'Growth switching, motility and application of *Bdellovibrio bacteriovorus'*

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Abstract

Bdellovibrio bacteriovorus, is a small mono-flagellate Gram-negative delta-proteobacterium, which has a bi-phasic lifecycle, consisting of a predatory phase; in which they invade on other Gram-negative bacteria and digest the prey cell's content to grow and septate, or host independent phase; in which they can grow and septate in media rich in amino acids as well as vitamins and cofactors. As *B. bacteriovorus* can kill other Gram-negative bacteria including pathogens, they have potential to be used as a 'living antibiotic'.

I have been part of this field since 2004, a time at which the first *B. bacteriovorus* genome (HD100) had just been sequenced and made available, and only one study into making deletion mutants had been published. During my time in this field, the research has expanded almost exponentially, with the understanding of core pathways and systems that make *B. bacteriovorus* so novel being highlighted and greatly understood. In addition new techniques and methodologies never before attempted in *B. bacteriovorus* research have been made possible and I have been lucky to be a part of this and carried out some of the work myself.

In particular I have worked on the mutation and phenotype testing of genes encoding pathways for motility, prey cell lysis, *B. bacteriovorus* intra-cellular signalling, and bi-phasic growth switching. These advances from my work including an animal trial into the predatory nature of *B. bacteriovorus* have laid the foundation for its use as a novel 'living antibiotic' in the future.

List of Published Papers

1. Characterizing the flagellar filament and the role of motility in bacterial prey-penetration by *Bdellovibrio bacteriovorus*

Carey Lambert, Katy J Evans, Rob Till, Laura Hobley, Michael **<u>Capeness</u>**,

Snjezana Rendulic, Stephan C Schuster, Shin-Ichi Aizawa, R Elizabeth Sockett

Mol Microbiol. 2006 April; 60(2): 274–286.

Words: 7956

2. A predatory patchwork: membrane and surface structures of *Bdellovibrio bacteriovorus*.

Lambert C, Hobley L, Chang CY, Fenton A, **<u>Capeness</u>** M, Sockett L.

Adv Microb Physiol. 2009;54:313-61. Review.

Words: 14321

3. The First Bite— Profiling the Predatosome in the Bacterial Pathogen *Bdellovibrio*

Carey Lambert, Chien-Yi Chang, Michael J. **<u>Capeness</u>**, R. Elizabeth Sockett

PLoS One. 2010; 5(1): e8599.

Words: 9080

4. Three *motAB* Stator Gene Products in *Bdellovibrio bacteriovorus* Contribute to Motility of a Single Flagellum during Predatory and Prey-Independent Growth

Karen A. Morehouse, Laura Hobley, Michael **<u>Capeness</u>**, R. Elizabeth Sockett

J Bacteriol. 2011 February; 193(4): 932–943.

Words: 8027

5. The *Bdellovibrio bacteriovorus* twin-arginine transport system has roles in predatory and prey-independent growth.

Chang CY, Hobley L, Till R, **<u>Capeness</u>** M, Kanna M, Burtt W, Jagtap P, Aizawa S, Sockett RE.

Microbiology. 2011 Nov;157(Pt 11):3079-93

Words: 8579

6. Effects of Orally Administered *Bdellovibrio bacteriovorus* on the Well-Being and *Salmonella* Colonization of Young Chicks

Robert J. Atterbury, Laura Hobley, Robert Till, Carey Lambert, Michael J. **<u>Capeness</u>**, Thomas R. Lerner, Andrew K. Fenton, Paul Barrow, R. Elizabeth Sockett

Appl Environ Microbiol. 2011 August; 77(16): 5794–5803.

Words: 8993

7. The Structure of an Unconventional HD-GYP Protein from *Bdellovibrio* Reveals the Roles of Conserved Residues in this Class of Cyclic-di-GMP Phosphodiesterases

Andrew L. Lovering, Michael J. **<u>Capeness</u>**, Carey Lambert, Laura Hobley, R. Elizabeth Sockett

mBio. 2011 Sep-Oct; 2(5): e00163-11.

Words: 5284

8. Genome analysis of a simultaneously predatory and prey-independent, novel *Bdellovibrio bacteriovorus* from the River Tiber, supports in silico predictions of both ancient and recent lateral gene transfer from diverse bacteria.

Hobley L, Lerner TR, Williams LE, Lambert C, Till R, Milner DS, Basford SM, Capeness MJ, Fenton AK, Atterbury RJ, Harris MA, Sockett RE.

BMC Genomics. 2012 Nov 27;13:670

Words: 7990

9. Activity of *Bdellovibrio hit* Locus Proteins, Bd0108 and Bd0109, Links Type IVa Pilus Extrusion/Retraction Status to Prey-Independent Growth Signalling.

Michael J. **Capeness**, Carey Lambert, Andrew L. Lovering, Kaoru Uchida, Roy Chaudhuri, L.J. Alderwick, David J Lee, David Swarbreck, Rob Till, Shin-Ichi Aizawa and R. Elizabeth Sockett

PLoS One. 2013 Nov 5;8(11):e79759

Words: 14651

Total Words: 84881

Longer Abstract

Bdellovibrio bacteriovorus is a novel delta-proteobacterium that can carry out predation upon other Gram-negative bacteria, including pathogens, during which it grows and replicates inside the prey cell using the host cells content for its own metabolic purposes. Due to this it is thought that *B. bacteriovorus* has applications as a 'living-antibiotic'.

B. bacteriovorus is a small, mono-flagellate bacterium that swims at high speeds to locate potential prey. Its genome contains an array of genes responsible for this motility and predatory lifecycle, but also contains all the necessary genes for growth free of prey cells. Conversion from Host Dependency (HD) to Host Independency (HI) is due, in most cases, to genetic mutation, though obligatory HI cells do exist in nature.

During my time employed at The University of Nottingham, I have been had roles as a part-time undergraduate degree student, as well as a part-time research technician in the School of Life Sciences (formerly Biology) and from this I have also gone on to carry out work at the Ph.D. level. All these roles I have undertaken in Prof. Sockett's lab. As such, the work I have carried out over the last 9 years is both progressive and vital to the expansion of the *Bdellovibrio* research field.

My Role

I began in 2004, when the first *B. bacteriovorus* genome sequence (HD100) had

just been published, prior to this very few genetic studies had been carried out in the field due to the limitation of not having the genome sequence.

From this time the field has greatly expanded and is now possible to carryout genetic manipulation such as insertion/deletions and now markerless deletions to study gene functions. The genomic sequence also made expression analysis possible from simple RT-PCR, to QPCR and microarrays and finally RNAseq. This multitude of genetic capabilities has greatly increased the amount of understanding and depth to the physiology of *B. bacteriovorus* over my tenure in the lab and I am pleased to say that I have been involved in all of their use and development.

With greater resources to draw from the physical techniques of working with *B. bacteriovorus* has also had to adapt and progress. Growth of the bacterium to isolate RNA, calculation of predation efficiency, and the analysis of other phenotypes using microscopy or growth assays arising from genetic manipulation have all been developed during my time over the past 9 years and I have, together with my colleagues, been at the forefront of this '*Bdellovibrio* renaissance'.

Bdellovibrio research is not without its limitations, it has not received the attention other organisms have, so there is very little research, relative to the *E. coli* field, being carried out on it so the background and pace of the research is reduced. As the bacterium has two novel growth niches requiring either complex media (HI), or other bacteria which it uses for predation (HD) and it is an on-going trial relating the two lifecycles to each other, though definite progression is detailed here.

Growth and maintenance of the *B. bacteriovorus* cells is also not straight forward, HI cells are pleomorphic making it difficult to characterise morphological changes, while HD cells are very small making them difficult to image outside of electron microscopy. As *B. bacteriovorus* does have quite a novel lifecycle, its genome contains a large amount of genes specific to *B. bacteriovorus* with no homologues found in other bacteria, because of this, physiological understanding can be sometimes limited, while at the same time offers a great opportunity to characterise this genes previously unfounded in science. Due to these reasons it has often been difficult to work on *B. bacteriovorus* though ultimately rewarding and has been a programme involving innovation along with experimentation.

The Publications

Due to the success of the advancing field of *B. bacteriovorus* research it has been possible to publish various papers detailing this progression and the greatening understanding of the organism in question. As such the Sockett lab has become the world-leading experts on *B. bacteriovorus,* publishing more papers over the last 10 years than any other lab, 9 of which I feature as an author with another not being mentioned here.

Certain themes underpin the list of publications, although all are in the field of *Bdellovibrio* research, many papers can be placed in the following groups; Cell Motility (papers: **1**, **4**), Predation by *Bdellovibrio* (**1**, **2**, **3**, **5**, **6**, **8** and **9**), Secretion signalling in HI/HD growth phases (**2**, **3**, **5**, **7** and **9**) and finally a trial application of *Bdellovibrio* as a therapeutic (**7**). As can be seen there is also much overlap between the studies with many of the more recent studies using techniques or evidence from earlier papers to prove hypotheses or build upon them.

Motility

Motility is a key component in the predatory lifecycle of *B. bacteriovorus*; the mutation of this pathway makes it an easy target to spot phenotypes by way of reduction in swimming speed/percentage of the population that is motile.

My first contributed work was to the second paper to come out of the Sockett lab on *Bdellovibrio* looking at flagellar motility (**1**). In this paper the foundations for many subsequent papers are laid, many of which I also feature on as an author, in particular the experimental techniques in this publication, such as RNA isolation and time course analysis is one that features predominantly in *Bdellovibrio* research and something that I helped pioneer (see papers **2**, **3**, **4**, **5**, **7, 8** and **9**). Based on this work it was possible to further explore the motility of *Bdellovibrio* and how vital it is to the organism and in later publications add increasing knowledge to a fundamental system in the lifecycle of *Bdellovibrio* (**4**).

Therapeutics

One of the greatest leaps in *Bdellovibrio* research is that exploring its use as a therapeutic, something which is perhaps the 'end-goal' of *Bdellovibrio* research, and came in the publication using *Bdellovibrio* to treat *Salmonella* infections in chickens *in vivo* (**6**).

This very much relied on all previous work so far to get to this trial application and it is hard to imagine it happening without the prior publications into understanding the fundamentals of *Bdellovibrio* growth. The work was a huge success and at the time represented the first *in vivo* use of *Bdellovibrio* as a therapeutic and has

such served as a basis for its *Bdellovibrio's* hypothetical use to treat of bacterial related ailments.

Regulation

Due to the advances in techniques and understanding in the *Bdellovibrio* research field across the past 9 years, the areas of study have begun to become more focused as time has progressed. This essentially made it possible to go from research into whole pathways to very specific genes due to the gained knowledge and techniques accrued over the previous years. An example of how the research has narrowed to look at more specific roles in *Bdellovibrio* is represented by obtaining the crystal structure for a specific protein; Bd1817 (**8**). This was a great achievement, not just because it was only the third crystal structure for a *Bdellovibrio* protein ever obtained but also it because it was the first HD-GYP protein of its kind to have its structure solved, a large goal in that field of sensing.

The most recent papers published (**7** and **9**) show just how far the field of *Bdellovibrio* has advanced during my time. From the start of my time at the University, when no genomic sequence was available, to a time where we have second genome published (**7**) and we are looking at more specific mechanisms and protein interactions underlying core events in the *Bdellovibrio* lifecycles (**9**). In particular the latest paper (**9**), which I am first author on, goes quite a way to solve the mystery of a cryptic gene; *bd0108,* that has been singled to have a role in growth phase switching in *Bdellovibrio*, though until now has never been

known how it works or other genes it is involved in. This paper also builds from work hypothesised in a previous publication (**2**).

Not only has the work on which I featured advanced the research within the lab, but also that of the wider *Bdellovibrio* research community. Currently the work on which I am an author has been cited 104 times since the first publication in 2006 until the latest publication at the end of 2013 (13 citations/year). The works have not only been cited by just those that work on *Bdellovibrio*, but also those that work on specific functions in other bacteria, such as motility, signalling and the use of bacteria as 'living antibiotics'.

There are currently 35 papers on *Bdellovibrio* from Prof. Sockett's lab and I'm proud to say I have helped author and contribute work to 9 of those and that my research has formed a basis for others both at the time of their undertaking and for the future researchers both in the Sockett lab and in the wider research fields.

Acknowledgements

First and foremost I would like to thank Prof. Sockett without who I simply could not have achieved one iota of my potential without her endless patience, intelligence, encouragement and friendship. From the doe-eyed boy at 17 to someone writing a thesis for a Ph.D. I never would have thought I was capable of any of it and I owe it all to her. No one could ask for a better supervisor and I hope I can repay her one day.

My second thanks goes to the members of C15, both past and present, I have had the great honour to work with the finest set of people one could hope over the last 9 years. These include (in no particular order): Carey, Rob T, Laura, David, Sarah, Emma, Tom, Andrew, Katy, Richie, Rowena, Max, Mike 2, Davy, Rob A, Karen, Mark, John and Marilyn. Thank you for all help in the lab, the free ranging philosophical questions, the odd crossword and the advice on what to have for dinner. You've all given me countless laughs and entertainment and I am eternally grateful.

I would also like to thank the A-Team for making every experiment possible as well as for keeping me up to date on all the departmental gossip and giving me the occasional biscuit. I think everyone should have a group of people like you to brighten up their work place.

A great thank you goes to all of the people I've had the great fortune to collaborate with, in particular Prof. Shin-Ichi 'Chi' Aizawa and Dr. Andrew 'Andy'

Table of Contents

Abbreviations

%	percentage
°C	degrees centigrade
α	Alpha
ABC	Adenine-5'-triphosphate Binding Cassette
Å	Angstrom
ADP	Adenosine DiPhosphate
Amp	Ampicillin
Apra	Apramycin
ATP	Adenosine-5'-TriPhosphate
AUC	Analytical UltraCentrifugation
BLAST	Basic Local Alignment Search Tool
β	Beta
bp	single nucleotide base-pair
CCCP	Carbonyl Cyanide m-ChloroPhenyl hydrazone
ChIPseq	CHromation Immuno Preciptation Sequencing
Da	dalton
Δ	delta – gene deletion
DNA	deoxyribonucleic acid
e.g	'for example'
EM	Electron Microscopy
flp	Fimbrae-like Proteins
FU	Colony Forming Units
g	gram
GFP	Green Fluorescent Protein
GMP	Guanosine MonoPhosphate
GTP	Guanosine-5'-TriPhosphate
HD	Host Dependent
HI	Host Independent
hit	Host InTeraction locus
i.e.	'that is'
IM	Inner Membrane
IPTG	Isopropyl β-D-1-thiogalactopyranoside
J	Joules
kb	Kilobase, 1000 base pairs
kDa	Kilo-daltons
Kn	Kanamycin
KO	Knock-out 'gene deletion
l	Litre
LGT	Lateral Gene Transfer
M	Molar
m	Metre
m	mili 10^{-3}
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionisation-Time Of Flight
Mb	megabase, 1,000,000 base pairs
mCherry	monomeric 'Cherry' Fluorescent protein
μ	micro 10^{-6}
μg	microgram
μl	microliter

min	minutes
mJ	milliJoule
ml	millilitre
mm	millimetre
MOI	Multiplicity Of Infection
MS	Mass Spectrometry
mTFP	Monomeric 'Teal' Fluorescent Protein
MW	Molecular Weight Marker
n	nano 10^{-9}
nm	nanometres
OD	Optical Density
OD_{600}	Optical Density at 600 nm
OM	Outer Membrane
ORF	Open Reading Frame
p	pico 10^{-12}
PCR	Polymerase Chain Reaction
PFAM	Protein Families Database
PFU	Plaque Forming Units
PG	Peptidoglycan
pH	negative log10 of the hydrogen ion concentration
PAML	Phylogenetic Analysis by Maximum Likelihood
PRED-TAT	Prediction of Twin-Arginine and secretory signal peptides
PTA	Phospho-Tungstic Acid
Q-PCR	Quantitative Real-Time Polymerase Chain Reaction
RNA	RiboNucleic Acid
RNAseq	RiboNucleic Acid Sequencing
rpm	Revolutions Per Minute
RSD	Reciprocal Smallest Distance
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis
TAT	Twin Argenine Transport/er
TEM	Transmission Electron Microscopy
URA	Uranyl Acetate
UV	Ultra-Violet
V	Volts

Overview of Contributions

Characterizing the flagellar filament and the role of motility in bacterial prey-penetration by *Bdellovibrio bacteriovorus*

Carey Lambert, Katy J Evans, Rob Till, Laura Hobley, Michael **Capeness**,
Snjezana Rendulic, Stephan C Schuster, Shin-Ichi Aizawa, R Elizabeth Sockett

Words: 7956

Paper Summary

Before the publication of this paper, *B. bacteriovorus* were known to be motile via the rotation of a single polar flagellum, and it was hypothesised that the force generated from this swimming was how they entered into prey cells. The paper focuses on the characterisation of the flagellar filament in the *B. bacteriovorus* strain 109J. *B. bacteriovorus* not only have an unusual flagellum as it is surrounded in a sheath, but also 6 flagellin genes predicted to encode proteins that compose the filament and are connected to the outer sheath membrane. The 6 flagellin genes were each mutated by insertion of a kanamycin resistance cassette and had the resulting phenotypes assessed using electron microscopy. The paper identifies how each of the flagellin genes contributes differently to the formation of the flagellum, analysing changes in both length of the flagellum and wavelength morphology in each of the *fliC* mutants. The most important finding of the paper is that *fliC3* mutant did not have any flagella, and so were non-motile but could still invade prey, thus challenging of the core believes about the role of

motility in predation. The paper also assesses the role correct flagella formation plays on motility and also how well each of the flagellin mutants can invade prey thus entering into the predatory cycle compared to a wild-type. Another finding was that flagellar mutants that have a shorter flagellum and so encounter prey at a lower occurrence, and therefore enter into the predation cycle less efficiently. For the first time RNA analysis by RT-PCR and Q-PCR across the predatory lifecycle of *B. bacteriovorus* was used to investigate the changes in expression of the different flagellin genes, and also how the expression of the flagellin genes change in different flagellar mutant backgrounds. SDS-PAGE and mass spectrophotometry work was also used to determine the composition of the flagellar filament.

The paper represents a significant increase in the understanding motility in *B. bacteriovorus*, and also laid the foundation for many methods that would emerge constantly as core experiments in subsequent research. The paper also marked the first instance the Sockett lab have collaborated with Prof. Aizawa in this field and also remains one of the highest cited papers to come from the lab.

Author Contribution

Carey Lambert

As first author Dr. Lambert undertook the main manuscript writing with Prof. Sockett and contribution from other authors. He also designed the cloning strategies and carried out the mutation of *fliC2*-6 genes by the insertion of kanamycin cassettes. He also carried out all the Q-PCR to assess the copy number of the different *fliC* gene transcripts across the *B. bacteriovorus* predatory lifecycle to find there is a reduction upon entry to the prey, but an

increase in expression of the *fliC* transcripts before prey lysis. Dr. Lambert also aided in the design of the some of the experiments used to phenotype the *fliC* mutants.

Katy J Evans

Katy, as well as being an author on the manuscript, carried out the electron microscopy of the different *fliC* mutants and assessed the phenotype of the flagellar mutant and found that different flagellar mutants have different flagellar morphologies. Katy also carried out the SDS-PAGE gels investigating the differences in flagella composition for each of the *fliC* mutants by subsequent mass spectrophotometry. Katy also verified that predation was possible with the non-motile *fliC3* mutant by using fluorescent microscopy and therefore showed predation was not flagella mediated.

Rob Till

Rob's main contribution to the manuscript was as help to Carey in the cloning of each of the *fliC* genes and their downstream mutagenesis and insertional activation by the addition of a kanamycin cassette.

