localization near to other transport systems, including ABC transporters which may have roles in exporting many of these enzymes out towards prey, or in importing the products of their protease actions. Similarly, nucleases analyzed in this study were also found localized near to ABC transporter systems and other generalized secretory pathway proteins in addition to other hydrolytic enzymes. This study predicted roles for these nucleases in end stage GP degradation of DNA, as well as during the AP. Analysis of the gene conservation between *B. bacteriovorus* strains HD100 and Tiberius found a greater protease compliment encoded in the HD100 genome. Similarly, many peptidases were shown to be poorly conserved between *B. bacteriovorus* HD100 and *B. exovorus* JSS, with the latter lacking 65 hydrolytic enzymes compared to HD100, these enzymes were hypothesized to be of greater importance in intraperiplasmic predation and of lesser importance for the epibiotic lifestyle exhibited by *B. exovorus*.

4.0 Discussion

4.1 Isolation of novel bacterial predators from the GI tract of animals

4.1.1 Background

The reasoning behind this study was informed by the increasing threat antibiotic resistant *Salmonella spp.* pose for the food industry, in particular pork production, where contaminated food products can lead to human infections and mortality. The additional threat of infections within the animals (pigs) themselves and the impact this has economically and on health and wellbeing of these animals was also all considered. This highlighted the need to develop and investigate alternatives to antibiotics, with *Bdellovibrio bacteriovorus* and other predatory bacteria being proposed as such a solution. The work of Atterbury and co-workers (Atterbury et al., 2011a) laid the ground work for this project, by successfully using *B. bacteriovorus* to reduce *Salmonella* in chicken GI tracts. This study did highlight however, that the effectiveness of the HD100 strain of *B. bacteriovorus* was hindered by the physiology of the chicken gut: varying pH, higher temperature than the optimum for *B. bacteriovorus* and lowered oxygen concentrations all contributed to less effective predation than in the optimum conditions created in a laboratory setting. Therefore, this project set out to find novel *Bdellovibrio* predators that would be isolated from the environment in which they could one day be used prophylactically. During this study samples were taken from the GI tract of pigs as well as the feces of pigs, reindeer and sheep, in the hope that characterizing any resulting isolated bacterial predators could reveal answers to the following questions: Would these novel predators have specialized adaptations to the physiological stresses faced in the GI tract making them distinct from laboratory strains of *B. bacteriovorus*? If these isolates show a level of adaptation, does this mean they are part of the natural flora of that environment and not just transient? Would these novel isolates follow a periplasmic lifestyle or exhibit alternatives methods of predation such as epibiotic (such as seen by *Bdellovibrio exovorus* and *Micavibrio spp*.) or wolfpack (as seen in *Myxococcus spp*.)?

4.1.2 *Bdellovibrio spp*. Rudolphii – a novel predatory isolate

Although a novel isolate which we putatively named *Bdellovibrio spp*. Rudolphii was isolated from reindeer, time constraints as a result of the ongoing COVID-19 pandemic meant the isolate remains to be fully characterized beyond the few initial observations made in this study. The bacteria were observed to grow better in liquid cultures incubated statically rather than in a shaking incubator, with assays of predatory efficiency also showing this. Therefore, the next questions and hypothesis' to investigate would be whether this isolate is capable of tolerating a microaerophilic or anaerobic atmosphere? In the static cultures used in this study, shaking was avoided to avoid the introduction of oxygen, however this could have decreased the chances that a predator would encounter a prey cell, causing it to take longer to clear the prey population in the culture than if the culture was shaken in a microaerophilic environment. It would be interesting to compare the predatory efficiency of *Bdellovibrio spp*. Rudolphii in static and shaken cultures when both are incubated in a microaerophilic environment. The motility of *Bdellovibrio spp*. Rudolphii was observed in these cultures to be erratic, with a small proportion of the bacterial cells seen swimming at any time point, this would also decrease the probability of collisions with prey. However, growth in microaerophilic or anaerobic cabinets would open the opportunity to not only investigate the ability of this isolate to grow at different atmospheric oxygen concentrations, but to see the affect shaking may have on increasing the collisions of predator with prey, possibly decreasing the time taken for the isolate to clear a culture of prey.

4.1.3 Genome sequencing of *Bdellovibrio spp.* Rudolphii

If *Bdellovibrio spp*. Rudolphii was found to efficiently complete predation when incubated in either a microaerophilic or anerobic cabinets, this would allow for sufficient predator cells to be harvested for DNA extraction and subsequent genome sequencing, allowing for both phylogenetic characterization (allowing us to determine whether this isolate is truly a *Bdellovibrio spp*.) and for further analysis of the genomic content which may elucidate the genetic causes for the properties of Rudolphii which make it distinct from lab isolates. If Rudolphii can successfully tolerate the lower oxygen atmospheres during microaerophilic or anerobic growth, one would predict a potential difference in

the electron transport chain and final electron acceptors, which a sequenced genome may provide the answers to. The reduced motility seen in this isolate may also be explained by determining the complement of flagella-related genes, determining whether there is a lack of *fliC* genes (knowing that *B. bacteriovorus* HD100 has 6 homologues of the flagellin gene *fliC* (Lambert et al., 2006a) and three pairs of the flagella motor proteins MotAB (Morehouse et al., 2011) or other integral flagella genes. And if these are present then why is the swimming motility different compared to HD100? Could this isolate predominantly use different motility methods more than HD100, such as gliding (Lambert et al., 2011a)?.

4.1.4 Purification of novel predatory bacteria isolates from the GI tract

The isolation of *Bdellovibrio spp*. Rudolphii has enlightened us on several steps in the purification process which could be improved in future studies for the isolation of GI tract predators. This will primarily account for the fact that predators of the gut have the possibility to be microaerophilic/anerobic and grow more efficiently at temperatures closer to the organism they were isolated from. The original isolation of Rudolphii and other isolates followed the standard procedure for isolating *Bdellovibrio* from aquatic and soil based environments, where 29 ^{o}C shaking incubators were used after plaques of novel predators were picked into 2mL liquid predatory cultures. Before this study, a microaerophilic or anaerobic *Bdellovibrio* isolate had never been reported. Between each purification stage it would be typical to have a purer sample after subsequent rounds of purification, with more plaques of the novel predators on the overlay plates, and less contaminating colonies from other organisms in the culture. In this study however, this was not the result, instead each round of purification would typically result in the same few plaques with a great deal of background GI tract flora still present. Filtering was partly successful in reducing background flora, but growth in a highly oxygenated environment of a shaking incubator may have inhibited novel predator growth due to their potential microaerophilic nature, decreasing the yield of novel predator plaques on overlay plates in subsequent purification rounds, with only the Rudolphii isolate being fully and successfully purified. In future studies, growth in microaerophilic or anerobic cabinets would be preferred to traditional aerobic shaking

incubators to reduce the exposure of these novel predators to oxygen during the early stages of isolation. These isolates would also be incubated at 37 ^{o}C rather than 29 °C which has previously been shown to be the optimum temperature of *B. bacteriovorus*. This change is suggested as Rudolphii was found to have higher viable cell counts during predation at 37 ^{o}C compared with 29 ^{o}C, this is notable as this is closer to the body temperature of reindeer (38 ^{o}C), which was the animal this novel predator was isolated from, providing evidence for potential adaptation to this temperature range.

4.1.5 Quantitative studies of predation

More comprehensive characterization of *Bdellovibrio spp*. Rudolphii and any future novel predatory isolates would be achieved in future studies using more quantitative methods. Important information to understand about these isolates would be their predation efficiency at a wider range of temperatures, within a differing pH range replicating the GI tract environments from which they were isolated as well as their tolerance to pH extremes. Measurement of optical density of prey over time through a plate reader would allow for generation of experimental data about these parameters, plaque assays to measure growth of the predator would also be implemented. More quantitative studies would also allow for determination of the prey range of these isolates: there are known Gram-negative bacteria that are easily preyed upon by *B. bacteriovorus* HD100 which are well documented in the literature, but the microaerophilic nature of these novel predatory isolates could expand the prey range of known *Bdellovibrio* to extend to microaerophilic and possibly even anaerobic pathogens on which *B. bacteriovorus* cannot usually prey upon. These could include pathogens causing food borne disease and impacting on the health & welfare of the animals including *Campylobacter spp., Salmonella spp.* and *Brachyspira spp.* (a causative agent of swine dysentery). For use in therapeutics, the impact *Bdellovibrio* and other novel predators have on the host microflora is also a key factor to consider: many bacteria in the GI tract of pigs and other farm animals are Gram-negative including *E. coli* and *Shigella* spp., therefore they would act as potential targets for predation. Measuring the impact on the reduction of the Gram-negative flora by predators and how this impacts the balance of the gut microbiome would need to considered in future studies, as an

imbalance could lead to overgrowth of Gram-positive pathogens including *Clostridia spp.* leading to potential dysbiosis.

4.1.6 Microscopy studies of predators to determine predatory mechanisms

Microscopy would be valuable for both visualizing motility and determining the nature of the predatory methods these predators adopt. Although the genome would reveal answers about motility at a molecular level, phase-contrast microscopy has already shown that cells of *Bdellovibrio spp*. Rudolphii have reduced motility compared to *B. bacteriovorus* HD100: cells would display low percentage motility, and those cells that did swim did not swim smoothly resulting in cells which moved much slower than *B. bacteriovorus* HD100. Electron microscopy (EM) could reveal morphological details of the flagella structure and then determine whether the isolates are monoflagellate like HD100 or have multiple flagella. Microscopic analysis of the flagella during growth in different conditions, such as pH, temperature, microaerophilic and anaerobic conditions, would help determine if these have any impact on the phenotype of the flagella and the resulting motility of the cells. Fluorescence microscopy where predators are grown with fluorescently-labelled prey would allow for the determination of predatory lifestyle, showing whether these cells are periplasmic predators like *B. bacteriovorus,* epibiotic like *B. exovorus* or secrete enzymes in a 'wolf-pack' method like *Myxococcus spp*.. Rudolphii cells in this study were seen to be attached to prey cells, but as this is a snapshot of predation, no assumptions can be made about this being evidence of epibiotic predation, as this could be the initial engagement of Rudolphii with a prey cell before potentially entering the prey cell to complete periplasmic predation. It was also observed in this study that rounded bdelloplast-like structures in Rudolphii cultures did not have Rudolphii-like cells attached to them.

4.1.7 Future studies

If given more time beyond the remit of the MRes there are several experiments which would be integral for gaining more knowledge into the effectiveness of Rudolphii as a novel therapeutic in the future. This would include recreating the *in vivo* conditions of the reindeer gut in the lab, simulating correct pH, temperature and oxygen levels,

allowing for analysis of Rudolphii in its 'natural setting' by measuring predator and prey numbers during the course of predation. Similarly, using an *ex vivo* approach using GI tracts from animals such as pigs (the GI tract of pigs can be easily obtained from commercial abattoirs as it is a waste product of the slaughtering process). The studies with *ex vivo* gut sections would allow for analysis of predation by *Bdellovibrio spp*. Rudolphii in a mixed environment, by introducing them alongside the Gram-negative prey (for an infection model) *Salmonella*, into the naturally occurring microbiome within the GI tract. The natural microbiome would include other Gram-negative species as well as Gram-positive species, this would allow us to gain insight into the effect predation could have on the microbiome including the potential overgrowth of the naturally occurring Gram-positive bacteria, but to also understand if these non-prey organisms could also interact with the predators directly acting as decoys, as has been previously described for *B. bacteriovorus* with *E. coli* and *B. subtilis* (Hobley, King and Sockett, 2006).