Laura Hobley

Laura used the Hobson BacTracker to phenotype the swimming speeds and tumbling phenotype of the *B. bacteriovorus fliC* mutants to find differences between the different flagellar mutants. Laura also contributed to the writing of the manuscript.

Michael Capeness

I prepared the RNA samples from the different *fliC* mutants with guidance from Dr. Lambert, as well as verifying the samples were synchronous and DNA free. I carried out the RT-PCRs shown in the paper to investigate the expression of the different *fliC* genes in both wild-type and the *fliC* mutant backgrounds and found that there is a characteristic expression pattern for the *fliC* genes in wild-type, but which is altered in the flagellar mutant backgrounds. I also contributed to the writing of the manuscript.

Snjezana Rendulic

Snjezana provided the lab with the sequences of the *fliC* genes before the publication of the *B. bacteriovorus* HD100 genome paper in 2004 as was involved in their isolation from the contiguous DNA data.

Stephan C Schuster

Prof. Schuster supervised the sequencing of the *B. bacteriovorus* HD100 genome from which the *fliC* gene sequences were given to us before the publication of the HD100 genome paper.

Shin-Ichi Aizawa

Prof. Aizawa both contributed to the writing of the manuscript, and helped to visualise different flagellar mutant strains of *B. bacteriovorus* by electron microscopy and interpret different flagella phenotypes including altered wavelengths and flagella length.

R Elizabeth Sockett

Prof. Sockett with Dr. Lambert was the main author of the manuscript and devised the hypotheses, and helped design all of the experiments and research behind the whole paper as well as interpreting data.

A predatory patchwork: membrane and surface structures of *Bdellovibrio bacteriovorus*.

Lambert C, Hobley L, Chang CY, Fenton A, **Capeness** M, Sockett L.

Words: 14321

Paper Summary

The Predatory Patchwork book chapter is a review on the different membrane and surface structures found in *B. bacteriovorus* using both genomic prediction and physiological experiments. It features in the "Review Journal": Advances in Microbial Physiology, and represents a summary on what was currently known about different transportation systems, periplasmic and outer membrane proteins and metabolism, and the role of flagella, chemotaxis and pili in predation. The work featured in the chapter, was a mixture of previously published data as well as unpublished data based on bioinformatics of the HD100 genome.

The chapter itself lays the foundation for areas of research that were eventually undertaken by Prof. Sockett's lab, such as peptidoglycan synthesis and further work on type IV pili.

Author Contribution

Carey Lambert

Dr. Lambert, as lead author of the manuscript oversaw how each chapter fitted together and developed the overarching theme and continuity as well as writing

the section of the chapter on the Sec system and outer membrane proteins. The chapter features bioinformatics analysed by Dr. Lambert on the HD100 genome analysing the presence/absence of various proteins involved the Sec system and outer membrane transport as well as RT-PCR analysis on some of the implicated genes.

Laura Hobley

Laura wrote the part of the chapter focussing on the role of flagella and chemotaxis in predation. The chapter mainly included a summary of previous work by our lab on the flagellar filament and the deletion of one of the MCPs and their effect on predation. The chapter also mentions some bioinformatics on other chemotaxis genes that have been yet unstudied.

Chien-Yi Chang

Dr. Chang wrote the part of the chapter about autotransporters in *B. bacteriovorus,* which is mainly a collection of bioinformatics on their presence and predicted function in predatory and host independent lifecycle.

Andrew Fenton

The main focus of the chapter section that Andrew wrote was on peptidoglycan chemistry and metabolism, and features a review of previous biochemistry data that was carried out early in the history of *B. bacteriovorus* as well as new data theories and data based on bioinformatics.

Michael Capeness

I wrote the section of the chapter about the role of Type IV pili in predation, which mainly summarised the previously published work on pili by our lab and incorporated some unpublished theories. I also included data on comparisons to

pili in *Myxobacteria*, flp pili, and bioinformatics to compose a predicted structure of the type IV pili. I also carried out some of the RT-PCR analysis featured in Dr. Lambert's section on the Sec system and outer membrane transport that had not been published previously.

Liz Sockett

Prof. Sockett along with Dr. Lambert was one of the main authors and overseers of the writing and editing process, helping with the theme and continuity.

The First Bite— Profiling the Predatosome in the Bacterial Pathogen *Bdellovibrio*

Carey Lambert, Chien-Yi Chang, Michael J. **Capeness**, R. Elizabeth Sockett

Words: 9080

Paper Summary

The paper focusses on array data showing the up-regulation of genes at thirty minutes into the predatory lifecycle of *B. bacteriovorus* HD100. At this point the *B. bacteriovorus* has entered the prey and begun the degradation of its cytoplasmic content, and so any up-regulated genes that are involved in the export of digestive enzymes. An array for host independent growth was also used, and comparisons between the two data sets highlight the differences between genes used in predatory growth and those used in host independent growth. Implicated genes were further investigated by either RT-PCR or by Q-PCR to confirm both their change at the 30 minute time point or in the host independent growth phase. This is the first time that any arrays have been carried out on *B. bacteriovorus* and led to a significant increase in the implication of genes involved specifically in predatory or in host independent growth. For example, ~7% of genes encoded by the HD100 genome were found to be involved in the establishment of the invading cell and the degradation of the prey, as they were up-regulated after 30 minutes incubation compared to not being up-regulated in host independent isolates. Roughly 5% of the genes from HD100 were found to be down-regulated at 30 minutes, suggesting a role in the free

swimming phase of the lifecycle. Many downstream research projects and proposals were based on the results featured in this paper.

Author Contribution

Carey Lambert

Dr. Lambert designed both the arrays and carried out the analysis to separate up/down regulated genes sets to implicate each gene sets to either a role in predation, the free swimming phase, or the host dependent lifecycle. He was also involved in the synchronous infections of *B. bacteriovorus* with *E. coli* for RNA samples and the preparations of the RNA samples and their subsequent quality verification. As well as that Dr. Lambert also carried out the Q-PCRs to confirm down/up-regulation of implicated genes. He is also the lead author to the manuscript along with Prof. Sockett.

Chien-Yi Chang

Like Dr. Lambert, Dr. Chang was also involved in the process of carrying out of synchronous *B. bacteriovorus* predation time courses and the harvest of RNA samples from them. Together with Dr. Lambert he also investigated the data set comparison and had a role in manuscript writing.

Michael J. Capeness

I was mainly involved in RNA preparations from the samples of taken from the synchronous *B. bacteriovorus* time course as well as testing their synchronicity and verifying they were DNA free, these were later used for both the array experiments and the RT-PCRs to confirm some of the findings. I also contributed to the writing of the manuscript and helped to interpret some of the array data.

R. Elizabeth Sockett

Prof. Sockett designed the experiments and research and along with the rest of the authors wrote and edited the manuscript as well as forming hypotheses for the correlation of returned data and the predicted gene functions of those differently regulated.

Three *motAB* Stator Gene Products in *Bdellovibrio bacteriovorus* Contribute to Motility of a Single Flagellum during Predatory and Prey-Independent Growth

Karen A. Morehouse, Laura Hobley, Michael **Capeness**, R. Elizabeth Sockett

Words: 8027

Paper Summary

The research question for this paper was to assess the role each of the 3 possible motor proteins (MotAB1-3) has on the predatory and host independent lifecycle of *B. bacteriovorus*. This was achieved by the insertion of a kanamycin cassette of the 3 different *motAB* genes. Each of the MotA and MotB proteins were also assessed for their homology to PomA and PomB which are alternative Mot proteins that are driven by sodium ions rather than by protons. This was done by bioinformatics and also by motility assays of the *motAB* mutants and wild-type in the presence of chemicals that inhibit proton or sodium channels. Motility assays were done by the use of a Hobson BacTracker, and predation assays were used to investigate the rate at which each of the mutants could lyse a co-culture of *E. coli.* Host independent growth was also investigated at to test if mutation of any of the *motAB* genes had an effect on non-predatory growth over the course of 20 hours. Electron microscopy was also undertaken to investigate any morphological phenotypes the mutants presented. RT-PCR expression studies of each of the *motA* and *motB* genes were also used to investigate

expression across the predatory lifecycle and differential regulation of other *motA* and *motB* genes in mutant backgrounds.

The outcome of these experiments showed that deletion of any 3 of the *motAB* genes was not sufficient to stop motility in the mutant strains. It was also showed that there are separate expression profiles for each of the *motA* and *motB* genes, and interruption of either *motAB* genes alters expression of the other *motA* and *motB* genes. In particular the expression of *motA1* and *motB1* correlated to the 4 hour mark in the predatory lifecycle. For each of the potential motor proteins (MotAB1-3) it was found that they responded to inhibition by CCCP, to show that they are proton driven and not sodium driven, therefore are true Mot proteins and not Pom proteins, which are sodium driven. It was also shown that only interruption of *motAB3* had an effect on the swimming speed, compared to wild-type and that no *motAB* mutant had altered wavelength or length of flagella. The predatory isolates of the *motAB1* mutant was also found to have a reduced escape time from the bdelloplast structure, indicating its importance as the initial MotAB protein at the escape phase of the lifecycle. Growing as a HI isolate all three *motAB* mutants reached a lower optical density than that of a wild-type suggesting that interruption of the genes produced a less fit host independent strain.

Author Contribution

Karen A. Morehouse

Dr. Morehouse originally cloned each of the *motAB* genes from *B. bacteriovorus*,

and inserted the kanamycin cassette into each of them, and successfully introduced the mutated version of *motAB1* and *motAB2* into separate *B. bacteriovorus* isolates. She also was one of the main authors of the manuscript.

Laura Hobley

Along with Dr. Morehouse, Dr. Hobley contributed to the writing of the manuscript but also carried out the motility tracking of the *motAB* mutants to show the effect each of the *motAB* mutations have on swimming speed, percentage motility, and their inhibition by CCCP and phenamil. She also carried out the predation assays and bdelloplast persistence assays and HI growth studies to look for defects in the different lifecycles of the *B. bacteriovorus motAB* mutants. Laura also did all of the electron microscopy work to show not alteration in flagellar morphology of the *motAB* mutants.

Michael Capeness

I both contributed to the writing of the manuscript and constructed and isolated the *motAB3* mutant in *B. bacteriovorus*. I was also responsible for showing the differences in expression of the *motA* and *motB* genes by RT-PCR analysis in the wild-type and mutant backgrounds. This included carrying out all of the RNA preparations including their testing of synchronicity, checking they were free of DNA and their quality control.

R. Elizabeth Sockett

Prof. Sockett designed the experiments and the research as well as contributing to the writing and editing of the manuscript as well as forming hypotheses of the data collected.

Bdellovibrio bacteriovorus twin-arginine transport system has roles in predatory and prey-independent growth.

Chang CY, Hobley L, Till R, Capeness M, Kanna M, Burtt W, Jagtap P, Aizawa S, Sockett RE.

Words: 8579

Paper Summary

The paper tested the role/s the TAT (Twin Arginine Transporter) pathway plays in the lifecycles of *B. bacteriovorus*. By the use of insertional mutation, each of the TAT genes; *tatA1*, *tatA2*, and *tatC*, were assessed for their essentiality in *B. bacteriovorus* in HI and HD isolates and the resultant mutants were tested for predatory efficiency and the rate of host independent growth. For predatory effects the persistence of bdelloplasts (post-invasion) in the predatory lifecycle was used as an indication of the mutations having an effect on growth. A list of TAT potential substrates with predicted function is also put forward, and fluorescent tagging of one of the proteins; Bd1802, was used to see it is exported by the TAT pathway and whether it is passed into the prey during predatory growth. The expression of *B. bacteriovorus* TAT-transporter substrates in *E. coli* was also used to test if they can be recognised by their TAT pathway. Complementation of *E. coli* TAT mutants was also attempted with the corresponding TAT genes from *B. bacteriovorus* using an SDS resistance assay, which has previously been shown to be a TAT-dependent process. RT-PCR analyses of TAT pathway genes and 3 *B. bacteriovorus* TAT substrates were

carried out across the predatory lifecycle of both the wild-type *B. bacteriovorus* and in the *tatA1* mutant.

The conclusions the paper draws are that *tatA2* and *tatC* are essential for viability in both HD and HI isolates of *B. bacteriovorus,* which is different to the majority bacteria such as *E. coli* where they are not, this is especially interesting as the HD100 genome contains a second *tatA* gene. The *tatA1* gene could be inactivated, but resulted in reduced predation efficiency and host independent growth. Expression of the *tatA1* gene was found to correlate to the entry of *B. bacteriovorus* to the prey cell, suggesting it has a role in the exportation of lytic enzymes into the prey cell cytoplasm. In the *tatA1* mutant background, changes in the expression of *tatB* and *tatC* were observed, suggesting a compensatory effect. Complementation of the *E. coli* TAT mutants with the *B. bacteriovorus* versions showed that only *tatA2* and tatC could complement their respective genes, whereas *tatA1* could not and *tatB* repressed growth of the *E. coli* mutant. Out of the predicted TAT substrates, eGFP-tagging of *bd1802* showed it was exported into the prey cell during the early stages of predatory growth inside the bdelloplast, though the gene was not essential to the viability of *B. bacteriovorus.* The work here has showed not only does the TAT pathway have a role in predation, but also that mutation of each of the genes has an effect of expression of the other genes involved in the pathway, and though the HD100 genome encodes a two *tatA* genes, both are required for effective predation.

Author Contribution

Chien-yi Chang

Dr. Chang was the main author of the manuscript, and also carried out the RT-PCR expression analysis of the TAT substrates and pathway proteins. Dr. Chang also cloned and mutagenized the TAT pathway genes; *tatA1*, *tatA2* and *tatC* to show the effect disrupting these had on the lifecycles in *B. bacteriovorus*.

Laura Hobley

Dr. Hobley as well as contributing to the writing of the manuscript she aided Dr. Chang in the cloning and subsequent mutation of the TAT genes in *B. bacteriovorus* and carried out both the HI growth and predatory assays for the TAT mutants in *B. bacteriovorus*.

Rob Till

Rob helped with the cloning of the TAT genes and their mutation as well as cloning the fluorescent tags to the TAT substrates. Rob also carried out the fluorescence microscopy of the *B. bacteriovorus* expressing the tagged TAT substrates and investigated *E. coli* expressing *B. bacteriovorus* tagged substrates.

Michael Capeness

I cloned the *tatC1* gene and mutated it in *B. bacteriovorus* by the insertion of a kanamycin cassette; I also initially carried out the expression of the TAT genes and their substrates by RT-PCR, confirming Dr. Chang's results. I also contributed writing of the manuscript and also carried out the initial mobilisation of the *apraR* cassette to make the pUC19:apraR construct.

Machi Kanna

Dr. Kanna carried out the electron micrographs of late stage bdelloplasts of the

tatA1 mutant to test any at any morphological changes at the later points of the predatory lifecycle.

William Burtt

William was an undergraduate student supervised by Dr. Chang and carried out the SDS resistance assay of the *E. coli* TAT mutants expressing *B. bacteriovorus* TAT proteins. William also carried the experiments to show if the *B. bacteriovorus* TAT components could complement the *E. coli* Tat mutants.

Pratik Jagtap

Dr. Jagtap was responsible for the bioinformatics side of the paper, listing the predicted TAT substrates and their predicted role in predation and/or host independent growth.

Shin-Ichi Aizawa

Prof. Aizawa, along with Machi was responsible for the electron micrographs of late stage bdelloplasts if of the *tatA1* mutant and interpreting any morphological abnormalities of the *B. bacteriovorus* strains or the bdelloplasts of their invaded prey.

Sockett RE

Prof. Sockett both contributed to the editing and writing of the manuscript as well as designing the research experiments and formulating the hypotheses from the data.

Effects of Orally Administered *Bdellovibrio bacteriovorus* on the Well-Being and *Salmonella* Colonization of Young Chicks

Robert J. Atterbury, Laura Hobley, Robert Till, Carey Lambert, Michael J. **Capeness**, Thomas R. Lerner, Andrew K. Fenton, Paul Barrow, R. Elizabeth Sockett

Words: 8993

Paper Summary

This paper was the first published use of *B. bacteriovorus* to test its antibacterial effects in live animals by the administration of *B. bacteriovorus* orally to young chickens in an attempt to reduce the amount of *Salmonella* colonisation. For this study, chicks were given *B. bacteriovorus* alone, as well as *Salmonella enterica* serovar Enteriditis and *B. bacteriovorus*, and the changes in gut flora in caecal and faecal samples were measured as a response to the treatment. This was measured by enumeration of the samples onto various selective agars and the changes in *B. bacteriovorus* numbers was assessed by their enumeration onto double layer overlay plates. Along with the wild-type treated chicks, one set were dosed with a strain, with a deletion of *pilA*; a gene known to be essential for predation. This was to test if predatory *B. bacteriovorus* were responsible for the change in gut flora levels and animal wellbeing. The outcome of this paper greatly underpins the use of *B. bacteriovorus* as therapeutic and biocontrol agent and answers some of the fundamental questions were it to be used as such. The paper shows that *B. bacteriovorus* was effective in reducing the amount of *Salmonella* colonisation in chickens. The paper also established that the although

the administration of *B. bacteriovorus* alone does alter the natural gut flora of the chickens shown by the decrease in the number of Gram-negative bacteria, and subsequent increase in Gram-positive bacteria, it does not affect the chicks wellbeing.

Author Contribution

Robert J. Atterbury

Dr. Atterbury obtained ethical licensing for all the experiments involving chickens. Dr. Atterbury along with Prof. Sockett and Dr. Hobley designed both the experiments and the research and contributed to the writing of the manuscript. He also was responsible for the dosing of the birds, the dissection of their caeca and collection of their faecal samples, and also the statistical analysis of results and experimental preparation.

Laura Hobley

Dr. Hobley, along with Dr. Atterbury designed and carried out the enumeration experiments and writing the manuscript also carried out the bird dosing and statistical analysis of results. Dr. Hobley also grew all of the strains used in the experiment and had a role in the preparation for the experiments, as well as isolating the *pilA* mutant of *B. bacteriovorus*.

Robert Till

Rob carried out enumeration of various bacteria from the faecal, caecal, and water samples onto various types of selective agar and *Salmonella* overlay plates. Rob also aided in the preparation for the experiments.

Carey Lambert

Dr. Lambert was responsible for the enumeration of faecal, caecal, and water samples to find the changes in numbers of different natural and introduced bacteria. Dr. Lambert also helped in the experimental preparation and contributed to the writing of the manuscript.

Michael J. Capeness

I was involved in the enumeration of various different bacteria such as *Salmonella*, *E. coli*, *Lactobacilli, Streptococci* and *B. bacteriovorus* from samples of faecal, caecal, and water, taken from chicks and their enclosure. I also helped in experimental preparation such as preparing plates and media and the transportation of sample. I also contributed towards the writing of the manuscript.

Thomas R. Lerner

Tom helped in the preparation for the experiments, and enumerated bacteria from fecal, caecal, and water samples.

Andrew K. Fenton

Andrew helped to prepare for the experiments and also helped to enumerate bacteria from faecal, caecal, and water samples. Andrew also hand a hand in experimental preparation.

Paul Barrow

Prof. Barrow hosted and facilitated the experiments involving the animal work.

R. Elizabeth Sockett

Prof. Sockett was responsible for experimental design and research as well the forming of hypotheses from the data. Prof. Sockett also had major role in the writing and editing of the manuscript and also had a role in chicken dissection and the enumeration of bacteria from faecal, caecal and water samples.

The Structure of an Unconventional HD-GYP Protein from *Bdellovibrio* Reveals the Roles of Conserved Residues in this Class of Cyclic-di-GMP Phosphodiesterases

Andrew L. Lovering, Michael J. **Capeness**, Carey Lambert, Laura Hobley, R. Elizabeth Sockett

Words: 5284

Paper Summary

The paper presents data on the first HD-GYP protein from any organism to have its crystal structure solved, and only the third crystal structure of a protein in the history of *B. bacteriovorus*. HD-GYP is a phosphodiesterase that breaks down cyclic-di-GMP, which is made by GGDEF proteins and normally degraded by EAL proteins. Work here has attempted to distinguish the activation characteristics of the HD-GYP protein from the EAL by an in-depth look at its crystal structure, and possibly shed light on why other structures of HD-GYP proteins have not been solved. This is of great importance as many bacteria use cyclic-di-GMP as secondary messenger to regulate genes involved in their lifecycles and also pathogenicity. Deletion study of the gene that encodes this HD-GYP protein; *bd1817* was also undertaken to assess its role in both the predatory lifecycle and the host independent lifecycle, and the Bd1817 protein was also fluorescently tagged. The paper reports the crystal structure of HD-GYP protein from *B. bacteriovorus*, the first of its kind to be solved and so has a wide impact on the field of GGDEF research. The structure was shown to possess a

binuclear metal centre and to interact with a phosphate molecule, thus showing how the turnover of cyclic-di-GMP occurs. Studies of the fluorescently tagged Bd1817 showed that the fluorescence was only detectable in host independent isolates, and at very low levels. Expression of *bd1817* gene across the predatory lifecycle showed that it peaks between the 3 and 4 hour points. Deletion of the *bd1817* gene had no adverse effects on the predatory or host independent growth of the mutant isolates of *B. bacteriovorus* compared to wild-type.

Author Contribution

Andrew L. Lovering

Dr. Lovering designed the biochemical side of the research and experiments, as well as carrying out all the protein expression of the Bd1817 and the determination of its structure. Andy also carried out all of the bioinformatics and contributed to the writing and editing of the manuscript.

Michael J. Capeness

I carried out the deletion of *bd1817* from the *B. bacteriovorus* HD100 genome and the phenotyping of the mutant by predatory and host independent growth assays. I also tagged the Bd1817 protein with a fluorescent tag and imaged its cellular location in *B. bacteriovorus* by fluorescent microscopy and showed that it was fluorescent albeit very weakly throughout the cytoplasm. I also contributed to the writing of the manuscript as well as preparing data and images. I also carried out the RT-PCRs investigating the expression of the *bd1817* gene confirming Dr. Lambert's expression profile.

Carey Lambert

Dr. Lambert, along with myself was involved in the screening of the *bd1817* potential mutants and the phenotyping of the acquired mutant by predatory and host independent growth assay. He also had a role in the writing of the manuscript and the RT-PCR analysis of the expression of *bd1817*.

Laura Hobley

Dr. Hobley had a role in taking of electron micrographs of the *bd1817* mutants to determine any differences in its morphology compared to wild-type *B. bacteriovorus* and also contributed to the writing of the manuscript.

R. Elizabeth Sockett

Prof. Sockett designed the physiological and genetic experiments as well as designing the research and the writing and editing of manuscript along with Dr. Lovering.

Genome analysis of a simultaneously predatory and prey-independent, novel *Bdellovibrio bacteriovorus* from the River Tiber, supports in silico predictions of both ancient and recent lateral gene transfer from diverse bacteria.

Hobley L, Lerner TR, Williams LE, Lambert C, Till R, Milner DS, Basford SM, **Capeness** MJ, Fenton AK, Atterbury RJ, Harris MA, Sockett RE.

Words: 7990

Paper Summary

This paper investigates a newly isolated strain of *B. bacteriovorus* from the river Tiber in Rome, called Tiberius. It was hoped that the analysis of the strain would also give further insight into the evolutionary history of the HD100 'lab-strain' and show a core gene set that defines a *Bdellovibrio*. The strain can grow simultaneously as both a HI isolate and HD despite not having typical prey-independent mutations in the *bd0108* homologue. Another example of how the strains differ is in the colour of the cells, with the HD100 being more yellow due to carotenoid presence, whereas the Tiberius is paler. Comparing the genomes of both the HD100 wild-type strain and the Tiberius showed great synteny despite being isolated from different sources (HD100 from soil, and Tiberius from estuarine water). There were differences in gene content indicating Lateral Gene Transfer (LGT) in the Tiberius strain had occurred, whereas this is at a very low level in HD100. Genes that had been acquired were analysed by bioinformatics to give a greater understanding of the ancestry of both the HD100 and Tiberius

isolates. The main conclusion of the paper was that although the Tiberius strains retains the genes required for predation, the environment in which it lives means that it can grow prey independently to acquire genomic content from other marine and freshwater bacteria and thus gives a great evolutionary insight into the *Bdellovibrio* sp. The paper itself represents a large genome project which involved many people in the analysis.

Author Contribution

Laura Hobley

Dr. Hobley was responsible for designing experiments and originally isolated the Tiberius strain from a sample of the Tiber water as well as isolating the genomic DNA of the pure culture that was used for sequencing. Dr. Hobley also headed the bioinformatics of the DNA sequences and helped join contiguous DNA sequences, by cloning and re-sequencing, after the initial 454 sequencing. Dr. Hobley also contributed to the microscopy of the Tiberius samples.

Thomas R. Lerner

Dr. Lerner helped in bioinformatics analyses of the Tiberius sequence and the cloning of the overlapping DNA sequences between contiguous reads from the 454 sequence.

Laura Williams

Dr. Williams carried out RSD (Reciprocal Smallest Distance) and PAML (Phylogenetic Analysis by Maximum Likelihood) to identify gene orthologues between the Tiberius and HD100 strains and determine rates of evolution respectively.

Carey Lambert

Dr. Lambert carried out bioinformatic analysis of the Tiberius genome and helped carry out the microscopic analysis of the Tiberius cultures.

Rob Till

Rob carried out bioinformatic analysis of the Tiberius genome and helped cloned the overlapping fragments to join the contiguous DNA sequences after the initial 454 sequencing

David S. Milner

David helped with the microscopy of the Tiberius cultures and helped carry out the bioinformatics analysis of the Tiberius genome.

Sarah M. Basford

Sarah helped with the bioinformatic analysis of the Tiberius genome.

Michael J. Capeness

I was both helped with the bioinformatic interpretation of the Tiberius genome, and grew the samples for characterisation of the differences between the carotenoid levels between the Tiberius strain and HD100 wild-type. As one of the initial annotators I helped modify and improve the process of investigating the different potential ORFs, in total I and looked at nearly 250 Kb of DNA from the Tiberius isolate and compared it to the HD100 genome.

Andrew K. Fenton

Dr. Fenton helped analyse the Tiberius genome using bioinformatics.

Robert J. Atterbury

Dr. Atterbury helped with the bioinformatic analysis of the Tiberius genome.

Maximillian A. Harris

Maximillian helped with the bioinformatic analysis of the Tiberius genome.

R. Elizabeth Sockett

Prof. Sockett was responsible for designing experiments, leading the bioinformatic analysis of the Tiberius sequence and supplying the sample of the water from the river Tiber.

Activity of *Bdellovibrio hit* Locus Proteins, Bd0108 and Bd0109, Links Type IVa Pilus Extrusion/Retraction Status to Prey-Independent Growth Signalling.

Michael J. Capeness, Carey Lambert, Andrew L. Lovering, Kaoru Uchida, Roy Chaudhuri, L.J. Alderwick, David J Lee, David Swarbreck, Rob Till, Shin-Ichi Aizawa and R. Elizabeth Sockett

Words: 14651

Paper Summary

The paper focusses on the role the previously little understood, *B. bacteriovorus*-specific gene *bd0108* has in both host independent growth and predatory growth of *B. bacteriovorus*. A strain of *B. bacteriovorus* that has a deletion of *bd0108* has been isolated and was compared to spontaneously isolated HI mutants and wild-type cells to test for changes in predation efficiency, HI growth, and cell morphology. In particularly the presence and absence of external pili was compared between different HI mutants by electron microscopy and also by SDS-PAGE, and the extent to which *bd0108* and *bd0108* genes have roles in the formation of these is assessed. Complementation of *bd0108* mutants was also undertaken to further reinforce the role *bd0108* has in *B. bacteriovorus*. Finally RNAseq was used for different HI isolates and wild-type HD100 to see the changes in expression of genes implicated it having a role in both in HI growth and the *bd0108* pathway. Interaction between Bd0108 and Bd0109 was also tested by a natural tryptophan quenching assay and a protease protection assay.

With all the data in mind, a model for the retraction and extrusion of pili and the subsequent effect of growth mode switching was put forward. The paper shows that pilus length alters depending on the mutation in *bd0108* the isolate has, with the longest observed pilus length correlating with the presence of the 42 bp deletion in *bd0108*, and that the markerless deletion of *bd0108* have very few or no pili at all. Only deletion of *bd0108* was possible, *bd0109* was found to be essential for *B. bacteriovorus* viability. The Δ*bd0108* strain could still complete predation when offered prey, and could complete predation within the same time frame as wild-type, despite growing as a host independent isolate. The HI growth of the Δ*bd0108* mutant and other HI isolates could be complemented by a plasmid expression of the *bd0108* wild-type gene returned the HI mutant to more dominantly host dependent state. As there was an interaction seen between Bd0108 and Bd0109 seen in the protease protection assay and the tryptophan quenching assay it was hypothesized this occurs in the periplasm, based on sequence analysis of the genes in question, and that this interaction alters the extrusion and retract of the pili on the surface of *B. bacteriovorus.* The work shows a significant leap forward in the role that *bd0108* has in HI/HD growth switching that has remained a cryptic puzzle since it was first implicated 20 years ago.

Author Contribution

Michael J. Capeness

My role lay mostly in the directed mutagenesis of *bd0108,* I was involved in the writing of the manuscript designing experiments and carrying out research. I

cloned and deleted the *bd0108* gene from *B. bacteriovorus* HD100. I was also involved in cloning and fluorescently tagging the *bd0108* and *bd0109* genes and their subsequent fluorescent microscopy in both *B. bacteriovorus* and *E. coli*. I cloned the *bd0108* gene and the 42 bp deletion variant into complementation plasmids and carried out the downstream phenotyping and comparisons using HI growth assays and predation assays and investigated the colony forming to plaque forming ratios (CFU:PFU). I have also had a role investigating the expression of *bd0108* and surrounding genomic genes by RT-PCR to show co-expression. I also measured the length of pili from electron micrographs taken by Dr. Lambert and carried out the statistical analysis.

Carey Lambert

Dr. Lambert's role lay mainly with the spontaneous *bd0108* mutants variants and had a role in the writing of the manuscript as well as assaying the *bd0108* mutant for predation in a fluorescent cell invasion assay. Dr. Lambert and I also undertook the predation, HI growth and the CFU:PFU ratio assays of the *bd0108* mutants and the complementation strains by growth and viable count assays as well as plate reader assays. Dr. Lambert has also analysed the RNAseq data for the HI mutants and the wild-type strain to show that there is different gene regulation in different spontaneous *bd0108* mutant backgrounds. Dr. Lambert and also carried out the SDS-PAGE and pili isolation experiments. Most of the electron microscopy pictures showing pili presence/absence counts have also been carried out by Dr. Lambert as have the *bd0108* and surrounding gene expressions RT-PCR analysis. Dr. Lambert has also helped in the screening of *bd0109* potential mutants.

Andrew L. Lovering

Dr. Lovering has had a role in the writing of the biochemistry and native protein interaction of the manuscript, designing research and looking at bioinformatics for the function and role of Bd0108 and Bd0109. Dr. Lovering also investigated the interaction between Bd0108 and Bd0109 by AUC (Analytic UltraCentrifugation) and subsequent fluorescence anisotropy to show Bd0108-109 interactions. Dr. Lovering also carried out the protease protection assays.

Kaoru Uchida

Dr. Uchida had a role in looking at the presence and absence of pili structures on the surface of *B. bacteriovorus* and different HI isolates by electron microscopy.

Roy Chaudhuri

Dr. Chaudhuri was responsible for the transforming of the raw RNAseq data into normalised data.

L.J. Alderwick

Dr. Alderwick ran the AUC and the florescence anistrophy to show Bd0108-Bd0109 interactions

David J Lee

Dr. Lee made the protein expression clones of both Bd0108 and Bd0109

David Swarbreck

Dr. Swarbeck collected the raw RNAseq data from the arrays.

Rob Till

Rob helped to clone deletion constructs of *bd0109* and attempted to delete *bd0109* from *B. bacteriovorus*.

Shin-Ichi Aizawa

Prof. Aizawa hosted and facilitated the electron microscopy to test for the

presence and absence of pili of various HI isolates.

R. Elizabeth Sockett

Prof. Sockett was responsible for the experimental design and research as well

as the forming of hypotheses and the writing and editing the manuscript.

1.0 Introduction

Bdellovibrio bacteriovorus is a small, mono-flagellate, Gram-negative bacterium belonging to the class of delta-proteobacteria. They were first discovered in 1962 by Stolp and Petzold (Stolp and Petzold, 1962) and were first named *B. bacteriovorus* by Stolp and Starr in 1963 to mean leech-like curve that eats bacteria (Stolp and Starr, 1963). *B. bacteriovorus* invades other Gram-negative bacteria, rounding them up to form a 'bdelloplast' structure by modifying the peptidoglycan of the prey cell, and residing in the prey's periplasm (**Fig 1. parts 1-8**) (**Fig 2A**). Once inside they kill the prey bacteria and release a hierarchical cocktail of lytic enzymes to utilise the prey's own cellular content, to grow and divide, with subsequent septated progeny escaping from the bdelloplast to repeat the life cycle. Such growth involving a prey is termed Host/prey Dependent (HD). *B. bacteriovorus* can also grow Host Independent (HI), in rich media, where they grow as filamentous cells which divide and grow (**Fig 1. parts a-c**) (**Fig 2B.**). HI growth, though possible, occurs only once in 10^6-10^7 cells (Cotter and Thomashow, 1992) with the majority of cells having a mutation in the *hit* locus in the gene *bd0108* though little is known about the pathway in which this protein encoded by the gene is found.

1.0.1 *Bdellovibrio* Isolation

First isolated in 1962 (Stolp and Petzold, 1962), *B. bacteriovorus* is a rapidly motile bacterium in liquids, with the requirement to attach to and invade other Gram-negative prey to replicate in a predatory manner, or grow host independently in rich media. *B. bacteriovorus* was first observed by the slow

emerging plaques on lawns of *E. coli* growing on the surface of an agar plate.

These were reminiscent of bacteria-phage, but light microscopy of infected prey

bacteria showed they contain a smaller bacterium that was growing and dividing

(Stolp and Starr, 1963).

B. bacteriovorus has also been isolated from soil, sewage, mammalian guts and

rivers. (Varon et al., 1974) (Jurkevitch et al., 2000). (Schwudke et al., 2001)

(Hobley et al., 2012b).
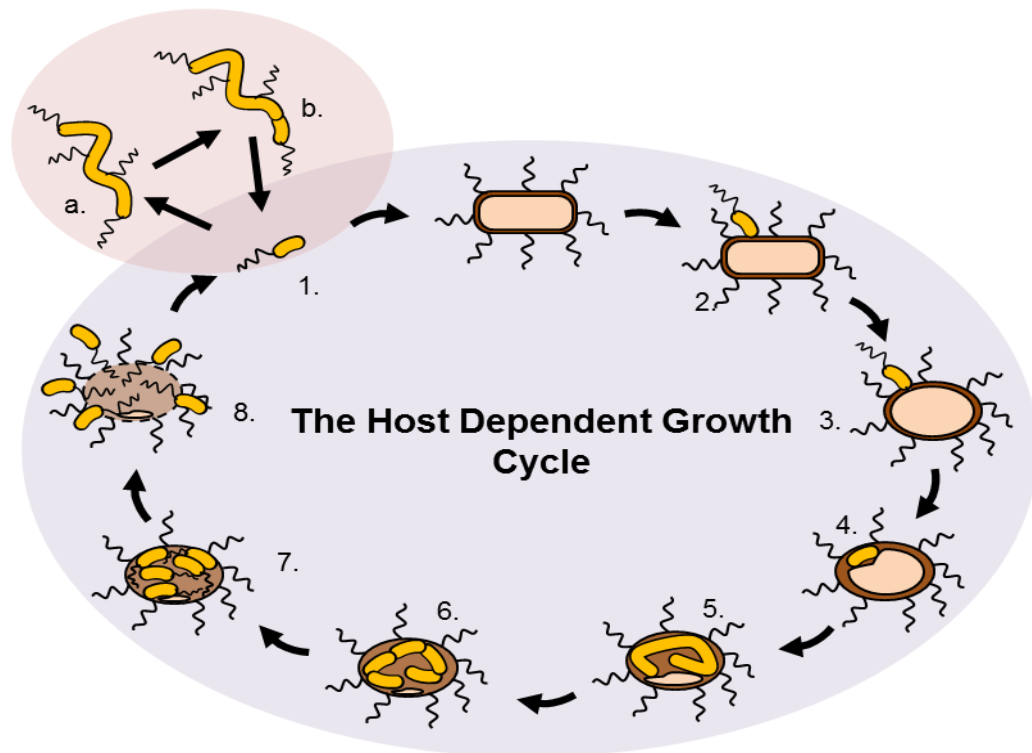
The Host Independent Growth Cycle

Fig 1. The bi-phasic lifecycle of *Bdellovibrio bacteriovorus*

The Host Independent Growth Cycle
Steps **1-9**. **1**. Attack Phase: A free swimming *Bdellovibrio* swims towards its potential prey using a complex array of MCPs and flagellar motility. **2.** Attachment: On contact with the potential prey the *Bdellovibrio* assesses whether it can invade using a series of yet to be discovered checkpoints. Should the prey not be eligible for invasion the *Bdellovibrio* can de-attach and swim away. **3.** Invasion: The *Bdellovibrio* creates a pore in the outside of the prey and releases de-crosslinking enzymes to break down the prey cell's peptidoglycan, rounding it up. The *Bdellovibrio* then invades using Type IV pili to pull itself into the prey's cytoplasm. **4.** Establishment: The *Bdellovibrio* begins the release of a hierarchical array of enzymes to breakdown the cell's content. **5.** Growth Phase: As the prey cell's content is broken down, the resultant monomers are taken up by the *Bdellovibrio* cell which uses it to grow filamentously. **6**. Septation. Once the majority of host cell's content is taken up, the *Bdellovibrio* filament septates into (on average) 6-9 cells. **7.** Flagellar synthesis: The *Bdellovibrio* progeny synthesize flagella. **8.** Release: The *Bdellovibrio* progeny escape from the now shell of a prey to begin the predatory life cycle once more.