4.1.8 Role of Predatory Bacteria in the Gut

An important question for predators which become resident in the gut microflora rather than transiently passing through the GI tract, is why are they there and what role do they play in animals and humans? One theory is *Bdellovibrio* species act as a 'balancer' within the gut, preventing an imbalance of flora known as dysbiosis. In humans with inflammatory bowel diseases (IBD) and coeliac disease, patients were found to have reduced numbers of *Bdellovibrio* and increased levels of gut microbiota (Iebba et al., 2013). The role of *Bdellovibrio* appears to be similar to predators in the animal kingdom, where predators are required to keep the prey numbers in check. Their presence in the reindeer GI tract may also follow the patterns seen in humans, with predators keeping potentially pathogenic prey under control to maintain the microflora balance, preventing dysbiosis and potential disease.

Although not fully characterised, the adaptations that have been observed so far in *Bdellovibrio spp*. Rudolphii, including a preference for microaerophilic environments and a growth temperature closer to the reindeer core body temperature, all point towards a predator which has evolved to cope with the physiological stresses in the GI tract that

commonly used laboratory strains such as *B. bacteriovorus* HD100 were unable to overcome, allowing *Bdellovibrio spp*. Rudolphii to become more than just another transient predator passing through.

4.2 Bioinformatics of the hydrolytic enzymes in *B. bacteriovorus* HD100.

4.2.1 Background

The hydrolytic enzyme catalogue of *B. bacteriovorus* HD100 was first analyzed and categorized back in 2004 when the first genome was sequenced (Rendulic et al., 2004). 293 hydrolytic enzymes were identified and were initially grouped by catalytic type and general function. The degree of accuracy to which these enzymes were identified and grouped was greatly limited by the technology of the time. 17 years later, there is greater availability of more accurate and comprehensive genomes for *B. bacteriovorus*, highlighting new enzymes which Rendulic and co-workers previously missed. Additionally, many of these enzymes were annotated incorrectly due to the limitations of bioinformatics software at the time. This data was however, a good entry point for probing the hydrolytic arsenal of *Bdellovibrio*. Previous comprehensive studies sought to understand the transport systems in *Bdellovibrio*. These studies identified transporters of many types including ABC transporters and the presence of Sec and Tat transporters which are integral for *Bdellovibrio* proteins to reach the periplasm and beyond (Barabote et al., 2007) Further study compared the transport systems of *B. bacteriovorus* HD100and *B. exovorus* JSS, determining transporters which may be more essential for the periplasmic lifestyle of *B. bacteriovorus* (Tajabadi et al., 2018). Beyond these, transcriptomic studies paved the way for individual molecular study, where expression data began to create a picture of when these enzymes act in the predatory lifecycle (Lambert et al., 2010; Karunker et al., 2013). Study of these enzymes has not only sparked interest to understand them in the context of the predatory lifecycle, but also to provide insight for their potential usage as a biotechnological tool (Bratanis et al., 2017; 2020; Bratanis and Lood, 2019).

The hydrolytic enzyme arsenal of *Bdellovibrio* is however incredibly vast, making systematic molecular studies of all of them not a sustainable approach for further

understanding. Instead, this study set out to further group the enzymes based on domain structure and to combine this with previously published transcriptomic data to elicit both enzyme function and role in the *Bdellovibrio* lifecycle to direct future molecular studies.

4.2.2 Proteases

4.2.2.1 S13 peptidases

The S13 peptidases are an excellent example of how transcriptional studies can guide future molecular studies (Lambert, Ivanov and Sockett, 2010; Lerner et al., 2012) Based on transcriptional data, both Bd3459 and Bd0816 would be predicted to have roles very early in the predatory process, due to the expression of both of these at 30 mins post infection of prey. The expression profile, combined with the known function of the S13 peptidases, suggested that both of these enzymes could play a role in cleaving the D-ala-D-ala bonds of peptidoglycan in the prey periplasm during the establishment of the bdelloplast. The third S13 peptidase, Bd3244, did not have altered expression 30 minutes after the onset of predation, suggesting that this enzyme was likely to be responsible for the maintenance of the *Bdellovibrio* cells own peptidoglycan. Molecular studies by Lerner and colleagues (Lerner et al., 2012) confirmed these predictions, showing that both Bd0816 and Bd3459 were responsible for the cleavage of the prey peptidoglycan resulting in the rounding up of the prey cell. In the same region of the genome to Bd3459, both Bd3458 and Bd3457 form the part of the LrgAB operon. Initial evidence from this study suggests that LrgAB proteins in *Bdellovibrio* are membrane localised. Interestingly, LrgA has sequence similarity with bacteriophage murein hydrolase-like transporter protein family proteins known as holins. Holins are lytic enzymes capable of degrading the cell wall of infected cells in preparation for the final stages of lysis and are controlled by anti-holin proteins to regulate the process (Brunskill and Bayles, 1996; Wang, Smith and Young, 2000). It has been previously shown in *Staphylococcus aureus* that due to high hydrophobicity of the LrgA and LrgB proteins, neither are hydrolases themselves but instead regulate the activity of murein hydrolases; increased hydrolase activity was detected in ΔLrgAB cells, while these levels returned to WT levels when complemented with complete copies of LrgAB (Groicher et

al., 2000). These findings indicates a role for LrgAB as antiholins. Other studies have implicated the Cid operon with regulating the *lrgAB* operon, with CidR acting as a positive regulator and CidA acting as a potential holin protein (van den Esker, Kovács and Kuipers, 2017; Claunch et al., 2018).

Lrg/Cid operons may also have a role in pyruvate uptake, possibly overlapping with the lytic functions (van den Esker, Kovács and Kuipers, 2017; Ahn et al., 2019). Although much of what is currently known about the LrgAB system has been determined in Gram-positive bacteria, it is possible *Bdellovibrio* may have similar or even more specialised functions for LrgAB directed to the predation process, by having possible involvement in the final lysis of the bdelloplast to release new progeny. It is unlikely however, that Bd3457 and Bd3458 are directly related to Bd3459 for murein hydrolase activity, but may all play a combinatory role in a holin/anti-holin system.

4.2.2.2 S8 peptidases

Notable in this domain type were the protease genes upregulated during AP and subsequently downregulated after 30 mins of predation, these included Bd1283, Bd2545. The exopeptidase and endopeptidase of these proteases could be important for initial invasion, breaking the peptide-cross linking in the prey cell wall. The presence of SPI signal peptides in both of these enzymes shows that both are at least transported to the *Bdellovibrio* periplasm, Bd2545 is likely to remain in the cytoplasmic membrane of the *Bdellovibrio* cell based on its domain structure, whilst Bd1283 may then be further transported into the prey cell. The function of Bd2545 also seems to play a dual role, with the PilZ domain also playing an important role with cyclic-di-GMP signaling, with Bd2545 potentially playing a role in the lifestyle switch from the highly motile cells seen in AP to growing and replicating within prey. An interesting observation in the S8 domain types is the co-localisation of other hydrolytic enzymes with them on the chromosome. For example, Bd1432 was found near one of the SNases Bd1431, and Bd1283 is located near an SLT domain containing enzyme (Bd1285). Many of the S8 proteases are also located close to transport system components; whilst all but three of the S8 proteases contain Sec-transport signal sequences directing these enzymes to the

Bdellovibrio periplasm where they may then be targeted by other transporters such as the ABC transporter located near Bd0449 and Bd1432.

4.2.2.3 M14 Peptidases

Of particular interest in this category was Bd3508. Bd3508 appears to be an enzyme involved in cell maintenance in attack phase and/or growth and replication of the *Bdellovibrio* because of its downregulation after 30 mins, but its high levels of expression during AP and GP. The presence of an SPI signal peptide suggests this protein is secreted into the *Bdellovibrio* periplasm. As carboxypeptidases function to remove the second alanine from D-ala-D-ala sequences found in new peptidoglycan chains, therefore Bd3508 could play a role in the maintenance of the *Bdellovibrio* peptidoglycan during attack phase, stabilizing the cellular structure during starvation, as well as being used in the creation of new peptidoglycan in the filamentously-growing *Bdellovibrio* cell in the bdelloplast.

4.2.3 Nucleases

4.2.3.1 SNase-containing nucleases

An interesting observation about the two enzymes is that despite having roles in both GP and AP for Bd1431 and Bd1934 respectively as described by the transcriptomic data, however gene deletion of Bd1934 resulted in no detectable phenotype (Lambert and Sockett, 2013), showing the potential redundancy of these enzymes. Both have predicted signal peptide sequences to reach the *Bdellovibrio* periplasm via the Sec-transport system, before possible transport into the prey periplasm or the external milieu where they would either act on the prey cell DNA or on extracellular DNA.

4.2.3.2 Endonuclease_1

All three endonuclease 1 containing enzymes appear to be involved in predation, with Bd1244 and Bd3507 having roles in AP. Further studies found similar results with conflicting reports of peak expression at 30 mins or 1 hr for Bd0934 and Bd3507 (Lambert and Sockett, 2013; Bukowska-Faniband, Andersson and Lood, 2020). These enzymes are also a great example for the redundancy of many *Bdellovibrio* enzymes, as

gene deletions of Bd0934 and Bd3507 had little effect on predation efficiency (Bukowska-Faniband, Andersson and Lood, 2020), with other nucleases likely being upregulated to maintain function, as is seen in flagella genes (Lambert et al., 2006a). All three endonuclease 1 containing proteins also contain a predicted sec secretion signal peptide, indicating that all are likely secreted out of the *Bdellovibrio* cytoplasm, suggesting that they will function outside of the *Bdellovibrio* cell, either acting on the prey DNA or on extracellular DNA such as that found in the biofilm matrix of many bacterial species. Thus, the lack of a detectable phenotype (Bukowska-Faniband, Andersson and Lood, 2020) may be that these enzymes act on extracellular DNA, and thus may have a role in degrading DNA in a biofilm matrix and are required for the *Bdellovibrio* to gain access to otherwise protected cells within a biofilm structure.

4.2.3.3 Exo_endo_phos

Bd1711 is the only enzyme containing the Exo_endo_phos domain to be upregulated during attack phase in *B. bacteriovorus*. As this is not upregulated during GP, or at 30 mins, it is possible that the activity of this enzyme is important in swimming attack phase cells prior to prey location and entry. If Bd1711 follows the general function of this domain type, then it is possible Bd1711 plays a role in DNA repair of these motile cells, or even in cell signaling preparing the cell to encounter new prey cells. The remaining 4 Exo_endo_phos enzymes in *B. bacteriovorus* appear to have a less important predatory role, being more involved in general *Bdellovibrio* 'housekeeping' duties, due to their upregulation during GP, suggesting they are required for growth and replication of the *Bdellovibrio* cell.

4.2.4 Conservation of genes in *B. bacteriovorus* HD100, Tiberius and *B. exovorus* JSS

The number of enzymes which are unique to HD100 and not found in Tiberius, another strain of *B. bacteriovorus,* was unexpectedly high. The partial HI lifestyle of Tiberius has made it more distinct than HD100; the higher prevalence of unique enzymes to HD100 originating from the proteases grouping elicits functions for these to be essential for acquiring nutrients from the prey periplasm, suggesting intraperiplasmic growth specificity for these enzymes. The downregulation or unchanged levels of

expression of many of these enzymes in HI strains of HD100 is also very compelling for the redundancy of these enzymes in HI lifestyles.

The high level of conservation between Bdt0871 and HD100 enzymes containing the DJ-1_Pfpl domain was interesting, as although there is conflicting literature about conserved regions and active site regions for enzymes with this domain, there are clear motifs and conserved regions which could act as active site binding pockets in these enzymes including the GXYXSE motif which was found. As this is present in both Tiberius, an intraperiplasmic predator that also can grow host-independently (without the need for a genetic mutation) and HD100, a true intraperiplasmic predator, this enzyme type must be essential to general predation and growth of both strains of *Bdellovibrio*. Similarly, the conservation of Bdt1340 with Bd1444, Bd2269 and Bd3238 follows a similar narrative. Strictly conserved active site pockets suggest these are integral to S8 enzyme function regardless of lifestyle.

The enzymes missing from *B. exovorus* which are highly expressed in attack phase cells of HD100 may be involved in preparing the *Bdellovibrio* cell for encountering a prey cell, or in the intial stages following contact with a prey cell, but as there was no change of expression seen at 30 mins post infection for many of these enzymes it is difficult to determine the precise timings of expression without full transcriptomic data at more timepoints during these early stages of predation. Additonally, the absence of these enzymes in *B. exovorus* shows their redundancy in epibiotic predation and possibly hints at a more integral role to an intraperiplasmic lifestyle. Similarly missing from *B. exovorus* is the corresponding enzymes which make up the LrgAB potential antiholins. In the epibiotic lifestyle, there is no jeopardy to the *Bdellovibrio* cell if prey cell lysis occurs as it is attached to the outside of the prey cell, therefore antiholins such as LrgAB which potentially regulate murein hydrolases would have a redundant role. Whereas, predators with an intraperiplasmic lifestyle would highly benefit from regulated murein hydrolase degradation of the prey cell wall, leaving them isolated in the periplasm in a favourable environment for *Bdellovibrio* growth.

4.3 Discussion summary and future study

The *Bdellovibrio spp.* Rudolphii isolates found in this study exemplifies the promise that *Bdellovibrio* isolated from the GI tract of farm animals have the potential to exhibit growth and good predation efficiency in microaerophilic environments, which has previously not been reported in any published literature. If more isolates like Rudolphii can be found and characterized, they would be much more appealing than the routinely used lab strains HD100 and 109J for testing the therapeutic potential of *Bdellovibrio* in more challenging environments *in vivo* such as the GI tract where there is not strict aerobiosis. Microaerophilic *Bdellovibrio* isolates in future studies could be assessed for predation on Gram-negative prey which currently used *Bdellovibrio* strains cannot prey upon, such as *Campylobacter,* as these favor an anoxic environment. If *Bdellovibrio* could be shown to prey upon *Campylobacter* for example this would be very advantageous not only as a therapeutic in human disease, but as a biocontrol agent of *Campylobacter* in farm animals which could prevent entry of *Campylobacter* into the food industry.

Potential predatory candidate genes which have been identified in this study could be new targets for molecular study, by introducing mutations into the genes of these enzymes to elicit a potential phenotypic change in predation for further understanding of their function. Alternatively, with a higher available budget, a comprehensive RNA-seq experiment would be very favorable for a more complete understanding of where these enzymes are expressed at each stage of the predatory cycle. Currently, transcriptomic data for these enzymes only confirms expression during AP, GP, HI growth or at 30 mins where GP is only represented as the 3 hrs timepoint. As the intracellular growth phase lasts for approx. 4 hours, RNA-seq data would fill the underrepresented time points between 30 mins and 3 hrs where enzymes may be expressed in this 2-3 hour window of predation and is currently not accounted for in the available data. Additionally the time point of 3 hrs to 4 hrs during the final stages of the predatory lifecycle before prey cell lysis is also not represented in the current published data. This time point is a major event in the life cycle of *Bdellovibrio* and likely involves

many of the hydrolytic enzyme arsenal. Further understanding of the key enzyme players in prey cell lysis are of great importance as these could form the basis for therapeutic treatment as antibiotic candidates in the future.

Together, these two parts of this study have added to our knowledge of the diversity of bacterial predators, both in their tolerance to different environmental conditions, and also the diversity in the proteins they encode and use during their predatory cycle. Both sections of this project have the potential to lead to further investigations that should help move the *Bdellovibrio* field further towards the use of *Bdellovibrio*, and their products, as alternative antibacterial therapeutics.

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6.0 Appendices

Appendix A – Subgrouping tables for proteases, nucleases, glycanases & lipases and other hydrolases

Table A1 – New subgroupings for Proteases based on functional domain (Red), including associated domains with each subgrouping and equivalent enzymes (with matching domain combinations) in *B. exovorus*, signal peptides are also noted (blue)

Key Domain sub-grouping	Additional domains of note	Proteins (*B. bacteriovorus* HD100)	Proteins (*B. exovorus*)
Peptidase_S8		Bd1283, Bd1432, Bd2428, Bd3087, Bd3857, Bd0449, Bd0521, Bd2321, Bd2832	A11Q_432, A11Q_1642, A11Q_1951, A11Q_1429, A11Q_2559, A11Q_284, A11Q_434, A11Q_1028, A11Q_2588
	PA PilZ CUB Bid_2 P_Proprotein	Bd2269, Bd3238, Bd1444 Bd2545 Bd0029 Bd2692 Bd0376	A11Q_1053, A11Q_946 A11Q_26 A11Q_1794,
Peptidase_S24		Bd0853	A11Q_743, A11Q_744
	Peptidase_S26 LexA_DNA_Bind	Bd0852, Bd0854 Bd3511	A11Q_742, A11Q_461
Peptidase_S49	CLP_protease S49_N, Coiled coil	Bd1067 Bd2188	 A11Q_1399
Peptidase_S9	S9_N AAA, BCA_ABC_TP_C, ABC_Tran PD40, peptidase_S15, DLH, Abhydrolase_6 DUF818, Hydrolase_4, Abhydrolase_6, Abhydrolase_1 DLH, Abhydrolase_2	Bd3175, Bd3466 Bd3388 Bd2519 Bd1031 Bd0982	A11Q_1061 A11Q_856
Peptidase_S13		Bd0816, Bd3244, Bd3459	A11Q_2041
Peptidase_S41	PDZ, TSPc PDZ, TSPc, coiled couil, DUF3340	Bd0169, Bd1239, Bd3534 Bd0967	A11Q_187, A11Q_517, A11Q_2245 A11Q_791

Peptidase_M24	AMP_N	Bd0649, Bd1338 Bd0202, Bd1372	A11Q_880, A11Q_1611, A11Q_136, A11Q_1149
Peptidase_M14	Zn_pept Zn_pept, DUF2817, AstE_AspA AstE_AspA AstE_AspA, DUF2817 DUF2817	Bd0306, Bd3234 Bd3426 Bd1776 Bd3508 Bd2418	A11Q_2300 A11Q_1444, A11Q_2038, A11Q_240 A11Q_1128. A11Q_463,
Peptidase_M10		Bd1265	A11Q_2167
	ZnMc	Bd0277	A11Q_1652
Peptidase_M13	M13_N, Coiled coil	Bd3547	
Peptidase_M20	Peptidase_M28 Peptidase_M42, M20_Dimer M20_Dimer	Bd3622 Bd2900 Bd0129	A11Q_1052, A11Q_1878, A11Q_1117
Peptidase_M22		Bd0636, Bd3788	A11Q_2360
Peptidase_M23		Bd2006, Bd3771	A11Q_128
	Coiled coil	Bd0398, Bd0168	A11Q_2465. A11Q_1218, A11Q_1327
Peptidase_M17		Bd2554	A11Q_188, A11Q_1180, A11Q_2212
	M17_N	Bd3755	A11Q_942
Peptidase_M15		Bd2654	A11Q_180
Peptidase_M48		Bd1287, Bd2798, Bd2068	
Peptidase_M1		Bd1518	A11Q_793, A11Q_792, A11Q_326
	DUF3458, DUF3488_C	Bd2521	
Peptidase_M3	M3_N	Bd3171 Bd3704	A11Q_952 A11Q_2001
Peptidase_M16	M16_C	Bd1552, Bd3869, Bd1077, Bd1078	
Peptidase_M4		Bd1084	A11Q_1547, A11Q_2572
Peptidase_M41	AAA AAA, FtsH_ext	Bd1928 Bd2667	A11Q_1373
Peptidase_M50		Bd1975	
	PDZ	Bd3787	A11Q_1307,
Peptidase_M74		Bd1032	A11Q_1338
Peptidase_S66		Bd1950	A11Q_1130
Tryp_SPc		Bd2332, Bd2923, Bd3481, Bd0706, Bd1043, Bd1898,	A11Q_1355

		Bd3482, Bd2334, Bd0564, Bd2535, Bd0629, Bd2627, Bd2630, Bd3415, Bd0994, Bd1541	
	Peptidase_S46	Bd1962	A11Q_2288, A11Q_1200, A11Q_137, A11Q_669, A11Q_486, A11Q_2385
Peptidase_A8		Bd3365	
	DiS_P_DiS	Bd0862	A11Q_2042, A11Q_1085, A11Q_2117
LON	AAA, Lon_C, Coiled coil AAA, Lon_C, coiled coil, Chll	Bd2144 Bd3749, Bd3876	
Lon_C	AAA, Chll, DnaB_C, ATPase	Bd2712	A11Q_2579, A11Q_176, A11Q_459
LON_Sybstr_bdg		Bd2218	
Transgly & Transpeptidase	S1, PCB_OVB BiPBP_C Coiled coil	Bd0160 Bd0297 Bd3351	A11Q_157,
Transgly		Bd2847, Bd0141	A11Q_585
Transpeptidase		Bd3522	A11Q_2333
	PBP_dimer	Bd2460	A11Q_2242
Peptidase_C1		Bd1427	A11Q_2030
	Peptidase_C1_2	Bd1649	
Peptidase_C15		Bd0879	A11Q_1024, A11Q_980
PmbA_TldD		Bd0699, Bd0958, Bd0700, Bd0959	A11Q_939
ClpB_D2-Small	zF-C4_ClpX, AAA, RuvB_N AAA, coiled coiled, Clp_N	Bd3753 Bd1288	A11Q_666, A11Q_667 A11Q_178
CLP_Protease		Bd3754	A11Q_1934, A11Q_1640 (without coiled-coil)
pfam: DJ-1_PfpI		Bd1521, Bd3678	A11Q_179
PHB	Band_7 Band_7, Band7_C	Bd2675 Bd2304	A11Q_2474 A11Q_553
Peptidase_U32	DUF3656 U32_C	Bd0923 Bd2328	
SpecificRecomb		Bd2271	
NfeD		Bd2305	A11Q_1243
RDD		Bd3273	
TGc	DUF3488	Bd3883	A11Q_1054, A11Q_1660, A11Q_80