The Host Independent Life Cycle
1. One in $10^6 - 10^7$ attack phase *Bdellovibrio* cells undergoes a genetic mutation that means it can grow free of prey cells and rely on acquiring nutrients from the media. **a.** The cell grows filamentously akin to stage 5. of the predatory growth cycle. **b.** At one end of the *Bdellovibrio* filament, a division event occurs. Allowing one *Bdellovibrio* progeny to septate from the filament to form an attack phase sized cell. This cell is then able to enter the predatory cycle, or as it still retains the initial prey independent making-mutation can grow filamentously again free of prey cells.
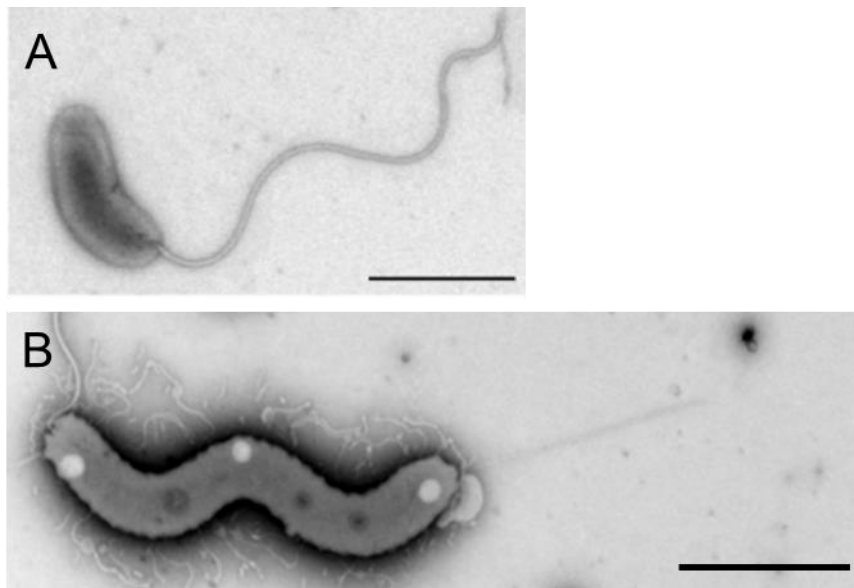
Fig 2. Electron Micrographs showing the difference in cell morphology between the Host Dependent strain of *B. bacteriovorus;* HD100 (**A**) and a Host Independent strain HID22 (**B**). Cells were stained with 0.5% uranyl acetate, pH 4. Scale bars show 1 μm. (**A**) is taken from (Hobley et al., 2012b).

1.1 Predation

B. bacteriovorus has a broad range of potential prey across Gram-negative bacteria including *Escherichia coli*, *Salmonella* sp. *Pseudomonas, Klebsiella, Enterobacter,* and *Acinetobacter* (Shilo, 1969), and strains are found in soil, fresh water, sewage, and in the human and animal gut (Varon and Shilo, 1980) (Jurkevitch et al., 2000).

Since the publication of the *B. bacteriovorus* HD100 genome in 2004 and the increasing ineffectiveness of conventional antibiotics, the potential for *B. bacteriovorus* to be used as a novel therapeutic has become more apparent. The broad range of Gram-negative prey that *B. bacteriovorus* can utilise implicates them being a useful tool to combat pathogens in either animal and plant systems as either a treatment, a preventative or food bio-security agent (Thomashow and

Rittenberg, 1979) (Lambert et al., 2003) (Rendulic et al., 2004) (Dashiff et al., 2011) (Saxon et al., 2014).

B. bacteriovorus HD100 contains a large genome (~3.8 Mb) for this size of bacterium (0.5 x 1.0 μm) within the genome is all the genes for essential lipids synthesis and *de novo* nucleotide synthesis, as well as all genes required to generate ATP from the glycolysis and fatty acid degradation pathway (Rendulic 2004). The HD100 genome contains genes for 150 proteases, 10 glycanases, 20 DNAases, 9 RNAases, and 15 lipases suiting the predatory niche that *B. bacteriovorus* occupies where prey cellular conversion is required. However the HD100 genome only contains genes for the synthesis of 11 amino acids and so requires prey precursors to do so (Rendulic et al., 2004) unless growing on complex media, containing amino acids, as HI isolates. This combined with the fact that predatory *B. bacteriovorus* does not replicate its genomic content outside of prey cells leads to the need to find a potential prey to replicate. (Hespell et al., 1974) (Thomashow and Rittenberg, 1979).

1.1.1 The Predatory Cycle

The first stage of the predatory cycle of *B. bacteriovorus* (**Fig 1 parts 1-8**) in liquid is a free swimming 'attack-phase' cell that in terms of DNA replication and cell division is (metabolically) mute. The attack-phase cell swims towards areas of potential prey, and can collide randomly with and attach and detach to biotic or abiotic surfaces, undergoing a yet uncharacterised recognition period to assess the potential prey.

Further work showed that *B. bacteriovorus,* preying upon *Pseudomonas* and *Erwinia,* could attach to prey cells, and penetrate through a pore, created by the *B. bacteriovorus,* to enter into the prey's periplasm, rounding up the prey cell in the process (Starr and Baigent, 1966). The prey-derived structure, known as the bdelloplast is osmotically stable (Thomashow and Rittenberg, 1978) and formed by the de-crosslinking of peptidoglycan chains by DD-endo/carboxypeptidases synthesised and secreted by the *B. bacteriovorus* just before prey entry (Lerner et al., 2012) (**Fig 3**). By the 15 minute timepoint of incubation with prey cells, the *B. bacteriovorus* begins establishment inside the periplasm and begins the hierarchical lysis of the prey cell contents and synthesis of its nucleic acids for replication of its own DNA (Starr and Baigent, 1966) (Matin and Rittenberg, 1972) (Lambert et al., 2010a). As uptake of the prey cellular content occurs the *B. bacteriovorus* lengthens and finally septates by synchronous, non-binary fission to produce up to 6-9 flagellated progeny (Fenton et al., 2010b). Once the *B. bacteriovorus* has depleted the bdelloplast of useable content i.e. not enough to make more *B. bacteriovorus* progeny, it leaves only a 'ghost' of the prey cell consisting of a mono-layer of just the outer membrane, the progeny destroys this ghost and leave by either flagellum mediated motility or gliding motility to start the lifecycle again. The whole process takes from 3-4 hours for *B. bacteriovorus* strain HD100 growing on *E. coli* growing in liquid culture, and can reduce the prey cell population from 10^{-8} ml^{-1} to 10^{-1} ml^{-1} within 24 hours (**Fig 4**).
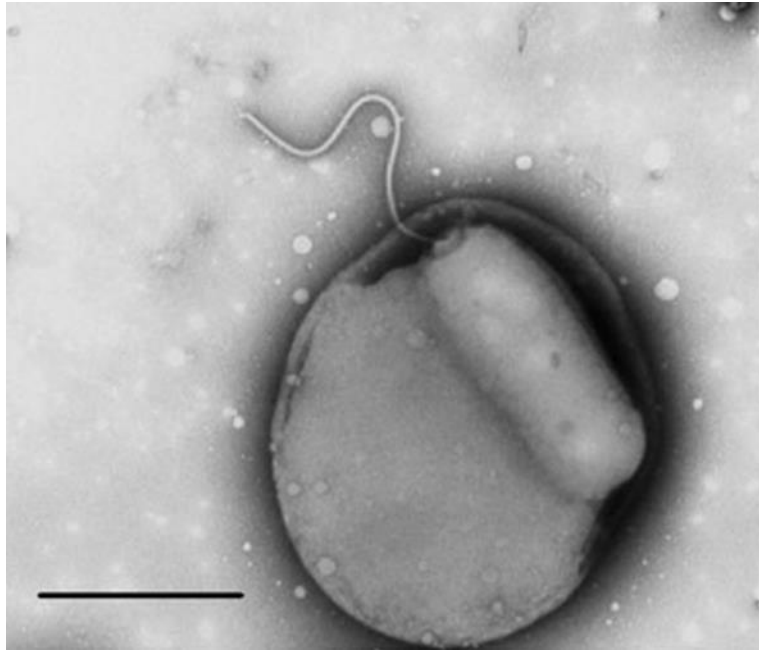
Fig 3. An Electron micrograph of *B. bacteriovorus* wild-type strain 109J inside a bdelloplast of a small *E. coli,* 20 minutes post-infection. Note the presence of the flagellum of the Bdellovibrio. 1% PTA stain. Bar = 1 μm. From **Paper 1**. (Lambert et al., 2006)
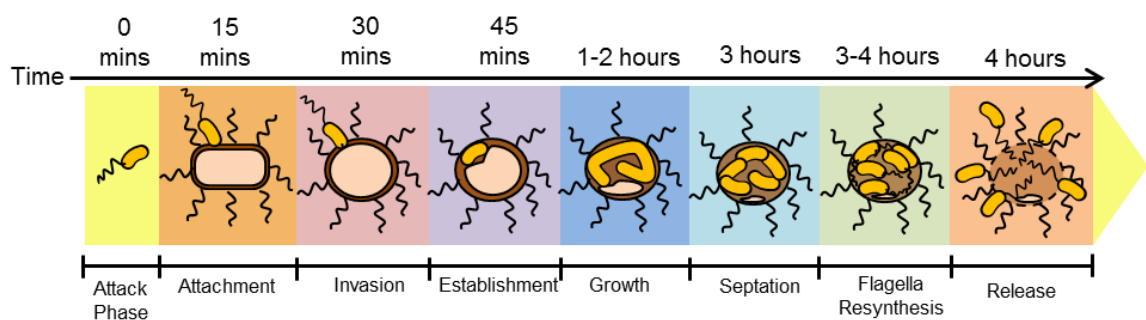
Fig 4. Timecoures of the HD lifecycle of *B. bacteriovorus*. Each timepoint represents a point at which samples are taken from the culture to be used for RNA isolation and from this it can be inferred the differences in gene expression at that specific time. Samples of the attack phase (*B. bacteriovorus* alone) and the *E. coli* alone are also taken to test against the presence of *E. coli* in the RNA sample.

1.1.2 The Process of Attachment

The attachment of *B. bacteriovorus* to a Gram-negative prey was once thought to be due to the speed at which the *B. bacteriovorus* swim, with the all the processes required for entry into the prey cell coming from physical collision between predator and prey (Starr and Baigent, 1966). This however is no longer

the case, based on work I was involved in (see chapter 2), insertion deletions of one of the six genes (*fliC3*) encoding a monomer that makes up the flagellar filament, abolishes production of the flagellum, though the mutant strain can still invade and fully lyse a prey cell albeit at a reduced rate (Lambert et al., 2006). The deletion of the *pilA* gene (*bd1290*) produces a mutant that is predation deficient, and can only grow as a HI isolate. The gene encodes the pilin monomer; PilA, involved in the production of a Type IVa pilus fibre, and this process was shown to be how *B. bacteriovorus* enters the prey cell (Evans et al., 2007). The pilus is extended on contact with the prey and enters through the pore which has been made enzymatically*,* and retracts, pulling in the *B. bacteriovorus* to the prey. Work in *Neisseria* has shown that pillus retraction can produced up to 100 pN of force (Maier, Potter, 2002). The pilus therefore provides enough force to pull the *B. bacteriovorus* through a tight pore in the outer membrane of the prey cell; with the binding tight enough that the *B. bacteriovorus* cells attached to prey cells cannot be separated by either vortex or light sonication (Burnham et al., 1968). Once inside the prey cell the pore is resealed. Varying Pili phenotypes have also more recently been shown to be involved HD/HI growth switiching as a potential feedback system to promote/inhibit growth (Capeness et al., 2013).

1.3 *B. bacteriovorus* as a Therapeutic

Despite the prevalence of *B. bacteriovorus* in nature, and its potential use as a novel anti-microbial very little research has been done to apply it to a biological system. The only research published at the time of my studies have been in fish, amphibians, isolated rabbit-ileum, and to treat infectious bovine

keratoconjunctivitis (Westergaard and Kramer, 1977) (Lu and Cai, 2010) (Boileau et al., 2011), and never *in vivo* until our publication on oral administration and chicken wellbeing (Atterbury et al., 2011) in which I was involved (see chapter 3).

With any microbial agent, resistance is a big problem and one that develops due to continual and overuse of the product, with *B. bacteriovorus* such maybe the case. Research into the recognition of potential prey by *B. bacteriovorus* has shown that there is no single mutatable receptor for the attachment and subsequent invasion unlike phage. This adds weight to the potential use for *B. bacteriovorus* as one of the major drawbacks of the use of both phage and antibiotics is that their target receptors are often modified to convey resistance, such as mutations in LamB conveying resistance to λ phage.

Recognition of Gram-negative prey cell surfaces by *B. bacteriovorus* seems more fundamental than specific cell surface receptors, given the broad spectrum of Gram-negative prey that *B. bacteriovorus* can prey upon, as receptors are not conserved across Gram-negative bacteria but predation by *B. bacteriovorus* is.

1.3.1 Resistance to *B. bacteriovorus*

There are a few examples of *B. bacteriovorus* not carrying out predation upon Gram-negative bacteria, such as the presence of an extra-cellular proteinaceous layer (S-layer) that can physically stop recognition between *B. bacteriovorus* and the LPS (LipoPolySaccharide) layer in *Caulobacter sp.* (Koval and Hynes, 1991). Another example is predation by *B. bacteriovorus* on alpha-proteobacteria, such as *Rhodobacter* which have an LPS that differs greatly from other Gram-negative bacteria (Strittmatter et al., 1983), and predation on these strains is generally slower. Though alpha-proteobacteria have different LPS that inhibit/slow

predation, studies in *E. coli* and *Salmonella* sp. of 'rough' mutants, defective and altered in LPS production produce an initial lag in predation that is eventually overcome, suggesting an adaptive population of *B. bacteriovorus,* and that LPS may not be the only factor influencing successful predation (Varon and Shilo, 1969a) (Schelling and Conti, 1986) (Gray and Ruby, 1991).

Though *B. bacteriovorus* can reduce the amount of prey cells in predatory culture by over 90%, there are some still surviving prey that are not preyed upon, these prey cell populations, once re-grown show a completely transient phenotype and can be preyed upon, with numbers being reduced to over 90% once more (Shemesh and Jurkevitch, 2004). This appears to be an apt strategy as complete elimination of the food source would be devastating for a bacterial predator that can only replicate inside a prey cell. Thus this gives the prey time to regrow.

As *B. bacteriovorus* requires a host to grow and replicate, they would be thought only to propagate when potential hosts were present and not in their absence. However, surprisingly 1 in 10^6-10^7 can grow in the absence of prey cells given nutrient-rich media. This growth and this may pose an issue if *B. bacteriovorus* were used as a therapeutic and administered to a subject as they could potentially overtake the system the *B. bacteriovorus* are initially aiding once the Gram-negative pathogens have been used up for example. This can be overcome by using predatory only mutants such as the Δ*bd1434* mutant that is obligatory predatory (Hobley et al., 2012a) but suppressor mutations to make the strain grow host independently could potentially arise in the strain.

1.4 HI Growth

Since the isolation of *B. bacteriovorus* in 1962, it was noted that they also can form saprophytic colonies on hard agar plates in the presence of heat-treated prey bacteria as well as prey supernatant, however this required a much higher number of predatory *B. bacteriovorus* cells compared to that of plaquing on prey lawns (Stolp and Starr, 1963). Further work by Shilo and Bruff (Shilo and Bruff, 1965) showed this required 10^6 - 10^7 *B. bacteriovorus* cells and could be achieved on rich protein containing media, completely free of treated prey cells. When given prey, the HI *B. bacteriovorus* cells didn't form clear, large plaque like those of HD cells but where small and turbid plaques that often contains colony growth in the centre of the plaque, but could still prey upon Gram-negative bacteria and complete the predation cycle (**Fig 1abc**). The frequency 10^6 - 10^7 was noted to be quite similar to the rate at which bacteria gained mutations (Schaaper, 1993) and it wasn't until the early 1990's that the HI phenotype was attributed to mutations in the *B. bacteriovorus* genome. The region that was mutated was dubbed the '*hit'* locus (host-interaction locus), though no function was assigned to it (Cotter and Thomashow, 1992). It was also noted that various mutations at the *hit* locus were possible, from insertion/deletions or larger deletions of 42 bp, with the latter being very common. For the 42 bp deletion mutant of the *hit* locus it was noted that the deleted region is flanked by 10 bp direct repeats that could account for the fragments excision. It should also be noted that not all HI isolates were mutated at the *hit* locus but possible in other genes involved in this growth switching pathway. Complementation with a wild-

type *hit* locus partially restored the plaquing phenotype to near wild-type (predatory) levels (Cotter and Thomashow, 1992).

HI cells are pleomorphic and are generally longer and much less motile compared to the 'comma shaped' HD cells (**Fig 2B**), but most isolates can still carry out predation, albeit at a reduced rate. Cells variably have numerous flagella, but a similar number per cell length in comparison to HD cells. The flagella are not always found at the pole as in HD cells, and the presence of flagella does not indicate presence or absence of motility. This morphology suggests that they are HD cells that have not divided successfully. The cell shape can be anything from long straight/coiled cells to short vibriods, typically measuring from 0.3 - 0.4 µm width to 1.0 - 10.0 µm in length (Seidler and Starr, 1969).

1.4.1 Bd0108

Even since the implication of the *hit* locus in growth switching by Cotter and Thomashow, (Cotter and Thomashow, 1992) and the publication of the *B. bacteriovorus* genome (Rendulic et al., 2004), only a little work has been done to assign function to the genes at the locus, the paper for which I feature on (Capeness et al., 2013) (see chapter 5.3). The gene which maps to *bd0108* in the genome sequenced HD100 is small and encodes a 101 amino acid protein, with a signal sequence at the N terminus from amino acid 1-23, and a cleavage sequence between position 23 and 24. The gene has no homologues outside the *Bdellovibrionaceae* family.

It was suggested by Rendulic (Rendulic et al., 2004) and Schwudke (Schwudke et al., 2005) that *bd0108*, the gene at the *hit* locus that was found to be mutated by Cotter and Thomashow, had a role in the formation of type IVb (flp) pili. Surrounding the *hit* locus, are genes encoding potential proteins in the flp pili pathway such as cell wall associated proteins (*bd0109*), pilin subunits (*bd0118*, *bd0119*) and pilus assembly proteins (*bd0110-0113*). Despite this no alternate pilus fibre proteins have been isolated apart from those of type IVa PilA associated proteins from cultures of predatory/non-predatory *B. bacteriovorus*. So no evidence other than the genome bio-informatics in these papers have implicated the *hit* locus and surrounding genes in flp pilus assembly until the publication on which I feature (Capeness, Lambert 2013).

As mentioned previously *bd0108* in *B. bacteriovorus* is commonly, but not exclusively mutated in HI isolates and is mutated in 89% of all HIs, with the 42 bp deletion in *bd0108*, previously found by Cotter and Thomashow (Cotter and Thomashow, 1992) and accounted for 46% of all *bd0108* mutations observed in a study of 53 HI isolates (Wurtzel et al., 2010) (**Table 1**). This suggested that not only is the 42 bp of importance as it occurs readily, but *bd0108* is the major 'hot-spot' for mutations to convey host independent growth. Other mutations of *bd0108* that have been described include insertions and single base deletions to produce a frame shift of the *bd0108* ORF resulting in truncation predicted proteins, or a complete change in amino acid sequence.