G_glu_transpept		Bd3478	
DUF3750		Bd0758	
Glycos_transf_2	Glycos_transf_2_3	Bd2270	A11Q_3436
Thioredoxin_8	Redoxin, AhpC-TSA	Bd2272	A11Q_1471
YtkA		Bd2273	A11Q_1885
Aminopep		Bd0755	
DUF45		Bd3704	A11Q_688
Acyl_transf_1		Bd3096	A11Q_1231
Abhydrolase_1, Abhydrolase_6, Hydrolase_4		Bd0282, Bd3128, Bd0910	A11Q_1321
	Ndr FSH1 DUF1057	Bd3480, Bd2017, Bd3462 Bd0283 Bd0873	A11Q_293 (+UPF0227)
Peptidase_S11	Beta-lactamase2	Bd2044	
Peptidase_M28	PD40, PA, PDZ	Bd2053	A11Q_1307, A11Q_1117
Trypsin	Trypsin_2	Bd0922, Bd2800	A11Q_129
~Misc~		Bd2268, Bd2079, Bd3378, Bd2674, Bd1271, Bd2274, Bd2275, Bd0751, Bd0754, Bd1171	

Table A2 – New subgroupings for nucleases based on functional domain (Red), including associated domains with each subgrouping and equivalent enzymes (with matching domain combinations) in *B. exovorus*, signal peptides are also noted (blue)

Key Domain sub-grouping	Additional domains of note	Proteins (*B. bacteriovorus* HD100)	Proteins (*B. exovorus*)
Endonuclease_1		Bd0934, Bd3507, Bd1244	A11Q_480
ABC_Tran		Bd2442, Bd0159	A11Q_987, A11Q_156
S1	Rnase_E_G CSP, RNB, OB_RNB	Bd3851 Bd0423	A11Q_504
GIYc	GIY-YIG GIY-YIG, Uvr, UvrC_HhH_N, HHH_5	Bd0254 Bd2311	A11Q_1037
Exonuc_VII_L	tRNA_anti_2, tRNA_anti-codon, coiled coil	Bd0197	A11Q_2304
Exonuc_VII_S		Bd0198	A11Q_2304
SNase		Bd1431	
	SNc	Bd1934	
Exo_endo_phos		Bd3524, Bd3670, Bd2451, Bd1711, Bd3586	A11Q_2426, A11Q_2243, A11Q_354, A11Q_1192, A11Q_521
TatD_Dnase		Bd1042	A11Q_828, A11Q_1014

DUF3108		Bd1501	A11Q_923
DHH	DHHA1	Bd2232	A11Q_1419
Methylase_S		Bd3695	A11Q_225 (+coiled coil)
Ribonuclease_3	Ribonuclease_3_3, DSRM, RIBOc	Bd1941	A11Q_1362
ExoIII	Rnase_T	Bd1346	A11Q_2520 (+DUF5051), A11Q_2043
Rnase_H		Bd2138	A11Q_1982
	Cauli_VI	Bd3131	
Rnase_HII		Bd2116	A11Q_1260
Rnase_PH	Rnase_PH_C	Bd2700	A11Q_1799
PDDEXK_1		Bd3140	A11Q_1988
	UvrD_C, UvrD-helicase, AAA, AAA_19	Bd3139	A11Q_1987
ENDO3C	HhH1, FES, HhH-GPD, ENdoIII_4Fe-2S	Bd0591	A11Q_2092

Table A3 – New subgroupings for glycanases and lipases based on functional domain (Red), including associated domains with each subgrouping and equivalent enzymes (with matching domain combinations) in *B. exovorus*, signal peptides are also noted (blue)

Key Domain sub-grouping	Additional domains of note	Proteins (*B. bacteriovorus* HD100)	Proteins (*B. exovorus*)
SLT		Bd2462, Bd0529, Bd3243, Bd1285, Bd3575, Bd3073, Bd0314	A11Q_562, A11Q_2040, A11Q_2160, A11Q_1263, A11Q_1641, A11Q_1805, A11Q_1098
	TPR_8 TPR_16, TPR_16 LysM (x3) SBP_bac_3	Bd1124 Bd2711 Bd1125 Bd3421	A11Q_890,
PLDc		Bd0448, Bd1516, Bd2389	A11Q_1018
Lipase_GDSL	Lipase_GDSL_2	Bd0340	A11Q_2180
Coesterase	Abhydrolase_3	Bd0664	
Esterase		Bd1622	
	Abhydrolase_2, FSH1, DLH	Bd3289	A11Q_767
LrgB		Bd3457	
Pfam 3D		Bd0519	
Abhydrolase_2		Bd3533	
	FSH1	Bd3206	A11Q_2022

Abhydrolase_1		Bd1121 Bd3609	A11Q_1227
Abhydrolase_6	Abhydrolase_1	Bd0737	A11Q_485, A11Q_28
Abhydrolase_4 & Abhydrolase_6		Bd1415	A11Q_293?

Table A4 – New subgroupings for other hydrolases based on functional domain (Red), including associated domains with each subgrouping and equivalent enzymes (with matching domain combinations) in *B. exovorus*, signal peptides are also noted (blue)

Key Domain sub-grouping	Additional domains of note	Proteins (*B. bacteriovorus* HD100)	Proteins (*B. exovorus*)
Metallophos		Bd3188, Bd2508	A11Q_2006, A11Q_1759, A11Q_683, A11Q_800, A11Q_66
	Metallophos_2	Bd0503, Bd3503, Bd3662	A11Q_2486
HDc *10 not 9	HD	Bd1426, Bd0407	A11Q_275, A11Q_2200,
	HD, HD_5	Bd2325, Bd2421, Bd1817	A11Q_992, A11Q_1025,
	DUF3552, coiled coil	Bd2166	A11Q_1384,
	tRNA_anti-codon	Bd3117	A11Q_1969
	DUF3552, KH, coiled coil, HD, KH-1	Bd1188	A11Q_1701
	RelA_SpoT, TGS, ACT_4,HD_4	Bd1570	A11Q_1536
	NTP_transf_2, GlnD_UR_Utase, HD	Bd1955	
	7TNR-HDED, 7TM-7TMR_HD, HD	Bd1817	
CN_Hydrolase		Bd1278, Bd2279	A11Q_2522, A11Q_2507, A11Q_777, A11Q_1644
Aamy	Aamy_C Malt_amylase_C	Bd1224 Bd2279	
Patatin		Bd0201, Bd1999, Bd2083, Bd3882,	A11Q_2581, A11Q_1329
NUDIX		Bd0654, Bd3179, Bd2755	
	NUDIX_4	Bd0714, Bd2220	A11Q_1415, A11Q_673
	Pfam: dCMP_cyt_deam_1, MafB19-deam	Bd0236	

Lactamase_B		Bd2896, Bd1301	A11Q_1847, A11Q_583, A11Q_266
	Lactamase HAGH Lactamase_B_3, Lactamase_B_2 Beta-Casp, RMMBL, Lactamase_B_2, Lactamase_B_6	Bd3353 Bd2204 Bd2858, Bd0901 Bd2036	A11Q_1683, A11Q_280
Lactamase_B_2		Bd2387	
FAA_Hydrolase		Bd0294	A11Q_455, A11Q_1657
Arginase		Bd1812, Bd3436	A11Q_2237, A11Q_2171, A11Q_500, A11Q_1075
	Coiled coil	Bd0356	
GTP_Cyclohydro2	GTP_CH_N DHBP_Synthase	Bd1533 Bd3038	A11Q_1751
dUTPase		Bd1553	A11Q_1546
FGase		Bd1603	A11Q_1515
AcetylCoA_hydro	AcetylCoA_hydro_C, CitF	Bd1620	
4HBT		Bd1827	A11Q_2390, A11Q_1954, A11Q_1380, A11Q_1080
TsaE		Bd1933	A11Q_1368
RF-1		Bd2164	A11Q_83, A11Q_1298, A11Q_913
GTP_cyclohydro1		Bd2522	A11Q_951
Amidohydro_1	Amidohydro_3	Bd2688 Bd2721	A11Q_496 A11Q_1813, A11Q_1787,
Amidohydro_3		Bd3303	A11Q_625
THF_DHG_CYH	THE_DHG_CYH_C	Bd3295	A11Q_632
MGS	AICARFT_IMpChas	Bd3002	A11Q_748?
Abhydrolase_1 & Abhydrolase_6		Bd0030, Bd1192	A11Q_28, A11Q_485
	Esterase	Bd3483	
DLH	DUF1100, BAAT_C	Bd3689	
Glyco_hydro_3		Bd0146	A11Q_145
Quef		Bd0087	A11Q_2018
HAD		Bd0205	A11Q_105, A11Q_2298
CDH		Bd0518	

HIT	DspS_C DcpS_C, Cwfj_1	Bd0570 Bd1069	A11Q_2099 A11Q_839
Fer4_15 & Fer4_13		Bd0590	A11Q_2093
CBAH		Bd0665	A11Q_1682
VWA		Bd0767	A11Q_887, A11Q_886
Hydrolase_3		Bd0963	A11Q_788, A11Q_1774
Hydrolase_2		Bd0992, Bd2766	
CBM_48	DUF3372	Bd1228	
AdoHcyase	AdoHycase_NAD, NAD_binding_7, VHP, THY, Cyt_BS, CarD_TRCF, ELFV_dehydrog_NAD, 2-HACID_dh_C, AlaDh_PNT_C, NAD_Binding_7, LLvN, TrkA_N, DIRP	Bd1339	A11Q_1610
UPF0054		Bd1487	A11Q_905
Hydrolase	E1-E2_ATPase E1-E2_ATPase, HMA, Hydrolase_3 HAD_2, HAD, Hydrolase_like HAD_2, Hydrolase_like HAD_2	Bd2297, Bd2609 Bd2224 Bd0379 Bd2040 Bd2289	A11Q_1221, A11Q_1716, A11Q_656, A11Q_1866 A11Q_2222, A11Q_1202, A11Q_1138,
HAD_2		Bd3427	A11Q_724, A11Q_2163, A11Q_221
Misc		Bd0304, Bd1072, Bd2417, Bd0982, Bd1094, Bd2476, Bd2706, Bd2777	N/A