HI Isolate	Mutation in bd0108	Resultant Amino Acid Sequence
HD100	Wild-type	MKRLLVLSILLTLGFSFAGTASADENANRPVNPGEDPNEAFRSTPFEATTSALGDCRECI AYRTGATTGKGSRRHDDTVSREIKGSSATPGGSEKAGTGRQ*
HID2	Wild-type	MKRLLVLSILLTLGFSFAGTASADENANRPVNPGEDPNEAFRSTPFEATTSALGDCRECI AYRTGATTGKGSRRHDDTVSREIKGSSATPGGSEKAGTGRQ*
HID6	CC insertion after base 217	MKRLLVLSILLTLGFSFAGTASADENANRPVNPGEDPNEAFRSTPFEATTSALGDCRECI AYRTGATTGKGSPVAMTTPFPEKSKAPARRLVDLRKPGLEDSNPICLET*
HID13	G->A substitution at base 3	IKRLLVLSILLTLGFSFAGTASADENANRPVNPGEDPNEAFRSTPFEATTSALGDCRECI AYRTGATTGKGSRRHDDTVSREIKGSSATPGGSEKAGTGRQ*
HID18	Deletion of the base G at base 211	MKRLLVLSILLTLGFSFAGTASADENANRPVNPGEDPNEAFRSTPFEATTSALGDCRECI AYRTGATTGKAPVAMTTPFPEKSKAPARRLVDLRKPGLEDSNPICLET*
HID22	42 bp deletion from base 210 to base 252	MKRLLVLSILLTLGFSFAGTASADENANRPVNPGEDPNEAFRSTPFEATTSALGDCRECI AYRTGATTGKGS--------------------------SATPGGSEKAGTGRQ*
HID26	Wild-type	MKRLLVLSILLTLGFSFAGTASADENANRPVNPGEDPNEAFRSTPFEATTSALGDCRECI AYRTGATTGKGSRRHDDTVSREIKGSSATPGGSEKAGTGRQ*
HD100 Δbd0108	Complete deletion of all but 5 codons	MKGRQ*

Table 1. Mutations in the *bd0108* gene of the various *B. bacteriovorus* strains and their resultant changes to the amino acid. Amino acids sequence in blue corresponds to the wild-type sequence of Bd0108, amino acids sequences in red are the resultant change to the sequence due to the mutation. Note that despite the 42 bp deletion in the HID22 strain, the reading frame is still maintained. Also of note is the similarity between the altered amino acid sequence (in red) between HID6 and HID18. From **Paper 9.** (Capeness et al., 2013).

Before my thesis the only fully sequenced strain of *B. bacteriovorus* was HD100, but now there is another; *B. bacteriovorus* Tiberius, an environmental strain isolated from the river Tiber (the paper for which I am an author) (see chapter 4.3). It too has a *bd0108* gene (*bdt0101*), and was remarkably similar in sequence to that of the HD100 gene, save for 3 amino acid changes. Tiberius can grow host dependently and independently within the same culture, and does not require a large number of cells for the conversion of HD to HI, with no subsequent mutations occurring in the *bdt0101* gene suggesting the population is wild-type for HI growth. Interestingly the region which undergoes the 42 bp deletion in *bd0108* is present and still flanked by the 10 bp repeats (Hobley et al., 2012b).

Apart from the paper on which I am an author (Capeness et al., 2013) the only direct mutation of *bd0108* has been by insertion of a transposon element, (Roschanski et al., 2011). The deletion of 193 bp (64 codons) of the 303 bp *bd0108* gene may not however been enough to fully deactivate the subsequent protein. The resultant strain grew host independently and was weakly predatory, giving small plaques on overlay plates containing *E. coli.* Complementation of the mutation *in trans* restored the plaquing phenotype to wild-type levels similar to that which was previously reported (Cotter and Thomashow, 1992).

1.4.2 Non-predatory *B. bacteriovorus* Mutants

As *B. bacteriovorus* can grow host-independently, genes required for predation can be studied by mutagenesis and the resultant phenotypes rescued by growing them in the absence of prey. A method for isolating predation-null mutants has been devised (Medina et al., 2008) and so has a system for the generation of transposon mutants that were unable to lyse cultures of *E. coli* (Tudor et al., 2008)*.* The genes discovered had roles in c-di-GMP signalling (*bd2325*), proteases (*bd2428*, *bd3534*) and amino acid and prolipoprotein transferases (*bd1131* and *bd3375* respectively), as well as many hypothetical proteins. These genes must be vital to the survival of the predatorily growing cells and either inhibit the invasion of the prey, or cause replicative stalling once inside.

Deletion of the gene *dgcA* (*bd0742*) from *B. bacteriovorus* HD100 also results in a HI only phenotype. DgcA is implicated in GGDEF signalling a process that appears to control motility in *B. bacteriovorus*, as *dgcA* mutants were unable to escape the bdelloplast structure by either gliding or flagellar mediated motility and thus cannot complete the predatory lifecycle (Hobley et al., 2012a). This is likely due to the absence of a functional flagellar filament akin to the *fliC3* mutant

(see chapter 2.1). Conversely another GGDEF gene, *dgcC* (*bd1434*), has the opposite phenotype when mutated and cannot form host independent derivatives so is obligatorily predatory (Hobley et al., 2012a).

1.5. Flagellar Mediated Motility

For a bacterium such as *B. bacteriovorus,* which is predatory and requires prey cells to invade and grow; motility is very important. Swimming towards areas of potential host cells, or away from areas devoid of prey cells is crucial to cell survival. Each *B. bacteriovorus* has one polar sheathed flagellum that rotates to propel the cell at speeds of up to 160 µm s^{-1} (Lambert et al., 2006). Each flagellum has a characteristic dampened waveform when viewed by electron microscopy (Thomashow and Rittenberg, 1985). Upon prey entry the flagellum can be shed, though not always (Shilo, 1969) (Thomashow and Rittenberg, 1979) (Lambert et al., 2006), with regeneration of the flagella occurring after septation of the *B. bacteriovorus* cells inside the bdelloplast structure. In liquid culture *B. bacteriovorus* can swim inside the hollowed bdelloplast structure late in the predation cycle (after septation), this aids in escape from the depleted 'ghost' (Flannagan et al., 2004). Once free of the lysed prey, each *B. bacteriovorus* flagellum continues to grow using either an internal store of nutrients or those from the surrounding media (Iida et al., 2009).

In a typical bacterium, the flagellar filament, made of FliC monomers, rotates by connection to the membrane-localised motor complex, which contains over 20 different proteins. The flagella rotor made of FliG and FliF subunits, is rotated by the MotAB complex, that changes conformation based on changes in proton (H$^+$)

or sodium (Na^+) gradients across the membrane (reviewed; (Thormann and Paulick, 2010) (**Fig 5**).

The *B. bacteriovorus* HD100 genome contains 3 *motAB* genes and 6 *fliC* genes, with the FliC composition of the flagellum being known (Iida et al., 2009). The genome also encodes 18 mcps (methyl-accepting chemotaxis proteins) and 1 aerotaxis protein; these types of sensory proteins respond to environmental signals to change flagellum rotation to swim towards areas of potential prey cells, or away from areas devoid of prey. Insertional inactivation of the *mcp2* gene in the *B. bacteriovorus* strain 109J (homologue is *bd1469* in HD100; 99% identity at the protein level) affected the rate of predation in liquid culture (Lambert et al., 2003). This underlines the importance for *B. bacteriovorus* to effectively locate potential prey. Interruption, by insertional inactivation of the *motAB* genes, (see chapter 2.2) showed that it also had an effect on predation, with inactivation of each different *motAB* (1-3) pair having a different, lessening effect on predation. There is redundancy seen in the *fliC* genes, as mutation of *fliC1* and *fliC2* did not have any effect on the flagellum morphology or swimming speed of the mutant strains (Lambert et al., 2006).
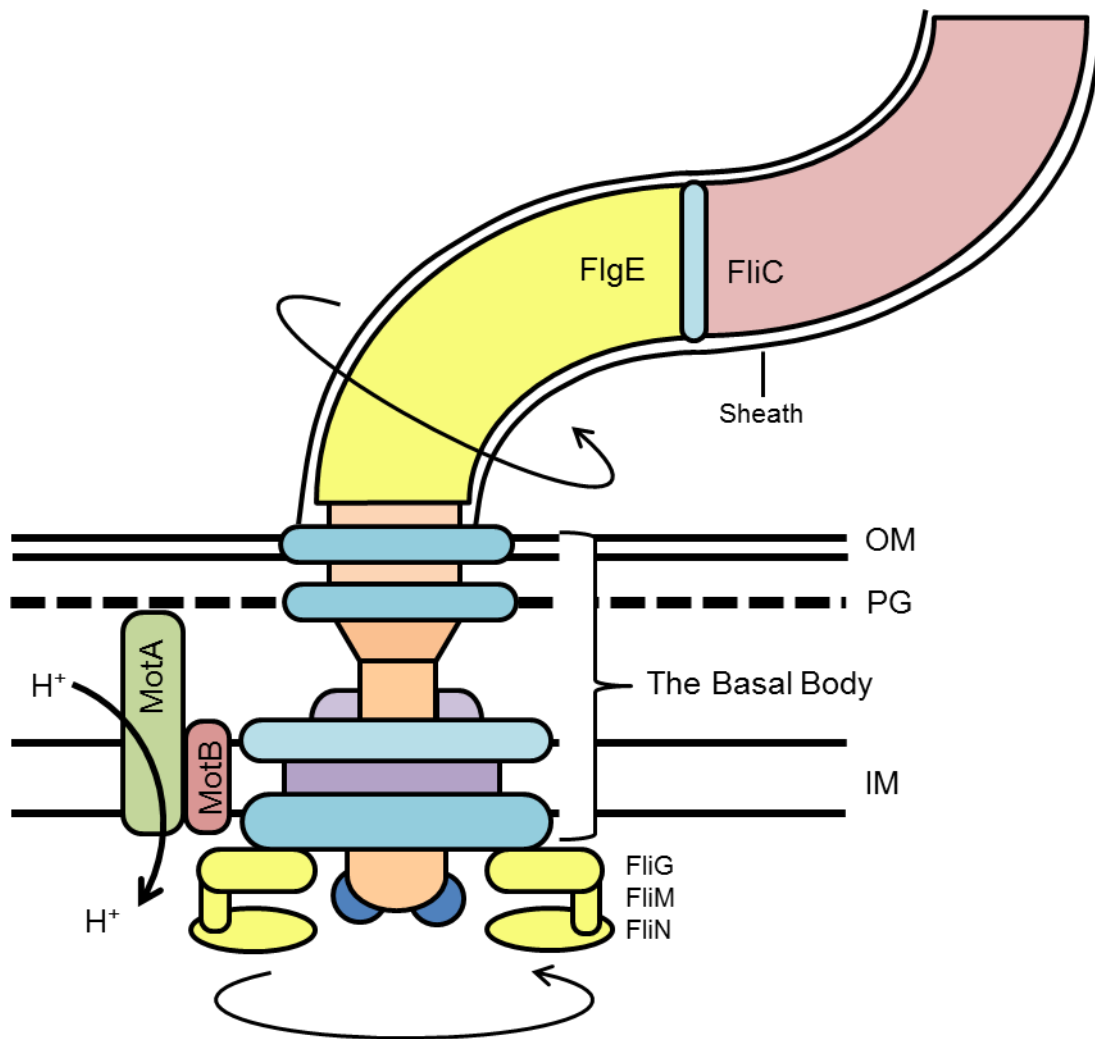
Fig 5. A proposed flagellum assembly in *B. bacteriovorus*. Influx of H$^+$ ions from the periplasm through the the MotAB complex into the cytoplasm causes a conformational change in the complex that interacts with the FliGMN complex causing rotation of the flagellum assembly. FliC (the flagellin) is connected to the assembly via FlgE (the hook). Note the additional sheath found encapsulating the flagellum filament and hook, unlike those found in *E. coli* and *R. sphaeroides*. Instead of H$^+$ ions some MotAB proteins utilise Na$^+$ instead.

In HI isolates of *B. bacteriovorus*, flagellar motility in liquid is still seen, albeit at a reduced level in the cell population, though this is not lost upon further subculturing on rich media in the absence of prey, indicating that flagellar motility is still required. Flagellar presence is also not conserved, with many HI *B. bacteriovorus* having more than one flagellum, and not always at the pole, this along with increased cell size reduces motility speed (Seidler and Starr, 1969).

1.5.1 Motility on Surfaces

Motility in *B. bacteriovorus* is not limited to flagellar mediated methods in liquid; gliding on surfaces has previously been described in both predatory *B. bacteriovorus* cells and Host Independent isolates and is completely independent of either flagella, or pili (Lambert et al., 2011). The speed of gliding was calculated as 16 µm hour^{-1} on a 1% agarose surface or 35 µm hour^{-1} when on the surface of a prey cell. *B. bacteriovorus* cells without flagella could still complete the predatory lifecycle, but had to glide out of the bdelloplast structure to find new prey. Gliding appears to be triggered by surface-sensing as there is a delay (of 1-2 hours) between the addition of *B. bacteriovorus* cultures to an agarose surface, on which they are immobilised, and the onset of gliding (Lambert et al., 2011). So far the only *B. bacteriovorus* regulatory gene absolutely confirmed to be required for gliding has been *dgcA* (*bd0367*) in HD100, a deletion mutant that cannot glide and is also non-motile by flagella, having only an empty sheath. In this mutant predation was also affected, the cells can still invade, but cannot escape the empty bdelloplast and so were only isolated as HI isolates. In *Myxococcus xanthus*, another predatory delta-proteobacterium, the Tol-like protein complexes were implicated in gliding, of which the *B. bacteriovorus* HD100 genome contains 4 operons of 8-10 genes (Mignot et al., 2007) (Mauriello et al., 2010) but so far only one of these genes has been studied; *bd0416*, and upon mutation gave no gliding related phenotype (Lambert et al., 2011).

Expressing flagellar mediated motility and gliding as well as a large array of chemotaxis genes, *B. bacteriovorus* is clearly primed to meet requirements to hunt out prey cells by various means, implicating the key need to find a prey cell and replicate within it.

1.6 Genetic Techniques in *B. bacteriovorus*

There is a plethora of different genetic techniques available to researchers in the *B. bacteriovorus* field and now so more than ever. Originally Cotter and Thomashow (Cotter and Thomashow, 1992) cloned fragments of the 109J genome for complementation of the 'HI phenotype' to restore the plaquing of HI isolates of *E. coli* plates. They consisted of large stretches of 109J genomic DNA in a plasmid, transferred to the *B. bacteriovorus* and integrated into the genome. This is how they identified the *hit* locus. The first directed mutant came in 2003 (Lambert et al., 2003) when an MCP gene was identified by screening a 109J cosmid library with a DNA probe for MCP conserved regions, the gene was sequenced and a kanamycin cassette was inserted into it. The resultant plasmid was conjugated into 109J and double crossovers were selected for. All this occurred prior to the publication of the first *B. bacteriovorus* genome sequence in 2004 (Rendulic et al., 2004).

Since the publication of the HD100 sequence, the Sockett lab has stopped manipulation of the 109J isolate (with a few exceptions) and concentrated on the HD100 strain to develop genetic techniques. With a fully sequenced genome, direct targeting of genes could be carried out. Initially all gene targets were amplified by PCR, with flanking regions and cloned into a plasmid and had an antibiotic cassette inserted into them, such as kanamycin, to disrupt/knockout the gene function, this allowed for the selection of ex-conjugates over wild-type cells (**Fig 6**) (**Fig 7**).
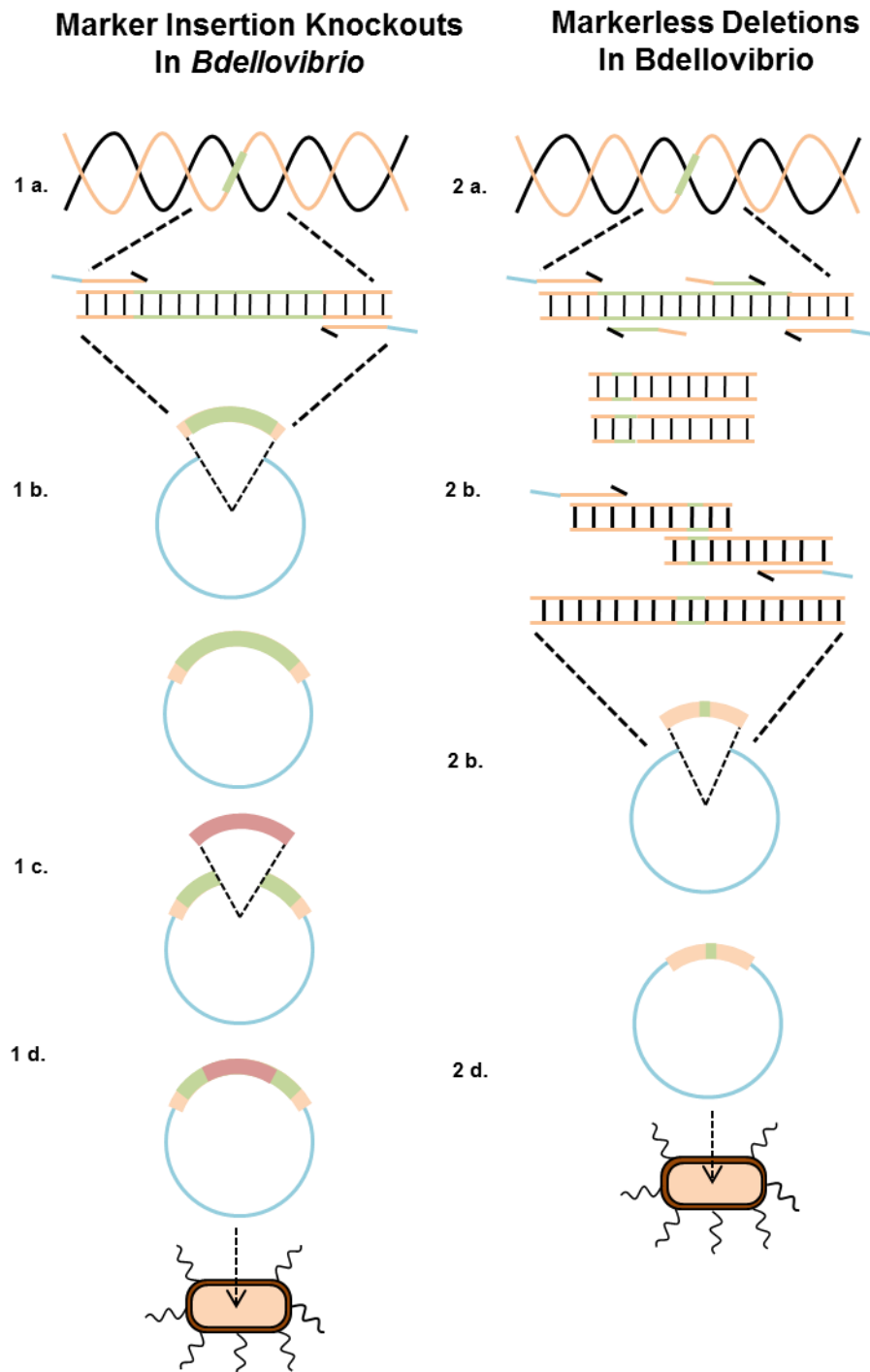
Fig 6. The two methods of gene disruption employed by the Sockett lab.
1. Marker insertion method: **1a**. Amplification of the desired gene, with flanking regions from the *B. bacteriovorus* genome. **1b**, insertion of the fragment into a cloning vector by restriction digest of the fragment and vector with compatible ends. **1c**, insertion of a antibiotic cassette into a unique site restriction site inside the gene of interest. **1d**, the plasmid is then transformed into a donor strain of *E. coli* for conjugation into *B. bacteriovorus.*
2. Markerless deletion method: **2a**, amplification of the flanking region of the gene of interest, producing to fragments with overlapping sequences. **2b**, amplification and fusion of the two PCR fragments to produce one fragment of the two flanking regions fused together. **2b**, restriction digests of the fragment into a compatible vector. **2d**, the resultant construction is then transformed into a donor strain of *E. coli* for conjugation *into B. bacteriovorus.*

The genome sequence also means greater understanding of the genes found in *B. bacteriovorus* as they can now be aligned against possible homologues from other bacteria, without the need for amplification or the sequencing of a cosmid from a library. The genome sequence also means expression arrays such as micro-arrays, or RNAseq can be carried out, to understand more about the physiological processes at the gene level to give greater insight into the transcriptional events at various points in and between the lifecycles of *B. bacteriovorus*.

Insertional knockout methods gave way to markerless deletions upon the publication of a method for markerless deletions in *B. bacteriovorus* (Steyert and Pineiro, 2007) (**Fig 6**). Now instead of using an antibiotic resistant cassette insertion to the gene, the target sequence is amplified by PCR so that the resultant sequence comprises of flanking regions to the gene in question joined together with 5 codons of the target gene, effectively deleting it. This is then cloned into a suicide vector and conjugated into the *B. bacteriovorus* to cross over into the genomic DNA, a selection is applied (in this case sucrose) and the plasmid along with the wild-type gene copy recombines and is removed from the cell, leaving only the deleted copy in the genome. The resultant cells are then tested for kanamycin sensitivity which would be conferred by the absence of the plasmid backbone.

Conjugation
Into *B. bacteriovorus*
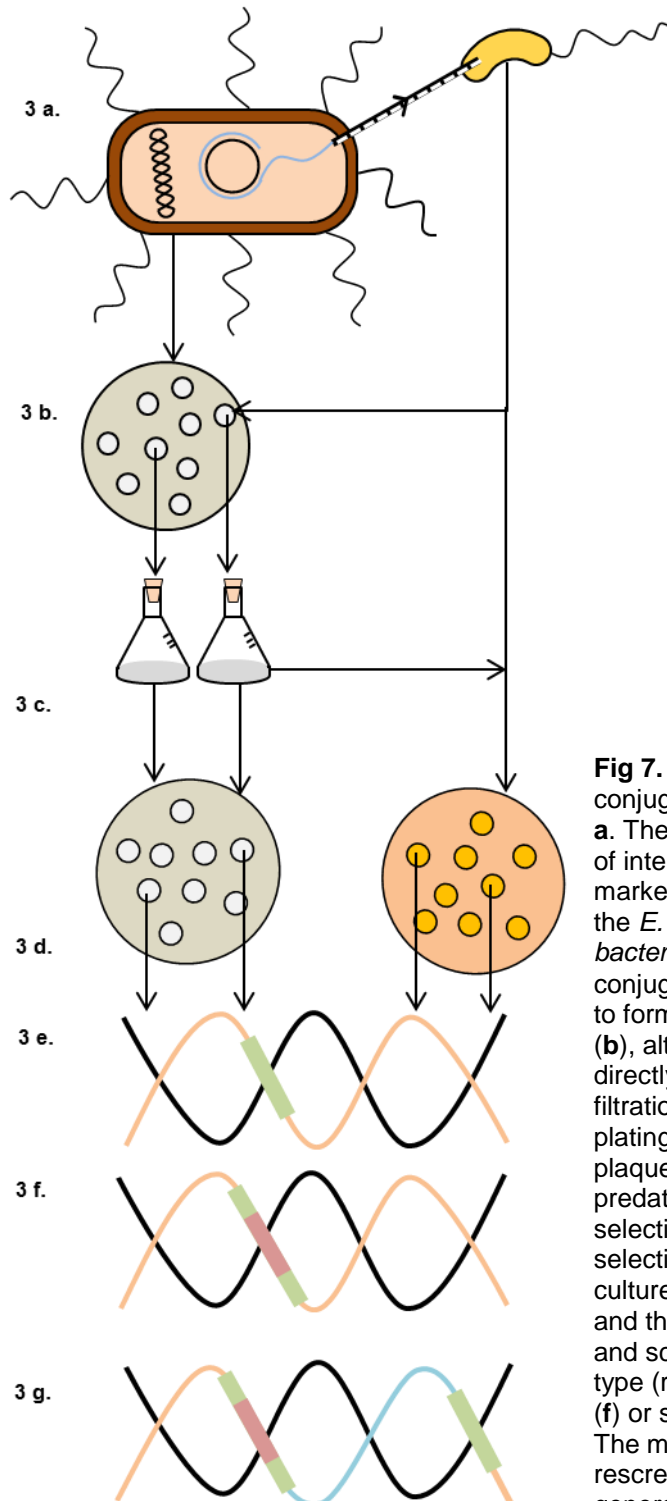
Fig 7. Conjugation and resultant ex-conjugants of *B. bacteriovorus*. **a**. The plasmid containing the fragment of interest (either marker insertion or markerless deletion) is conjugated from the *E. coli* donor strain to *B. bacteriovorus*, the resultant ex-conjugates are then overlayed on plates to form plaques under antibiotic selection (**b**), alternatively HIs can be made directly from the ex-conjugatants, by filtration out of the donor *E. coli*, and plating onto rich media. **c**. individual plaques are picked and grown in predatory culture under antibiotic selection (marker insertion) or no selection (markerless selection). **d**. The cultures are re-overlayed +/- selection and the individual plaques are regrown and screened resulting either in wild-type (revertant ; **e**) , double-crossovers (**f**) or single crossovers (merodiploid) (**g**). The merodiploids are then regrown and rescreened and can also be used for the generation of HI isolates.

Silent deletion has many advantages over the original cassette insertion method, such as there being no requirement for antibiotic selection, something which is metabolically intense on the cell. Multiple deletions in the same cell line can now also be carried out without requiring multiple different antibiotic selection markers. Currently the vast majority of mutants being made in *B. bacteriovorus,* particularly in the Sockett lab use this method.

The newest advance in terms of genetic tools is a plasmid that replicates stably in *B. bacteriovorus*, called pSUP404.2 (Roschanski and Strauch, 2011). With this *in trans* complementation of targeted markerless deletions can be carried out to verify phenotypes, this is something that has not been possible until recently, complementation required the single crossover method which has associated problems. Though somewhat in its infancy, complementation is a technique that will be more prominent as time goes by.

1.7 A Note on the Order of the Thesis

This thesis is one of progression as it spans a larger timeframe than most other theses. The work in this thesis started in 2004, with the first work being carried out on *B. bacteriovorus* 109J and using insertional gene knockouts, 109J eventually gives way to HD100 which is used in the majority of the papers. Insertional gene knockouts give way to markerless deletions, and finally complementation is used to verify genetic phenotypes.

The work in the thesis is grouped by topic, rather than in chronological order (though it is chronological inside each topic) and some of the work is published several years after some of the initial experiments were carried out in order to generate more experimental data.

2.0 Flagella Motility by *B. bacteriovorus*

B. bacteriovorus is a highly motile bacterium as explained previously, and it uses this motility to effectively hunt down and prey upon other Gram-negative bacteria. *B. bacteriovorus* can not only move by flagellar mediated methods, but also by gliding allowing it two separates methods to locate prey either in liquid culture or on a surface (Lambert et al., 2011). Thus the *B. bacteriovorus* HD100 genome shows that the bacterium dedicates a large amount of genomic content to motility with at least 2.62% of annotated genes (94/3584) having homologues in other bacteria implicated in flagellar mediated motility, though 34% of the genome has no predicted function (Rendulic et al., 2004).

The first work on motility in *B. bacteriovorus* involved investigating chemotaxis towards and away from the susceptible prey, identifying a variety of different organic compounds, including amino acids that act as attractants, (LaMarre et al., 1977) (Straley and Conti, 1977) (Straley et al., 1979). The flagellar filament has also previously been worked on, with some of the components of both the filament and the sheath it is contained in analysed and its content determined (Rendulic et al., 2004). Despite these studies there were no genes identified to the correlate with this data to, it wasn't until 2003 when the first genetic study of *B. bacteriovorus* began by the Sockett lab that showed mutation of one of the MCP genes in the lab strain 109J (*bd1469*) gave a reduction in predation.

It wasn't until the *B. bacteriovorus* HD100 genome paper that it was identified just how important flagellar motility is. In which it was discovered 20 MCP genes and 1 aerotaxis gene that have a role in cell response to surrounding conditions in the

media relating to the presence/absence of potential prey (Rendulic et al., 2004). The paper also showed that there were six predicted flagellin genes for the production of a sheathed flagellum, 3 predicted *motAB* genes which are the motor that drives the flagellar rotation as well as a full complement of other genes involved in the production of a full flagellum. Given this the field of genetics in *B. bacteriovorus* was now more approachable as the sequences for all the genes implicated in flagellar synthesis and motility were known it just remained for a research lab to work out the importance and role of these genes in *B. bacteriovorus.*

2.1 Paper 1.

Characterizing the flagellar filament and the role of motility in bacterial prey-penetration by *Bdellovibrio bacteriovorus*

Carey Lambert, Katy J Evans, Rob Till, Laura Hobley, Michael **Capeness**, Snjezana Rendulic, Stephan C Schuster, Shin-Ichi Aizawa, R Elizabeth Sockett

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My Contribution

For this first paper I was mainly involved in the production of total RNA from the flagellin mutants, and the RT-PCR analysis of the expression of the different *fliC* genes to characterise the difference in expression of the flagellin genes in the mutant backgrounds. This involved the isolation of RNA using a Promega SV total RNA isolation kit and the subsequent checking of whether the isolated RNA was free of DNA, by way of a Taq PCR. I also determined the RNA concentration and quality, by spectrophotometry (NanoDrop®) and Agilent Bioanalyzer®

respectively. This is the first time RT-PCR analysis of gene expression has been published in the *B. bacteriovorus* research field with the intent to compare both wild-type and gene mutants.

What the paper claims

The main aims of the paper were to identify each of the flagellin genes and inactivate them by insertion of an antibiotic resistance cassette into each of the highly conserved homologues in strain 109J. The resulting phenotypes would give an insight into the role each one of the genes plays in flagellar assembly by observation by electron microscopy and predation by luminescence predation assay. Given the sequences of the flagellin genes, Q-PCR and RT-PCR could be carried out to investigate different expression across the *B. bacteriovorus* predatory lifecycle, or indeed their expression in the HI growth phase.

The sequences of the flagellin genes in *B. bacteriovorus* HD100 were found to be conserved in another wild-type lab strain 109J (**Table 2**). In *B. bacteriovorus* 109J, there are six flagellin genes (*fliC1-6*, Accession Numbers: AJ851165, AJ748315, AJ748317, AJ748319, AJ748318, AJ748316, and in HD100 they correspond to CAE78570.1, CAE78572.1, CAE78395, CAE78397.1, CAE80817.1, CAE78149.1 respectively) found at 3 different locations on the genome.

Each of the six *fliC* genes were mutated by the insertion of a kanamycin antibiotic resistance cassette inserted into a unique restriction site in each gene (**Fig 8A**).

Strain HD100 GenBank Accession No.	Strain 109J EMBL Accession No.	Bdellovibrio gene/ organism consensus	Putative σ²⁸ consensus sequence in 109J fliC promoter (if any) with two known sequences for comparison
CAE78570.1	AJ851165	fliC1	–
CAE78572.1	AJ748315	fliC2	TAAA (N15) GCCGAAAG
CAE78395.1	AJ748317	fliC3	TAAA (N16) GCCTAGAT
CAE78397.1	AJ748319	fliC4	–
CAE80817.1	AJ748318	fliC5	TAAA (N15) GGCCATTA
CAE78149.1	AJ748316	fliC6	TAAA (N15) CGGCATTG
–	–	E. coli	TAAA (N15) CCCGATAA
–	–	R. sphaeroides	TAAA (N14) GCCGTTGA

Table 2. Accession numbers of the various flic genes in *B. bacteriovorus* strains HD100 and 109J, including the predicted putative sigma 28 promoter consensus regions for each flagellin gene compared to those of *E. coli* and *Rhodobacter sphaeroides fliC*. From **Paper 1**. (Lambert et al., 2006).

Of each of the six *fliC* genes, *fliC1*, *2*, *4*, *5* and *6*, were inactivated by insertion of an antibiotic cassette and cultured in the predatory phase of *B. bacteriovorus*. Only *fliC3* could not be inactivated in this phase and had to be mutated in the HI growth phase. This was found to be because the *fliC3* mutant gave a predatory-altered phenotype. In ex-conjugants when grown in predatory cultures, the *fliC3* mutant was outcompeted by merodiploid or revertant cells that had lost the suicide plasmid, in each case the out competing cells had a wild-type copy of the *fliC3* gene and were better at predation.

By growing the *fliC3* mutant as a HI isolate, in liquid predatory cultures containing *E. coli* it was shown that they did not lyse the prey, however when applied directly to stationary prey, in this case a fluorescent strain of *E. coli* S17-1 on an agarose surface, they could enter the prey cell, grow, septate and escape the bdelloplast. The predation efficiencies of the other *fliC* mutants (*fliC1-2*, *4-6*) were also assessed in a luminescent predation assay against the 'wild-type' 109JK strain (a kanamycin derivative of 109J). Of the mutants only *fliC4* and *fliC5* had an altered predation rate besides the *fliC3* mutant.
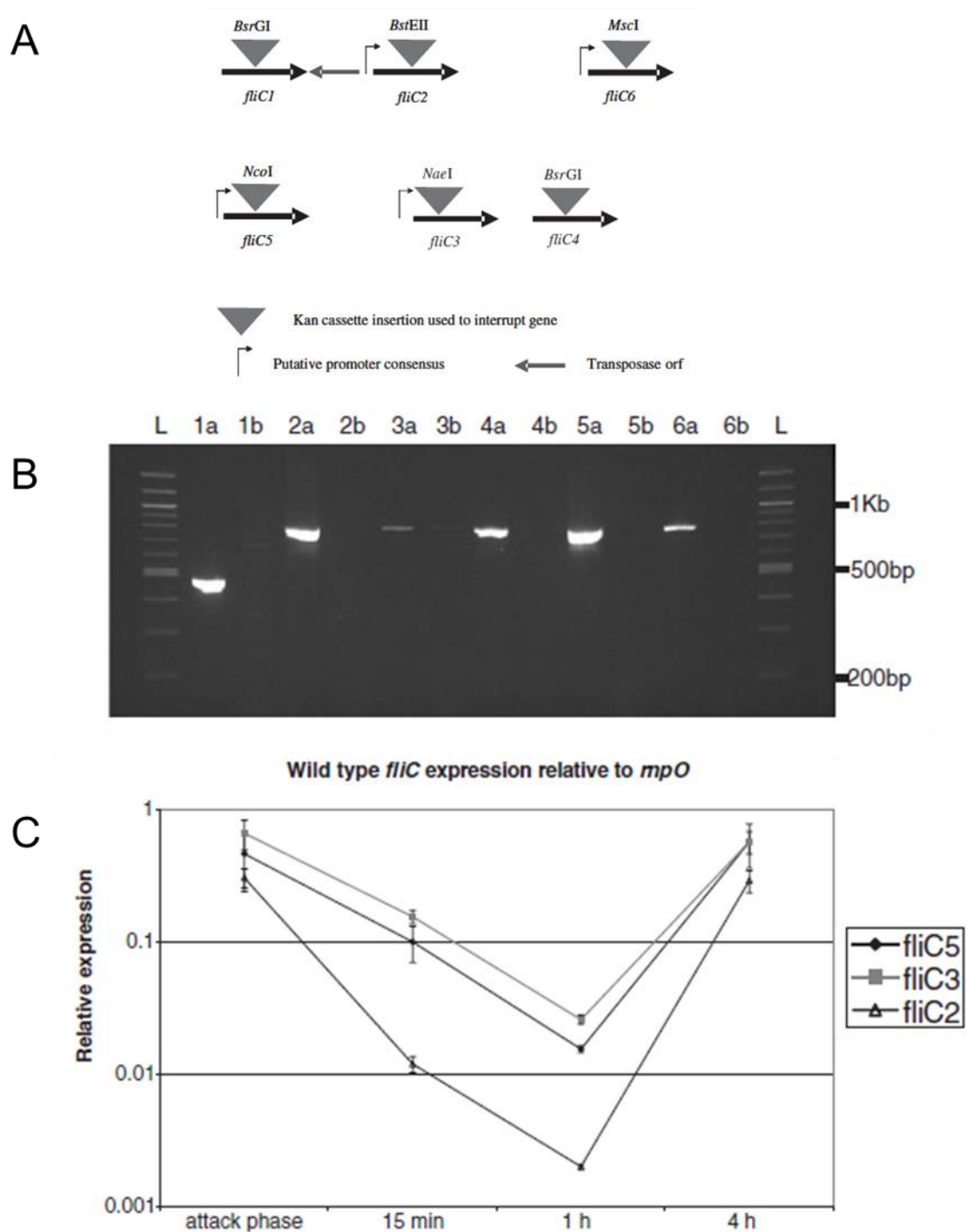
Fig 8. A. Annotation of the 6 flagellin genes in HD100 and 109J showing location and the method for insertion of a kanamycin cassette into each. **B.** RT-PCR using *fliC*-specific primers on total RNA from *B. bacteriovorus*
109J predatory culture. Lanes = 100 bp DNA ladder; 1–6: *fliC1– fliC6* (#a) and no reverse transcriptase (#b). C. Q-PCR for quantitative expression of wild-type flic genes (2, 3, and 5) in *B. bacteriovorus* 109J. Expression was normalised against *rnpO*. There is noticeable drop at the 1 hour post-infection time point. From **Paper 1**. (Lambert et al., 2006).

Motility behaviour was assessed in liquid culture by the use of a Hobson BacTracker that tracks movement in a 2-dimensional plane, against the 'wild-type' 109JK strain. It was found that the *fliC5* mutant, with the truncated flagellum, had a reduction to 80% of wild-type in swimming speed. The *fliC4* and *fliC1* mutants also showed a reduction in swimming speed to 85% and 92% respectively to that of wild-type levels. Separately the highest swimming speed of *B. bacteriovorus* HD100 was recorded at 160 μm s^{-1} and 35 μm s^{-1} for 109J.

Each of the *fliC* mutants were observed by electron microscopy, of the mutants; *fliC1*, *2*, *4*, 6 showed no discernible phenotype in respect to the length of the flagellum, or a change in the wavelength morphology (**Fig 9**). The *fliC5* mutant however did show a truncated flagellum length compared to the wild-type 109JK strain and as such had altered wavelength morphology as it lacked the distal dampened waveform repeat seen in wild-type cells. The *fliC3* mutant showed a complete absence of the flagellar filament with only the empty sheath present, which gives a reason for its lack of motility and predation in liquid culture.