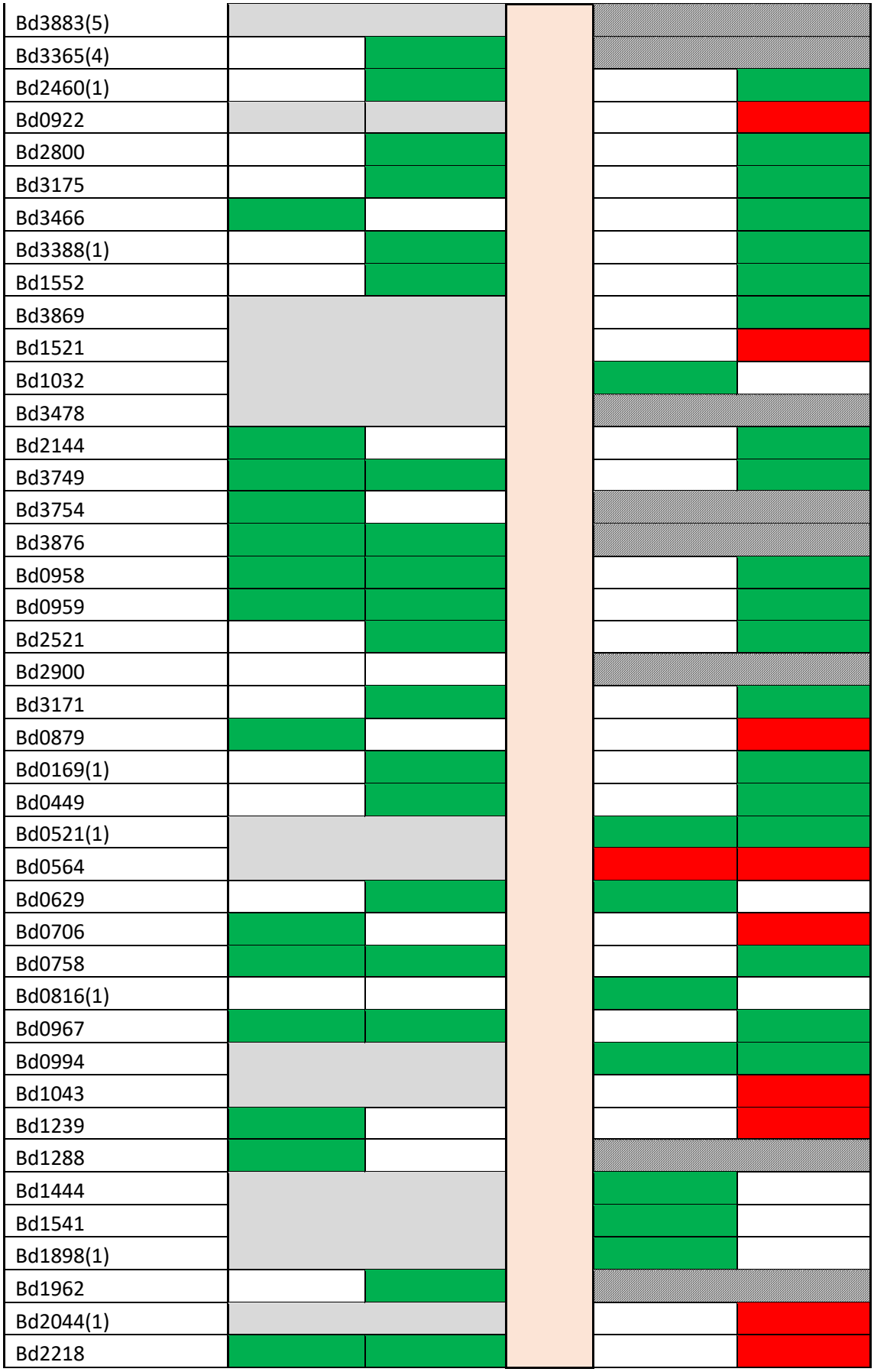
Appendix B – Transcriptomic data extrapolated from supplementary data from (Lambert et al., 2010; Karunker et al., 2013).

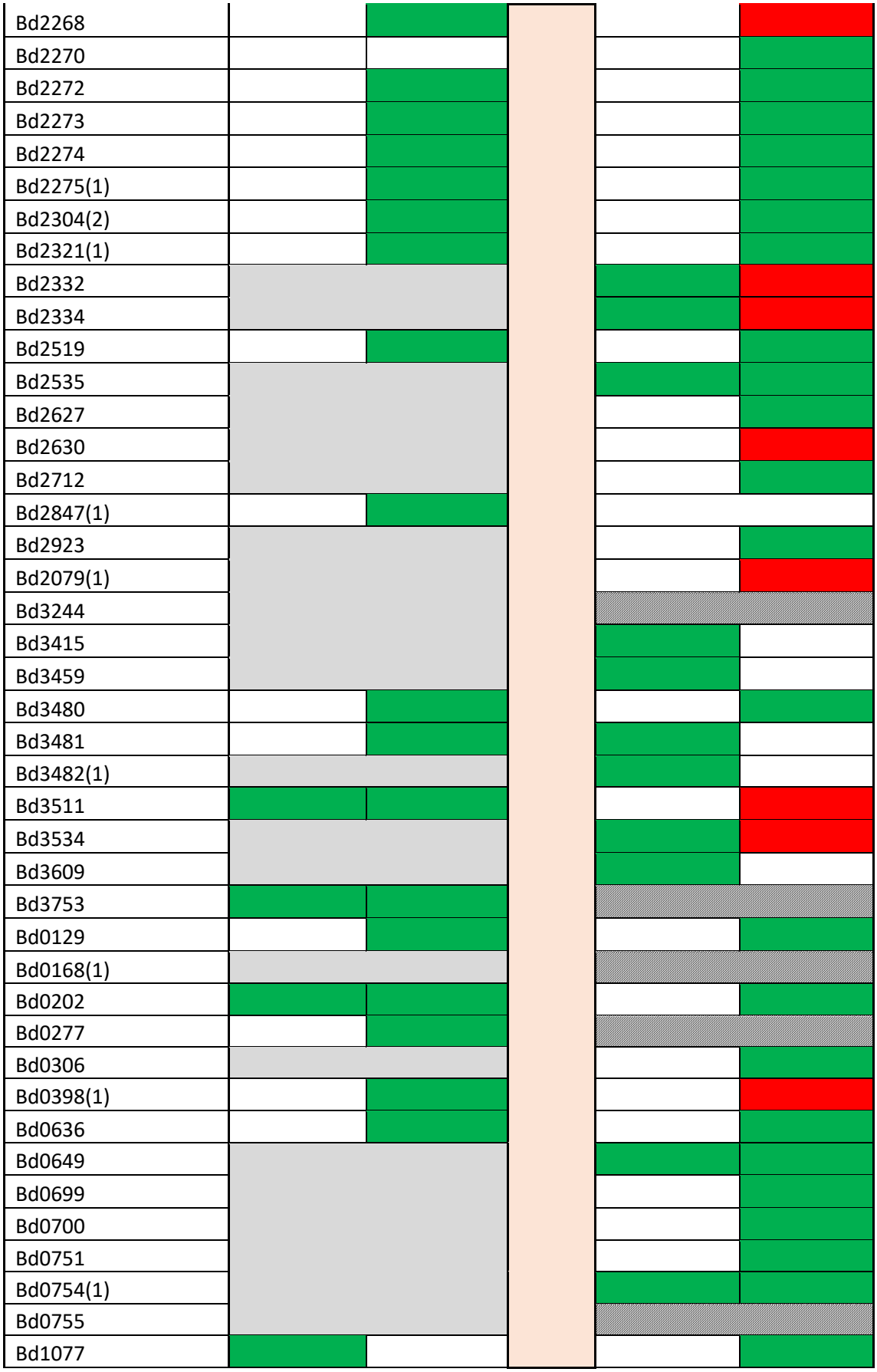
Table B1 – Key for transcriptional data

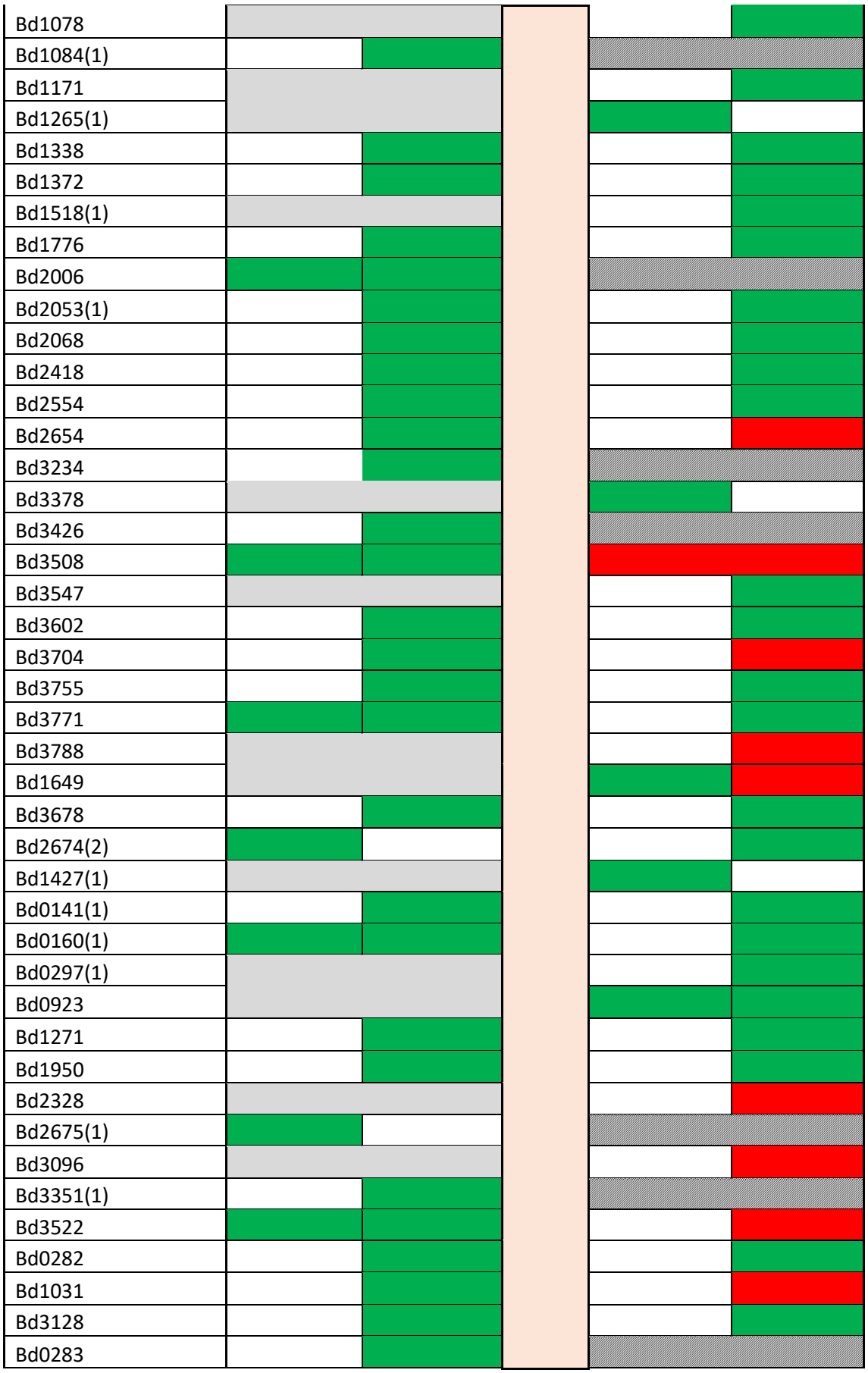
Key	
	Upregulated
	Silent
	Downregulated
	No Change