Electron microscopy of early bdelloplast structures in the *B. bacteriovorus* lifecycle of the mutants and 109JK wild-type also showed that in the flagellum is not always shed upon entry into the prey cell, and 80% of the invaded *B. bacteriovorus* still possessed flagella, which protruded from the prey cell (**Fig 10**).
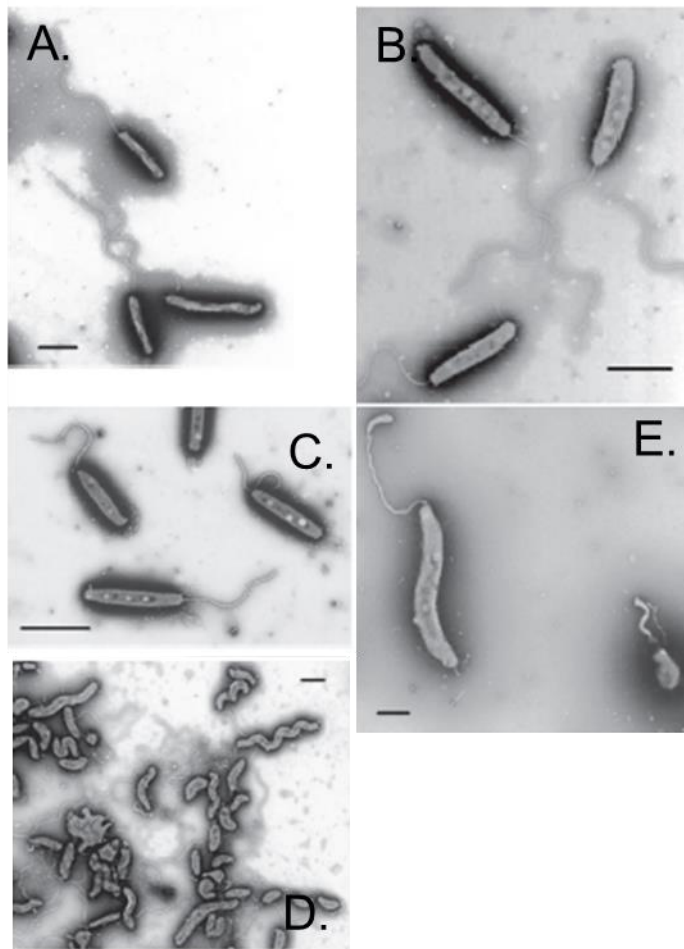
Fig 9. Electron micrographs of wild-type and *fliC* mutants of *B. bacteriovorus* 109J. **A.** 109JK wild-type showing typical length and waveform. **B.** 109J *fliC6* mutant showing to change in flagellum length or waveform (as is true for the *fliC1*, *fliC2* and *fliC4*.) **C.** 109J *fliC5* mutant showing a shorter flagellum and loss of waveform. D. HI 109J *fliC3* showing absence of flagella and just an empty sheath. E. HI 109J HIK3 showing typical flagella length and sheathing as well as variations in strain HI strains morphology. All samples were strain with 1% PTA. Scale bars = 1 µm. From **Paper 1**. (Lambert et al., 2006).
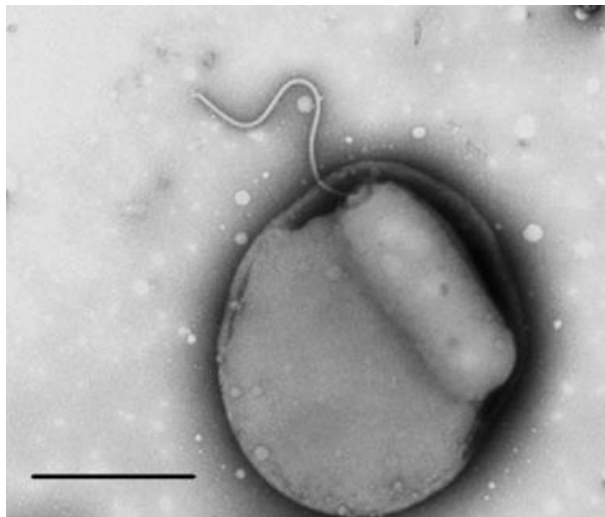
Fig 10. An electron micrograph of *B. bacteriovorus* wild-type strain 109J that has entered into an *E. coli DFB225* cell to form a bdelloplast at 20 minutes post-infection. The flagellum of *B. bacteriovorus* is still attached showing that it is not always shed upon invasion. The sample was stained with 1% PTA stain. Scale bar = 1 µm. From **Paper 1**. (Lambert et al., 2006).

Expression of the different *fliC* genes was also investigated by RT-PCR by myself, and Q-PCR by Dr. Lambert (**Paper 1 Fig 2C**). It was found that the not only do the *fliC* mutants lack their respective *fliC* gene expression (as expected),

but also that *fliC2*, *3*, and *5* expression is highest in the attack-phase stage of the lifecycle, compared to once the *B. bacteriovorus* cell had entered and become established in the prey cell. There is a return to high levels of expression of the aforementioned *fliC* genes at 4 hours as single progeny are formed and flagella synthesis occurs as the cells begin to escape the bdelloplast structure (**Fig 12**). Expression of the *fliC* genes in the fliC3 mutant background against the wild-type HIK3 was also tested for and showed an increase in the expression of *fliC2*, *5*, and *6*, compared to *fliC1* and *fliC4* which had similar levels of expression in the *fliC3* as in wild-type (**Fig 12**).
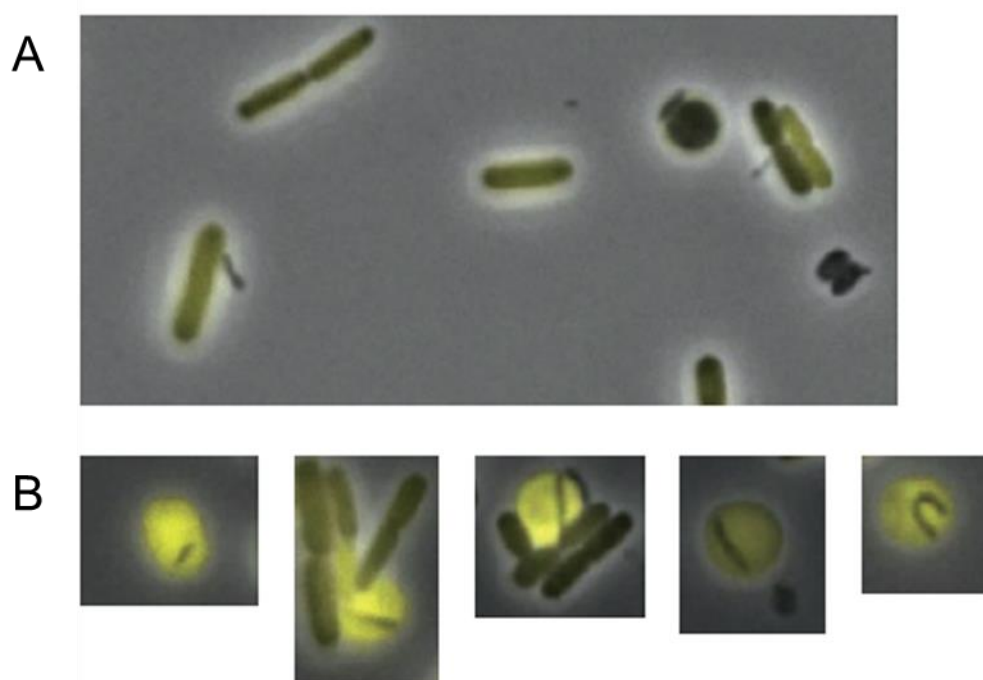
Fig 11. A. An example microscope image of the *B. bacteriovorus* 109J *fliC3* HI mutant predating upon YFP labelled *E. coli* S17-1:pZMR100. **B.** A selection of bdelloplasts by the same mutant showing that they do carry out predation despite lacking a functional flagellum. All samples were picked from overlay plates. From **Paper 1**. (Lambert et al., 2006).

Presence of the different FliC proteins in flagella shearing preparations was also found and downstream mass-spectrophotometry (Q-TOF MS) of total sheared flagellar proteins both the 109JK 'wild-type' and the 109J *fliC3* mutant showed that all but the FliC4 flagellin protein could be found in flagellar filaments (**Fig 12**). However the *fliC3* mutant; which has no flagellar filament and only the remaining flagellar sheath still contained FliC1, 2 and 5.
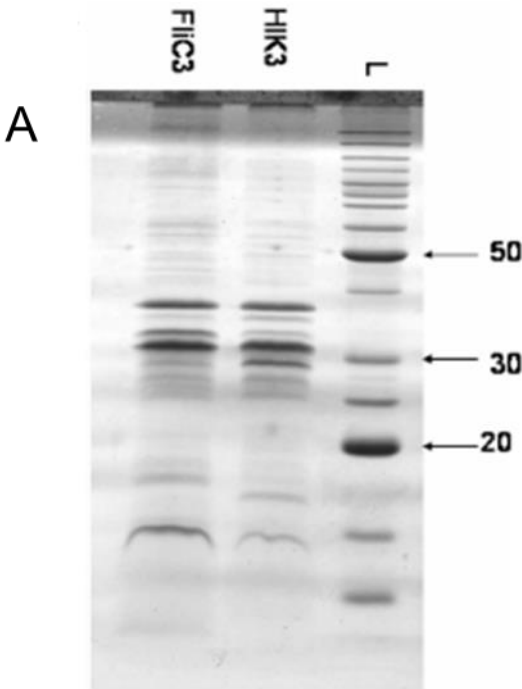
Fig. 12. SDS-PAGE of sheared flagella/ flagellar sheath preparations of the *B. bacteriovorus fliC3* HI mutant 'wild-type' HIK3 showing a difference in protein composition. L is BenchMark protein ladder.
B. RT-PCR that shows *fliC* expression levels using *fliC*-specific primers on total RNA from *B. bacteriovorus fliC*3 HI and wild-type HIK3 that is wild-type for flagella length and waveform. Expression products are seen for all *fliC* genes with the exception of *fliC*3 in the *fliC3* mutant strain. Lanes: L, 100 bp DNA ladder; 1–6: *fliC1–fliC6*, in the HI *fliC3* mutant strain; 7–12: *fliC1–fliC6* expression in the HIK3 'wild type' strain. From **Paper 1**.(Lambert et al., 2006).
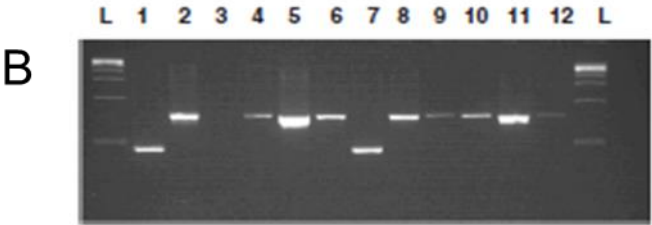
2.1.1 Conclusions of the Paper

The most important conclusion of this paper is that though the *fliC3* mutant does not have a flagellum, it can still invade prey and complete the predation cycle when placed directly onto prey. Since *B. bacteriovorus* was first observed by light microscopy it has always been thought that the speed at which they swim and the resulting collision with another Gram-negative bacteria is the reason they can enter the prey cell. The *fliC3* flagella null mutant however rejects this hypothesis and that there must be another system for predator entry such as pili, as suggested in the HD100 genome paper (Rendulic et al., 2004) and later confirmed by Evans (Evans et al., 2007). This along with electron micrographs showing that the flagella are not always shed on entry to the prey cell as suggested in early work (Thomashow and Rittenberg, 1979), this challenges the knowledge that was known about the role of the flagellum in predation.

One possible reason the *fliC3* mutant can invade into the prey cell and complete predation, though is not an effective predator in liquid culture is that along with the lowered efficiency of locating prey, once the prey cell is lysed, the mutant may struggle to escape the bdelloplast structure, thus lengthening killing time. This is something that is seen in MotAB mutants in a later featured paper (Morehouse, Hobley 2010) (see chapter 2.2).

Since the first studies on *B. bacteriovorus* had been carried out it was noted that they were highly motile, this together with the well characterised flagellar motility system in other bacteria, such as *E. coli* and *Rhodobacter*, led to the perhaps obvious choice to work on flagella production. With the HD100 genome made available the first attempts of experimenting on a gene set, in this case flagellin

production, were undertaken. Though this early work is carried out in another *B. bacteriovorus* wild-type strain, the genes were found to be highly conserved and it was only with the sequencing of the HD100 genome that the study could be carried out as the primer sequences were based on the sequenced strain though used on 109J. The fact that such high conservation is seen, not only between the *fliC* genes in the same strain, but also the *fliC* genes between the two different wild-type strains indicates that flagellin proteins are already highly tuned to the needs of *B. bacteriovorus* to be highly motile.

Since the publication of the HD100 genome paper, very few gene sequences from *B. bacteriovorus* had been made available and as such little genetic studies had been done with only one published targeted mutation of a gene having been done so far (Lambert et al., 2003). This paper took the number of published gene mutants in the field of *B. bacteriovorus* research from 1 to 8.

The paper highlights just how important motility is to *B. bacteriovorus*, as they contain multiple flagellin genes, one of the highest numbers for a bacterium, and an insertion-mutation into each has a different effect in the flagellum morphology, the swimming speeds and/or predation resulting in a less motile than wild-type cell. Particularly *fliC3* appears to be the most important of the flagellin genes as mutation of the gene results not only in the absence of the flagellar filament, but also a vast decline in predation in liquid culture, resulting in them only being isolated in the host independent growth phase. This suggests that though flagella are not critical for predation they are important for locating potential prey in liquid culture and as such the genome contains multiple copies of the flagellin genes as a 'back up' in case of deletion/inactivation of one or more of the flagellin genes.

The order in which the flagellar filament is assembled could also be suggested from the different morphologies seen when each of the *fliC* genes were mutated. As the flagellar filament grows from the distal end and is assembled hierarchically, flagellin proteins found more proximal to the cell are laid down first, and are required for the laying down of flagellin proteins found more distally. As such mutations in *fliC1* 2, 4, and 6 that had no change in either flagellum length of wavelength morphology may have their respective flagellin proteins found at the distal end. The *fliC5* mutant that had a shortened flagellum length, but still a flagellar filament present may encode a flagellin found part way between the distal and proximal end. The *fliC3* mutant however must encode a protein found at the very proximal end of the flagellum, as no flagellar filament is formed, despite secretion of other flagellin filaments (FliC1, 2, and 5).

The only expression studies that have been carried out in *B. bacteriovorus* previous to this paper were to investigate the co-expression of SOS response gene operons (Campoy et al., 2005). Our paper showed the first time course expression studies in *B. bacteriovorus* that compared the gene mutants against wild-type by RT-PCR and how they differ in mutant background. Also expression of different *fliC* genes across the time course using Q-PCR has showed that they are not constantly expressed throughout the predation lifecycle, and although expected, showed that flagellin gene expression is less when the cells do not require motility. This is something that I had the biggest role in including formulating the RNA production methods and RT-PCR reaction conditions and interpreting the results, as well establishing *fliC* expression as a marker for synchronicity in RNA preparations for forth coming *B. bacteriovorus* expression studies.

Another pioneering technique used in this paper was predation by *B. bacteriovorus* on fluorescent *E. coli* to distinguish the presence of bdelloplasts against uninfected *E. coli* cells, and also the non-fluorescent predator from its prey. It was observed that the *B. bacteriovorus* growing as a filament inside the periplasm of the fluorescent prey cell as well as when the filament has septated to give single progeny. Because of this it is possible to gauge the stage of the lifecycle the invaded *B. bacteriovorus* is at by how long the filament is or whether there is more than one dark cell shape, corresponding to possible septation. This features in many future publications from the Sockett lab.

2.1.2 Since the Publication and Impact

Our paper has been cited 47 times since 2006 and as such is the third most cited paper solely on *B. bacteriovorus* by the Sockett lab to date, the first being the *B. bacteriovorus* HD100 genome (118 citations) (Rendulic et al., 2004) and the second is MCP gene knockout paper (49 citations) (Lambert et al., 2003). Citations including work on other mono-flagellates (such as *Caulobacter* and *Shewanella*) and flagella/motility orientated reviews as well as *B. bacteriovorus* work by other labs.

The main successor to this work was jointly published between the Sockett lab and the Aizawa lab that further determined the constituents of the flagellar filament in the *fliC* mutants, and the 109J wild-type (Iida et al., 2009). They found that the flagellin proteins were arranged proximal to distally in the following order: FliC3, FliC5, FliC1, FliC2 and FliC6. However like with the aforementioned publication only small amounts of FliC4 were found and it was not possible to

characterise its position. They also further determined the quantities of each of the flagellins in the filament, and found that it was mainly comprised of FliC5, FliC1 and FliC6, with only small amounts of FliC3, FliC4 and FliC2. As well as this they noted that prolonged cultivation of each of the mutants also effected flagella length and wavelength morphology which differed when compared to wild-type in the previous paper. It was found that this was because upon their release from the bdelloplast, the flagellar filament of the *B. bacteriovorus* progeny can grow despite being in low nutrient media over the course of 5 days, with altering flagellar filament constituents.

The collaboration between the Sockett lab and the Aizawa lab on the first flagellar filament publication resulted in the start of a long running research partnership between the two labs, currently standing at 6 publications since the flagellar filament characterisation and also the awarding of two grants to continue and strengthen Anglo-Japanese collaboration, one of which, the Diawa Award (for Anglo-Japanese research), I attended the ceremony of at the Royal Society as part of the research team.

In another publication it was found that an insertion deletion of RpoE-like sigma factors in *B. bacteriovorus* had an effect on the flagellin filament length (Lambert et al., 2012). Mutation of *bd0881* gave a shorter flagellum length, but increased swimming speed, though the mutant showed diminished predation efficiency compared to wild-type. Expression of *bd0881* also matched flagellin expression across the predatory lifecycle, though deletion of *bd0881* didn't alter the expression of *fliC1*. So Bd0881 could have a role in pre-septation of *B. bacteriovorus* progeny, around the same time that time the flagellin genes are expressed. Another RpoE-like sigma factor was also mutated; *bd0743*, and had a

longer flagellum, and reduced swimming speed, though no decrease in predation efficiency was seen. Between the two mutants it could be said that different flagellar filament components affect the rigidity or torque of the flagellum, causing altered swimming speeds, where longer does not mean faster, and faster swimming speed does not mean better at predation.

Deletion of the *dgcA* gene in *B. bacteriovorus* HD100 has also been showed to have an effect on flagella synthesis (Hobley *et al.* 2012). The mutant strain had only an empty sheath containing no flagellar filament. This seems akin to the *fliC3* mutant from this study, as the protein DgcA is predicted to be involved in cycle di-GMP signalling in other bacteria it is possible that this may regulate flagella synthesis.

Techniques

The technique for RNA isolation across a predation lifecycle, and subsequent sub saturating-RT-PCR analysis is something that was pioneered (which I had a major role in) in this paper and as such their protocols are used in a vast majority of downstream publications by the Sockett lab. RT-PCR analysis are one of the initial steps when working on different genes to get an insight on where in the predation lifecycle they are expressed and perhaps where a phenotype of the gene mutant may manifest itself. The Sockett lab also uses the characteristics of the flagellin gene expression to test the synchronicity of the RNA samples taken over a time course to test whether the *B. bacteriovorus* is not stalled or growing erratically throughout the predatory cycle.

Another technique used in this publication that had downstream implications was the use of the Hobson BacTracker, a microscope mounted with a camera used to

track and record both swimming speed and tumbling characteristics for bacteria. Previously this had only been used for *Rhodobacter*, *Helicobacter* and *E. coli* not something as small as *B. bacteriovorus* (Karim et al., 1998)(Jeziore-Sassoon et al., 1998). Nevertheless, the methodology was defined here, and used in later publications involving the mutation of genes involved in flagella motility (Morehouse et al., 2011) (see chapter 2.2) (Lambert et al., 2012).

The fluorescent microscopy used to show predation of the *fliC3* mutant also had further large impacts after the publication. In a study investigating the role of the *pilA* gene in *B. bacteriovorus*, insertion mutation led to a strain that could only grow as a HI. Though this mimics the *fliC3* mutant, the *pilA* mutant could not complete predation even when directly placed on susceptible prey in a manner as described in the flagellin paper (Evans et al., 2007). Other downstream effects of this paper include the use of synchronous infections of fluorescent *E. coli* to investigate *B. bacteriovorus* septation by time-lapse microscopy and also the use of fluorescent protein tags in *B. bacteriovorus* themselves, both of these were adapted and devised from the microscopy carried out in this publication (Fenton et al., 2010c).

Something which the original *B. bacteriovorus* flagellin paper does not mention is how the non-motile *fliC3* mutant can escape the bdelloplast to further invade other *E. coli* cells. Only is it suggested in the HD100 genome paper that *B. bacteriovorus*, like *Myxococcus xanthus*, has gene homologues that are implicated in adventurous gliding motility, which is non-flagella or pili mediated. Research by the Sockett lab in 2011 showed that *B. bacteriovorus* can use gliding motility to escape the bdelloplast when predation occurs on solid surfaces rather than in liquid culture (Lambert et al., 2011). For the experiment they used

time-lapse microscopy of a deletion mutant of *fliC3*, rather than the insertion mutant, but found it could escape the bdelloplast by gliding motility. Because they had the previous knowledge that *fliC3* mutation is flagella minus, they determined that the gliding process on a solid surface was not by means of flagella.