Table B2 - Proteases

	Karunker		Lambert	
	AP	**GP**	**30 mins**	**HI**
Bd0029				
Bd0376				
Bd1283(1)				
Bd1432				
Bd2269				
Bd2428				
Bd2545(2)				
Bd2692				
Bd3087				
Bd3238				
Bd3857(1)				
Bd0852(1)				
Bd0853(1)				
Bd0854(1)				
Bd1067(1)				
Bd2188(1)				
Bd2271(6)				
Bd2305(6)				
Bd3622				
Bd1287(2)				
Bd1928(2)				
Bd1975(5)				
Bd2667(2)				
Bd2798(1)				
Bd3273(3)				
Bd3787(5)				
Bd0862(6)				


Bd3883(5)
Bd3365(4)
Bd2460(1)
Bd0922
Bd2800
Bd3175
Bd3466
Bd3388(1)
Bd1552
Bd3869
Bd1521
Bd1032
Bd3478
Bd2144
Bd3749
Bd3754
Bd3876
Bd0958
Bd0959
Bd2521
Bd2900
Bd3171
Bd0879
Bd0169(1)
Bd0449
Bd0521(1)
Bd0564
Bd0629
Bd0706
Bd0758
Bd0816(1)
Bd0967
Bd0994
Bd1043
Bd1239
Bd1288
Bd1444
Bd1541
Bd1898(1)
Bd1962
Bd2044(1)
Bd2218


Bd2268
Bd2270
Bd2272
Bd2273
Bd2274
Bd2275(1)
Bd2304(2)
Bd2321(1)
Bd2332
Bd2334
Bd2519
Bd2535
Bd2627
Bd2630
Bd2712
Bd2847(1)
Bd2923
Bd2079(1)
Bd3244
Bd3415
Bd3459
Bd3480
Bd3481
Bd3482(1)
Bd3511
Bd3534
Bd3609
Bd3753
Bd0129
Bd0168(1)
Bd0202
Bd0277
Bd0306
Bd0398(1)
Bd0636
Bd0649
Bd0699
Bd0700
Bd0751
Bd0754(1)
Bd0755
Bd1077


Bd1078
Bd1084(1)
Bd1171
Bd1265(1)
Bd1338
Bd1372
Bd1518(1)
Bd1776
Bd2006
Bd2053(1)
Bd2068
Bd2418
Bd2554
Bd2654
Bd3234
Bd3378
Bd3426
Bd3508
Bd3547
Bd3602
Bd3704
Bd3755
Bd3771
Bd3788
Bd1649
Bd3678
Bd2674(2)
Bd1427(1)
Bd0141(1)
Bd0160(1)
Bd0297(1)
Bd0923
Bd1271
Bd1950
Bd2328
Bd2675(1)
Bd3096
Bd3351(1)
Bd3522
Bd0282
Bd1031
Bd3128
Bd0283

Bd0873						
Bd0910						
Bd0982						
Bd2017						
Bd3462						
Bd2832						
Bd0072						
Bd0035						
Bd1391						
Bd3488						

Table B3 – Nucleases

	Karunker			Lambert		
	AP	**GP**		**30 mins**	**HI**	
Bd0934						
Bd3507(1)						
Bd1244						
Bd2442						
Bd0159						
Bd0197						
Bd0198						
Bd0254						
Bd0591						
Bd1042						
Bd1431(1)						
Bd1501(1)						
Bd1934						
Bd2232						
Bd2311						
Bd3139						
Bd3140						
Bd3524						
Bd3670						
Bd3695						
Bd1941						
Bd3851						
Bd0423						
Bd1346						
Bd2036						
Bd2116						
Bd2138						

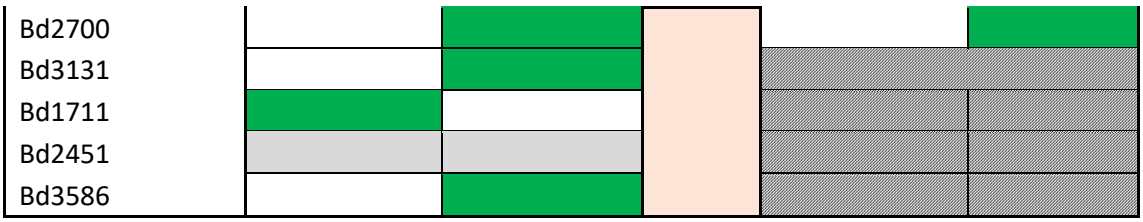
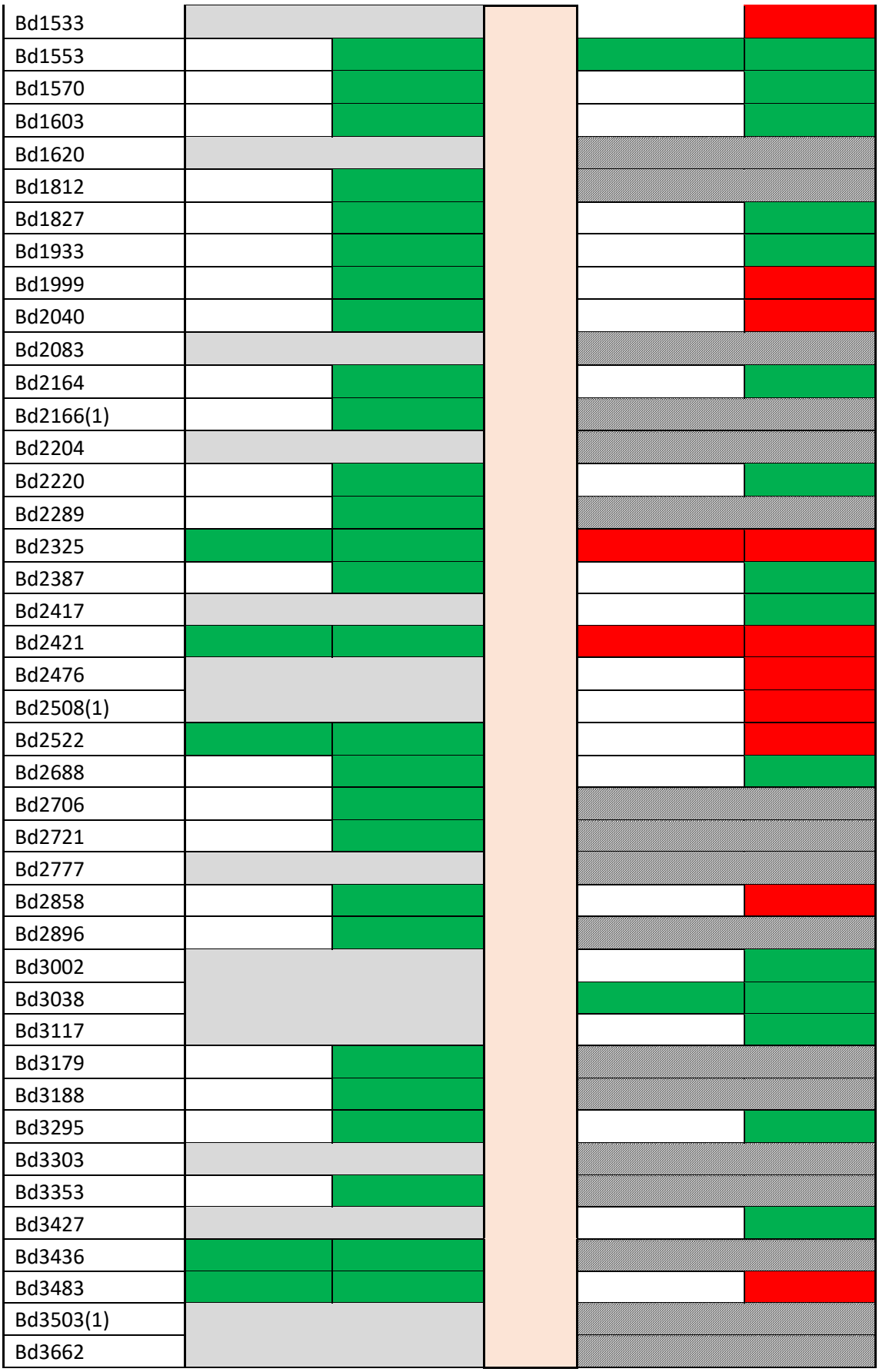


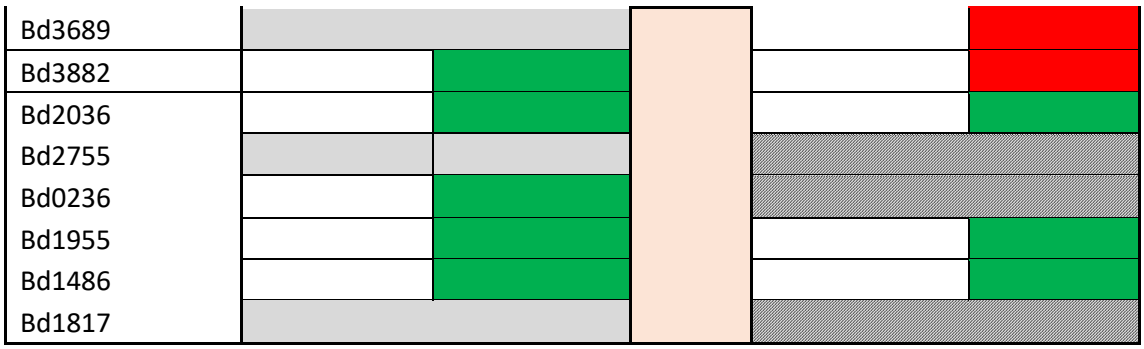
Table B4 – Glycanases and lipases

	Karunker		Lambert	
	AP	**GP**	**Bd**	**HI**
Bd3457(4)				
Bd1124				
Bd1285(1)				
Bd2462				
Bd2711(1)				
Bd3575(1)				
Bd0519				
Bd0529				
Bd1125				
Bd3243				
Bd0340				
Bd0448(1)				
Bd0664(2)				
Bd0737				
Bd1121				
Bd1415				
Bd1516				
Bd1622				
Bd2389				
Bd3206				
Bd3289				
Bd3533				
Bd0314				
Bd3421				
Bd3073				

Table B5 – Other hydrolases

	Karunker			Lambert	
	AP	**GP**		**30 mins**	**HI**
Bd1224					
Bd0503(4)					
Bd1278(7)					
Bd2224(5)					
Bd2297(7)					
Bd2609(7)					
Bd0146					
Bd2279					
Bd0030					
Bd0087					
Bd0201					
Bd0205					
Bd0294					
Bd0304					
Bd0356					
Bd0379					
Bd0407					
Bd0518(1)					
Bd0570					
Bd0590					
Bd0654					
Bd0665(1)					
Bd0714					
Bd0767					
Bd0901					
Bd0943					
Bd0963					
Bd0978					
Bd0992(1)					
Bd1069					
Bd1072					
Bd1094					
Bd1188(1)					
Bd1192					
Bd1228(1)					
Bd1301					
Bd1339					
Bd1426					
Bd1487					


Bd1533
Bd1553
Bd1570
Bd1603
Bd1620
Bd1812
Bd1827
Bd1933
Bd1999
Bd2040
Bd2083
Bd2164
Bd2166(1)
Bd2204
Bd2220
Bd2289
Bd2325
Bd2387
Bd2417
Bd2421
Bd2476
Bd2508(1)
Bd2522
Bd2688
Bd2706
Bd2721
Bd2777
Bd2858
Bd2896
Bd3002
Bd3038
Bd3117
Bd3179
Bd3188
Bd3295
Bd3303
Bd3353
Bd3427
Bd3436
Bd3483
Bd3503(1)
Bd3662



Appendix C – Genes unique to *B. bacteriovorus* HD100 compared to *B. bacteriovorus* Tiberius based on supplementary data from (Hobley et al., 2012b)

Table C1 – Key for unique genes to HD100 compared to Tiberius

Key	
	Not unique to *B. bacteriovorus* HD100
	Unique to *B. bacteriovorus* HD100

Table C2 – Proteases

Enzyme	Unique to HD100?
Bd0029	
Bd0376	
Bd1283(1)	
Bd1432	
Bd2269	
Bd2428	
Bd2545(2)	
Bd2692	
Bd3087	
Bd3238	
Bd3857(1)	
Bd0852(1)	
Bd0853(1)	
Bd0854(1)	
Bd1067(1)	
Bd2188(1)	
Bd2271(6)	
Bd2305(6)	
Bd3622	
Bd1287(2)	

Bd1928(2)	
Bd1975(5)	
Bd2667(2)	
Bd2798(1)	
Bd3273(3)	
Bd3787(5)	
Bd0862(6)	
Bd3883(5)	
Bd3365(4)	
Bd2460(1)	
Bd0922	
Bd2800	
Bd3175	
Bd3466	
Bd3388(1)	
Bd1552	
Bd3869	
Bd1521	
Bd1032	
Bd3478	
Bd2144	
Bd3749	
Bd3754	
Bd3876	
Bd0958	
Bd0959	
Bd2521	
Bd2900	
Bd3171	
Bd0879	
Bd0169(1)	
Bd0449	
Bd0521(1)	
Bd0564	
Bd0629	
Bd0706	
Bd0758	
Bd0816(1)	
Bd0967	
Bd0994	
Bd1043	
Bd1239	
Bd1288	
Bd1444	

Bd1541	
Bd1898(1)	
Bd1962	
Bd2044(1)	
Bd2218	
Bd2268	
Bd2270	
Bd2272	
Bd2273	
Bd2274	
Bd2275(1)	
Bd2304(2)	
Bd2321(1)	
Bd2332	
Bd2334	
Bd2519	
Bd2535	
Bd2627	
Bd2630	
Bd2712	
Bd2847(1)	
Bd2923	
Bd2079(1)	
Bd3244	
Bd3415	
Bd3459	
Bd3480	
Bd3481	
Bd3482(1)	
Bd3511	
Bd3534	
Bd3609	
Bd3753	
Bd0129	
Bd0168(1)	
Bd0202	
Bd0277	
Bd0306	
Bd0398(1)	
Bd0636	
Bd0649	
Bd0699	
Bd0700	
Bd0751	

Bd0754(1)	
Bd0755	
Bd1077	
Bd1078	
Bd1084(1)	
Bd1171	
Bd1265(1)	
Bd1338	
Bd1372	
Bd1518(1)	
Bd1776	
Bd2006	
Bd2053(1)	
Bd2068	
Bd2418	
Bd2554	
Bd2654	
Bd3234	
Bd3378	
Bd3426	
Bd3508	
Bd3547	
Bd3602	
Bd3704	
Bd3755	
Bd3771	
Bd3788	
Bd1649	
Bd3678	
Bd2674(2)	
Bd1427(1)	
Bd0141(1)	
Bd0160(1)	
Bd0297(1)	
Bd0923	
Bd1271	
Bd1950	
Bd2328	
Bd2675(1)	
Bd3096	
Bd3351(1)	
Bd3522	
Bd0282	
Bd1031	

Bd3128	
Bd0283	
Bd0873	
Bd0910	
Bd0982	
Bd2017	
Bd3462	
Bd2832	
Bd0072	
Bd0035	
Bd1391	
Bd3488	

Table C3 – Nucleases

Enzyme	Unique to HD100?
Bd0934	
Bd3507(1)	
Bd1244	
Bd2442	
Bd0159	
Bd0197	
Bd0198	
Bd0254	
Bd0591	
Bd1042	
Bd1431(1)	
Bd1501(1)	
Bd1934	
Bd2232	
Bd2311	
Bd3139	
Bd3140	
Bd3524	
Bd3670	
Bd3695	
Bd1941	
Bd3851	
Bd0423	
Bd1346	
Bd2036	
Bd2116	

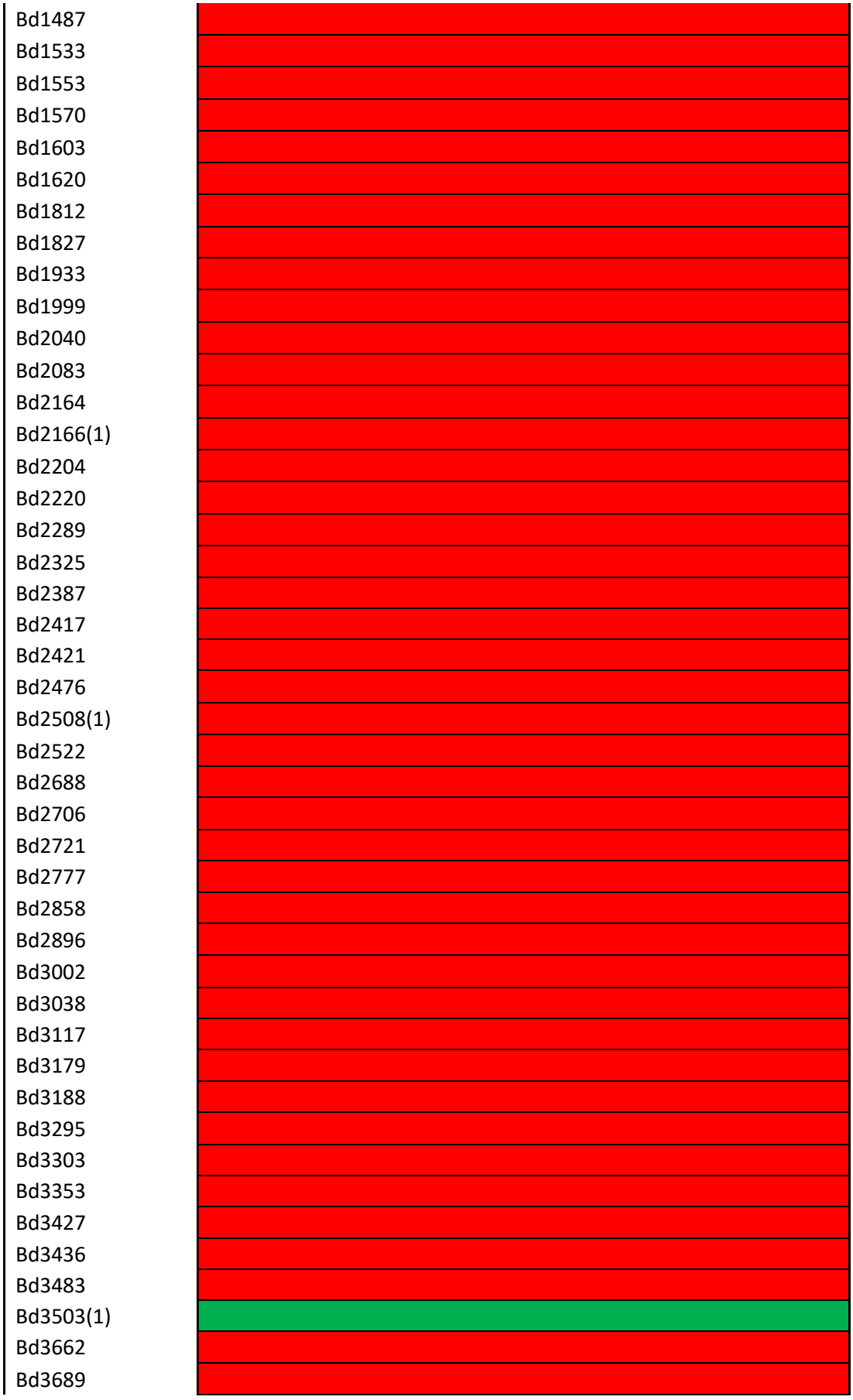
Bd2138	
Bd2700	
Bd3131	
Bd1711	
Bd2451	
Bd3586	

Table C4 – Glycanases and lipases

Enzyme	Unique to HD100?
Bd3457(4)	
Bd1124	
Bd1285(1)	
Bd2462	
Bd2711(1)	
Bd3575(1)	
Bd0519	
Bd0529	
Bd1125	
Bd3243	
Bd0340	
Bd0448(1)	
Bd0664(2)	
Bd0737	
Bd1121	
Bd1415	
Bd1516	
Bd1622	
Bd2389	
Bd3206	
Bd3289	
Bd3533	
Bd0314	
Bd3421	
Bd3073	

Table C5 – Other hydrolases

Enzyme	Unique to HD100?
Bd1224	
Bd0503(4)	
Bd1278(7)	
Bd2224(5)	
Bd2297(7)	
Bd2609(7)	
Bd0146	
Bd2279	
Bd0030	
Bd0087	
Bd0201	
Bd0205	
Bd0294	
Bd0304	
Bd0356	
Bd0379	
Bd0407	
Bd0518(1)	
Bd0570	
Bd0590	
Bd0654	
Bd0665(1)	
Bd0714	
Bd0767	
Bd0901	
Bd0943	
Bd0963	
Bd0978	
Bd0992(1)	
Bd1069	
Bd1072	
Bd1094	
Bd1188(1)	
Bd1192	
Bd1228(1)	
Bd1301	
Bd1339	
Bd1426	


Bd1487
Bd1533
Bd1553
Bd1570
Bd1603
Bd1620
Bd1812
Bd1827
Bd1933
Bd1999
Bd2040
Bd2083
Bd2164
Bd2166(1)
Bd2204
Bd2220
Bd2289
Bd2325
Bd2387
Bd2417
Bd2421
Bd2476
Bd2508(1)
Bd2522
Bd2688
Bd2706
Bd2721
Bd2777
Bd2858
Bd2896
Bd3002
Bd3038
Bd3117
Bd3179
Bd3188
Bd3295
Bd3303
Bd3353
Bd3427
Bd3436
Bd3483
Bd3503(1)
Bd3662
Bd3689

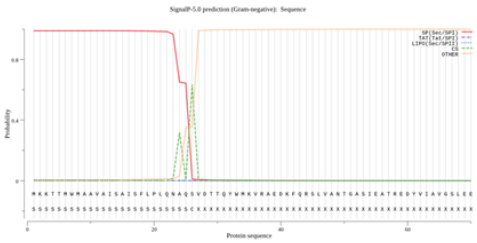
Bd3882	
Bd2036	
Bd2755	
Bd0236	
Bd1955	
Bd1486	
Bd1817	

Appendix D - SignalP-5.0 Confirmation of Signal peptides and TMHMM transmembrane regions

Proteases

Peptidase M14

Bd3234



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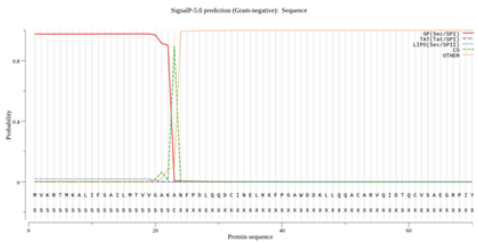
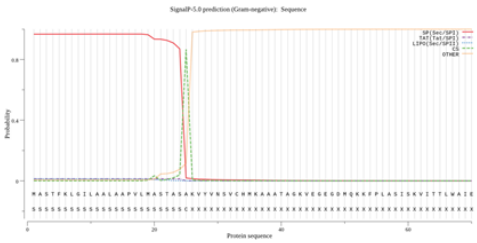


Peptidase S13

Bd0816



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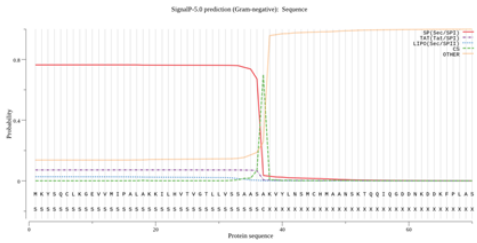
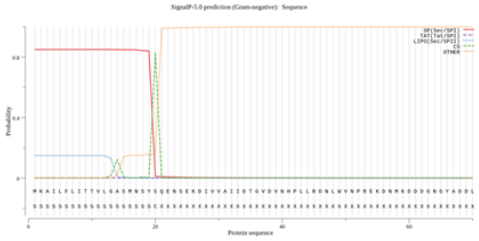


Peptidase S8

Bd1432



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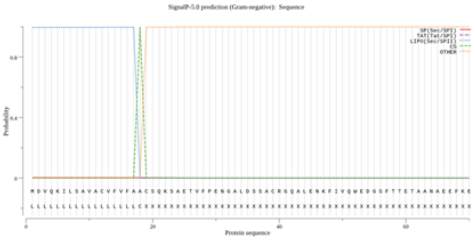

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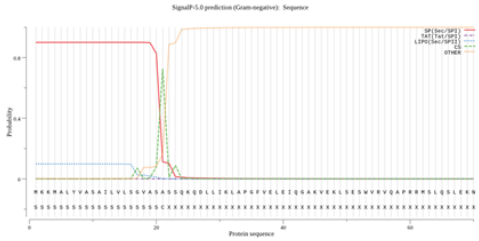

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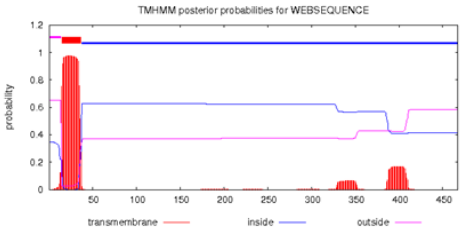

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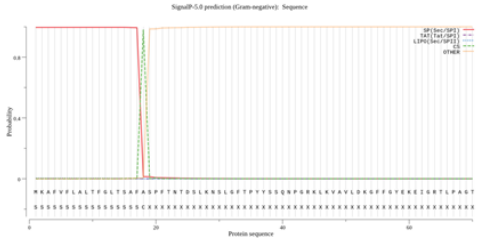
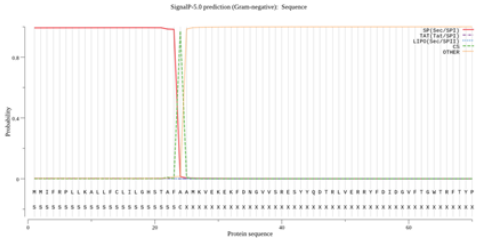


Bd0521



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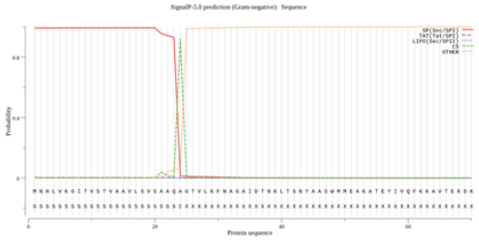

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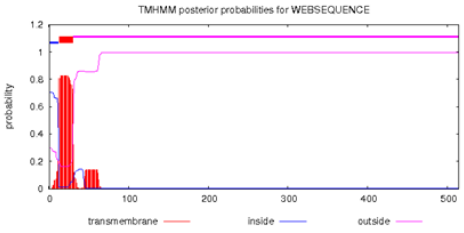

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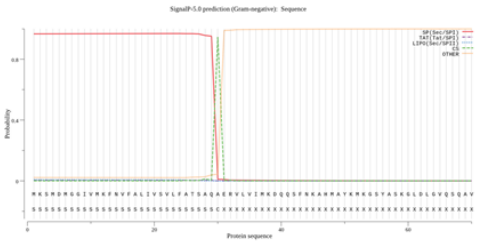

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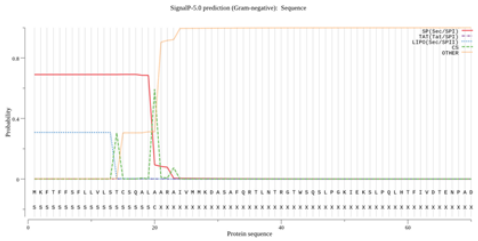

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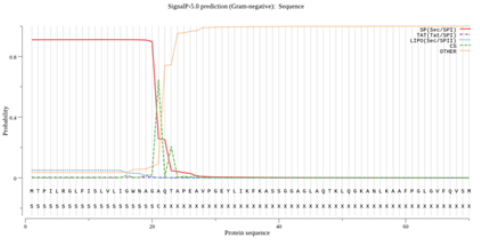

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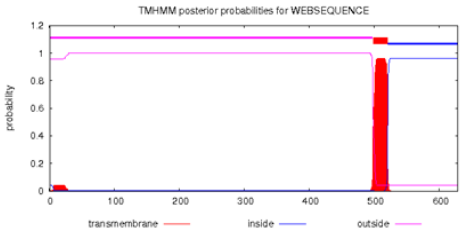

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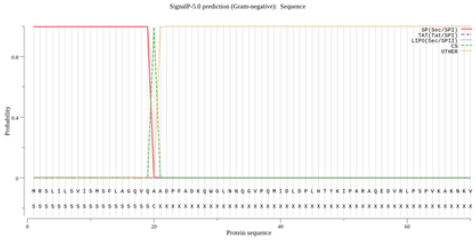

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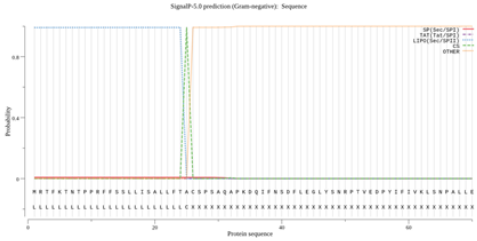
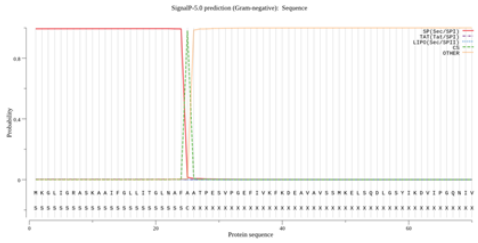


Bd0029



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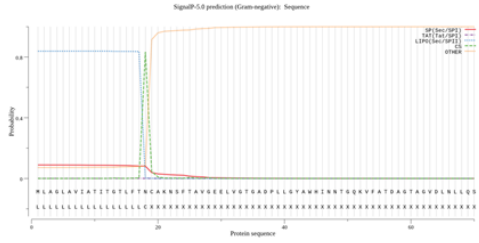

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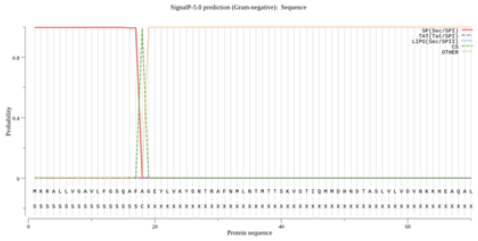
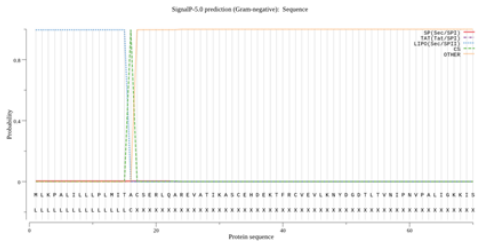


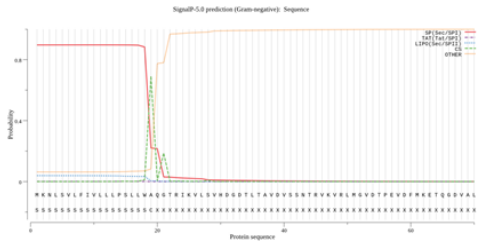
Nucleases

SNases

Bd1431

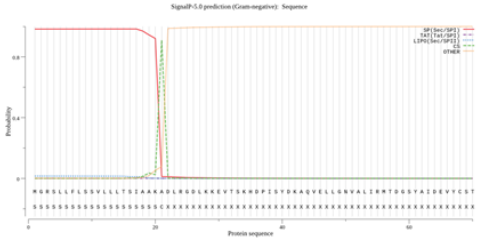


Bd1934



Endonuclease 1

Bd0934



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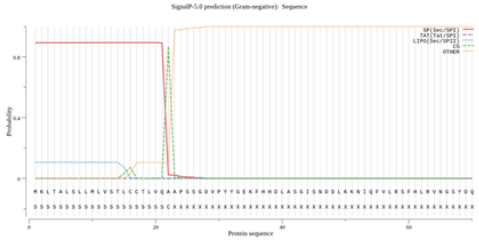

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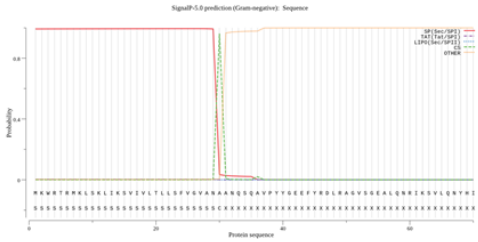

Exo_endo_phos

Bd3586