Summary

Our paper presents a significant leap in the understanding on the role of flagella in the predatory (and HI growth) phases of *B. bacteriovorus*. The paper challenges some of the central dogma about *B. bacteriovorus* and re-establishes now fundamental beliefs . Our paper also pioneers new techniques, many of which I was involved in the setting up of and that are now found throughout other publications in our lab, some of which have been adopted by other labs. It cements a relationship with our collaborator, Prof. Aizawa for future work, some of which is featured later in this thesis. Looking at the role of flagella in motility is akin to looking at a car's wheels to work out how it moves, and as such the motility field goes a lot deeper than just the surface. The paper opens up a vast field of possibilities for further research, some of which is mentioned later such as flagella composition, and the Mot proteins, which are the actual 'motor' that drives the process.

2.2 Paper 2. Three *motAB* Stator Gene Products in *Bdellovibrio bacteriovorus* Contribute to Motility of a Single Flagellum during Predatory and Prey-Independent Growth

Karen A. Morehouse, Laura Hobley, Michael **Capeness**, R. Elizabeth Sockett

My Contribution

For this paper I was responsible for the generation and verification of the *motAB3* insertion mutation and also the purification of RNA from the *motAB* mutants and the subsequent RT-PCR analysis and interpretation of the different expression of the *motAB* genes in mutant and wild-type backgrounds.

What the Paper Claims

There are three *motAB* gene pairs in *B. bacteriovorus* HD100*,* and so three separate strains of insertion mutants of the three *motAB* genes were generated and found to be viable in both the predatory growth phase and the host independent growth phase. This showed that none of the *motAB* gene pairs is absolutely essential for predation or growth. All mutant strains could also swim, suggesting a flagellar motor made of different MotAB proteins.

Bioinformatical Analyses

Bioinformatics analysis of the *motA* genes from *B. bacteriovorus* HD100 found that they encoded an extended N-terminal sequence (*motA1*, *motA2*) or missing the first N-terminal transmembrane domain (*motA3*), however further analysis rectified this by finding an alternative start codon for each. When compared to

other MotA proteins from other bacteria these new sequences had better homology.

Further bioinformatics also compared the *motAB* gene products to both *E. coli* and *Salmonella* t*yphimurium motAB* genes, which are proton driven and the *pomAB* gene products of *Vibrio alginolyticus* that are sodium driven. It was found that MotA1 had homology to both Mot and PomA sequences from the aforementioned bacteria, while MotA2 was more homologous to that of a PomA, and MotA3 though was most homologous to a MotA, it did have a phenamil binding site that inhibits sodium driving. For the MotBs, sequence comparisons could not be made to either the MotB of *E. coli* or *S. typhimurium*, or the PomB of *V. alginolyticus*.
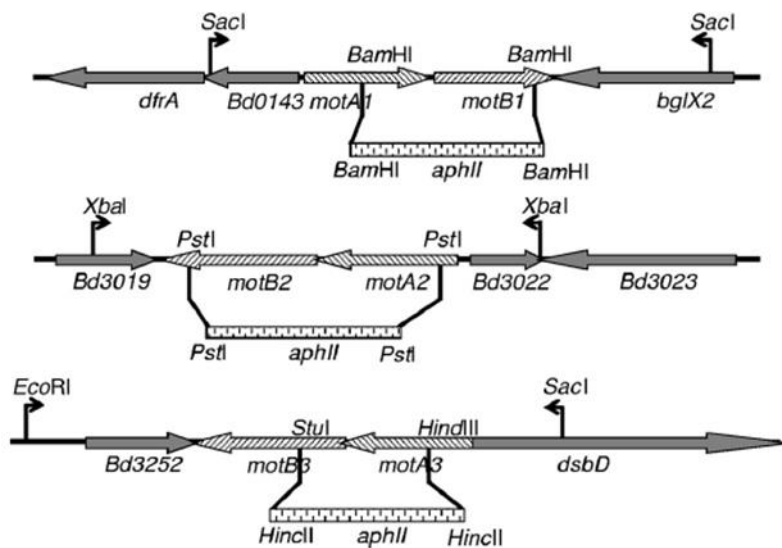
Fig 13. Insertion/deletion method for the mutagenesis of each of *motAB* pairs (hatched arrows) in *B. bacteriovorus* HD100 showing the primers used for the amplification of the region (black arrows) as well as the and the enzymes used for the insertion of a kanamycin cassette to delete function. From **Paper 4.** (Morehouse et al., 2011).

Predation and Growth Studies

Each of the *motAB* pair was successfully interrupted by the insertion of a

kanamycin cassette (**Fig 13**). For predation and HI growth studies, the *motAB* mutants were compared against a 'wild-type' HI *fliC1* merodiploid that was still an insertion mutant, though still retained a wild-type copy of the *fliC1* gene. Predation by each of the mutants over the course of 24 hours was affected differently, with *motAB3* mutant being slowest to the complete predation of the *E. coli* culture, followed by *motAB1* and *motAB2.* All of the *motAB* mutants were slower than the wild-type. Growing as a HI isolate, all the *motAB* mutants grew to a higher optical density than the *fliC1* merodiploid wild-type, while during the initial growth phase the *motAB3* deletion strain grew the slower than the wild-type and other *motAB* mutants (**Fig 14**).

In a separate experiment the *motAB1* mutant was compared against both the wild-type *fliC1* merodiploid and the *motAB2* mutant in which prey entry, bdelloplast persistence, and percentage swimming was measured over the course of 8 hours. It was found that all the mutants were comparable to wild-type in terms of prey invasion time, but *motAB1* mutant had bdelloplasts still present after 8 hours, whereas the control and *motAB2* mutant had completed the predation cycle between the 4-6 hour time points. This indicates a delay release phenotype for the *motAB1* mutant.
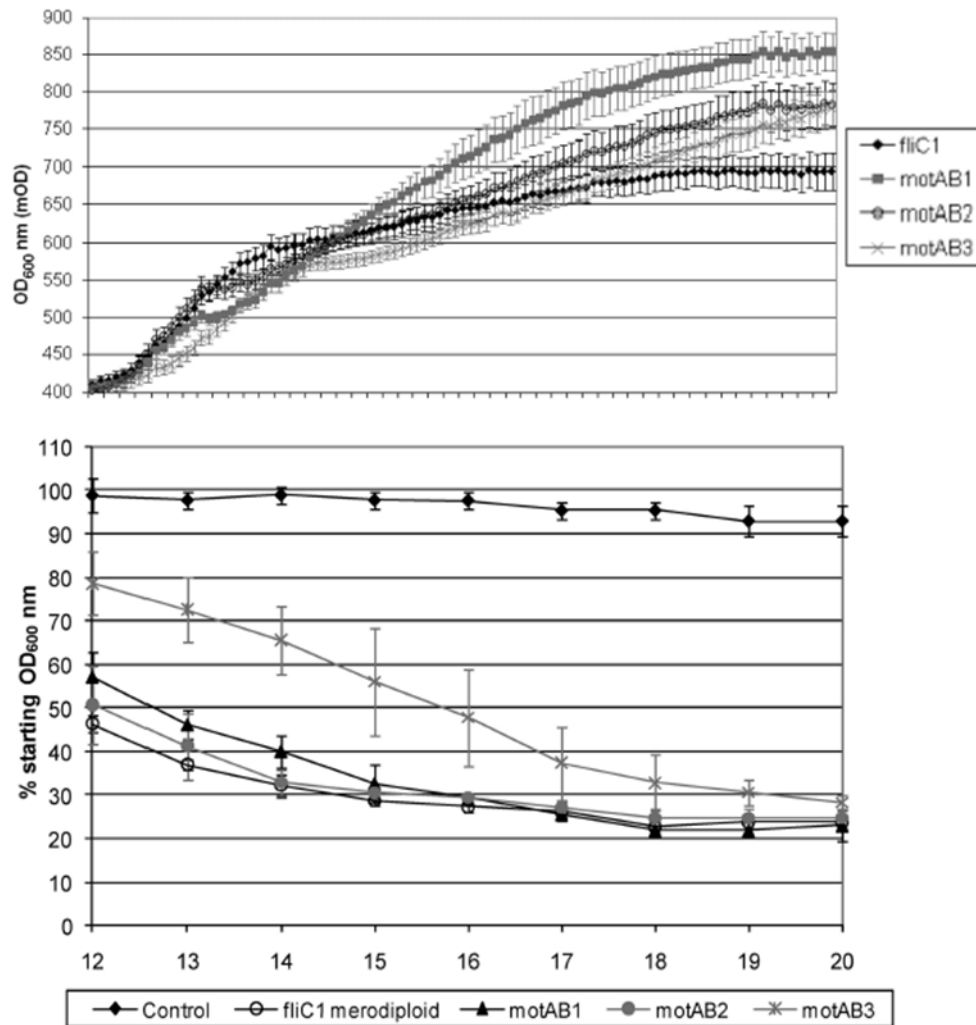
Fig 14. HI and predatory growth assays of the *motAB* deletion strains as well as the *fliC1* merodiploid wild-type control. Top: Change in optical density for each isolate from a starting density of 0.1. Bottom: Predation efficiency of the HD *Bdellovibrio motAB* deletion mutants and fliC1 merodiploid wild-type. A drop in optical density is inference of the bdelloplast lysis rate of each strain. In both cases error bars represent the 95% confidence interval. From **Paper 4.** (Morehouse et al., 2011).

Flagellar Morphology

Electron microscopy revealed that all *motAB* mutants were wild-type for presence

of flagella, and also the correct wavelength morphology indicating that mutation

of the *motAB* genes did not affect flagella synthesis (**Fig 15**). By the use of a

Hobson BacTracker, the swimming speeds and percentage motility of the

mutants were assessed and although all mutants were motile, *motAB3* had both

a slower swimming speed and percentage motility, while the *motAB2* and

motAB3 mutants only had reduced percentage motility. Using light microscopy it

was also shown that none of the *motAB* mutations had an effect on swimming in

the bdelloplast structure.
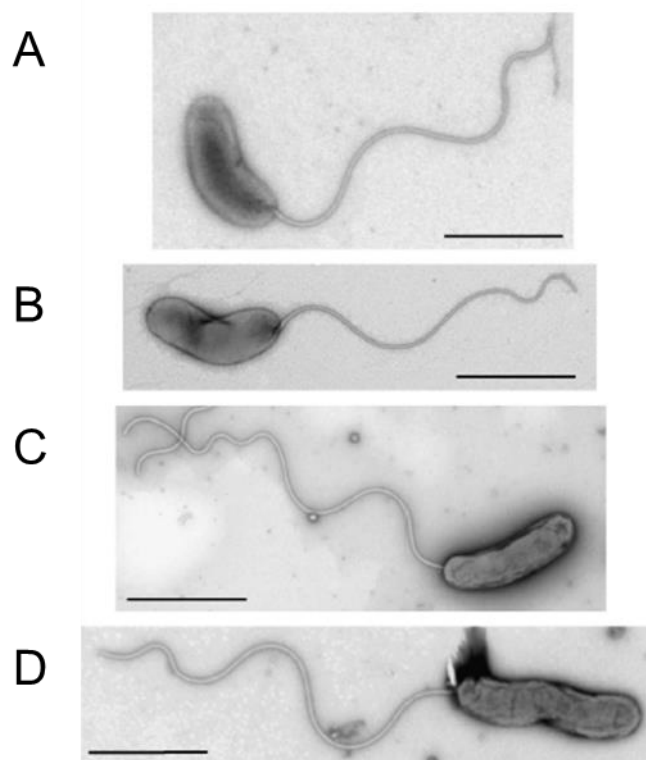
Fig 15. Electron micrographs of the HD100 *motAB* mutants. **A.** *fliC1* merodiploid wild-type; **B.** *motAB1* mutant; C. *motAB2* mutant; **D.** *motAB3* mutant. All samples were stained with 0.5% URA pH 4. Scale bars = 1 m. From **Paper 4.** (Morehouse et al., 2011).

Analysis of Swimming

Both swimming speed and percentage motility were assessed for each *motAB*

mutant strain versus the wild-type by the addition of the proton ionophore CCCP

(carbonyl cyanide m-chlorophenylhydrazone) or the sodium channel blocker

phenamil, and as a result it could also be found whether *B. bacteriovorus*

predicted Mot proteins are in fact Pom proteins and driven by sodium levels (**Fig**

16). It was found that neither the addition of phenamil or changes in the

concentrations of sodium affected motility in the *motAB* mutants. Interestingly the

percentage motility of the reconstituted wild-type (*fliC1* merodiploid control) did

decrease with the addition of 50 µM phenamil. Addition of 5 µM CCCP did completely abolish motility in all strains suggesting they all MotAB ion channels are driven by protons, rather than by sodium and that phenamil gave none specific effects.

		HD100 wild-type	*fliC1* merodiploid	*motAB1*	*motAB2*	*motAB3*
DMSO control	Swimming speed (µm/s)	78.6 ± 13.1	77.7 ± 11.9	66.1 ± 16.1	72.4 ± 18.2	26.5 ± 5.3
	% motility of culture	> 90%	> 90%	50-60%	80-90%	< 10%
Phenamil 50µM	Swimming speed (µm/s)	63.6 ± 16.2	66.3 ± 22.4	71.5 ± 14.3	74.1 ± 21.1	28.4 ± 5.1
	% motility of culture	~ 75%	~ 50%	80-90%	80-90%	~ 30%

[cccp]	[NaCl]	*fliC1* merodiploid	*motAB1*	*motAB2*	*motAB3*
0	0	79.8 ± 16.5µm/s >95%	71.9 ± 18.1µm/s ~75%	71.3 ± 21.3µm/s >95%	26.9 ± 4.0µm/s ~25%
	25mM	71.8 ± 16.4µm/s >95%	54.8 ± 13.8µm/s ~75%	76.4 ± 29.5µm/s >95%	29.2 ± 9.8µm/s ~40%
1µM	0	54.1 ± 12.3µm/s ~10%	62.5 ± 11.7µm/s ~50%	52.1 ± 12.9µm/s ~80%	23.5 ± 5.2µm/s ~10%
	25mM	56.0 ± 14.3µm/s ~10%	45.1 ± 12.2µm/s ~50%	55.0 ± 14.7µm/s ~80%	24.0 ± 6.3µm/s ~10%
2µM	0	36.9 ± 8.7µm/s ~5%	34.8 ± 9.3µm/s ~20%	43.3 ± 6.8µm/s ~50%	22.4 ± 4.7µm/s ~10%
	25mM	46.8 ± 14.3µm/s ~5%	32.5 ± 7.2µm/s ~20%	35.9 ± 8.9µm/s ~50%	19.9 ± 1.8µm/s ~10%
5µM & 10µM	0	Non-motile	Non-motile	Non-motile	Non-motile
	25mM	Non-motile	Non-motile	Non-motile	Non-motile

Fig 16. Motility of the *motAB* mutants and *fliC1* merodiploid wild-type in the presence of phenamil, NaCl, and CCCP. **A.** The mean swimming speed and percentage motility in the presence of the Na$^+$ channel inhibitor phenamil. **B.** Mean swimming speed in Ca-HEPES buffer (pH 8.2) with different concentrations of NaCl. Error bars show the standard deviation at the 95% confidence interval. **C.** The mean swimming speed and standard deviation and percentage motility in the presence of the H$^+$ channel inhibitor CCCP. From **Paper 4.** (Morehouse et al., 2011).

motAB Gene Expression

Expression of the different *motAB* genes were also investigated in the predatory

lifecycle by using RT-PCR analysis, and the genes were found to be constituently

expressed throughout, despite changes in the expression of the flagellin gene

fliC3, whose product is a component in the formation of the flagellar filament

(Lambert et al., 2006) (**Fig 12B**). It also shows that there is up regulation of

motA1 and *motB1* at the 4 hour mark, as the newly septated progeny being to

regrow flagella and escape the bdelloplast. Expression of each *motA* and *motB* in

the *motAB* mutant backgrounds was also investigated, and found that only the

levels of *motA3* and *motB3* decrease in the *motAB2* mutant strain (**Fig 17**) (**Fig**

18).
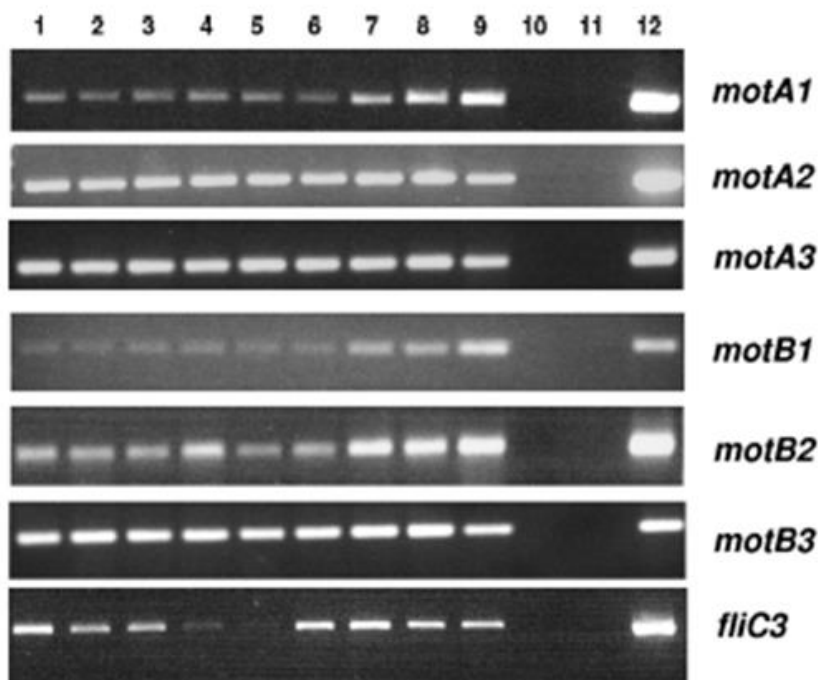
Fig 17. RT-PCR products of each of the *motA* and *motB* genes on RNA isolated from a synchronous predatory culture of *B. bacteriovorus* HD100. Also the *fliC3* gene, showing that expression synchronicity is achieved Lanes:1, attack-phase wild-type HD100; 2, 15 min post-infection; 3, 30 min post-infection; 4, 45 min post-infection; 5, 1 h post-infection; 6, 2 h post-infection; 7, 3 h post-infection; 8, 4 h post-infection; 9, HID13; 10, no-template negative control; 11, *E. coli* S17-1 only; 12, wild-type HD100 genomic DNA positive control. From **Paper 4.** (Morehouse et al., 2011).
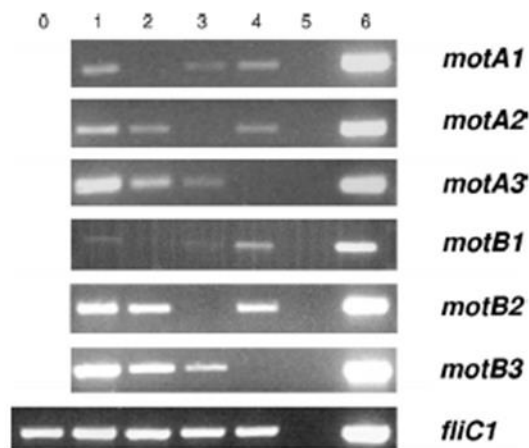
Fig 18. RT-PCR products of the three *motA* and *motB* genes on matched amounts of RNA templates from the attack-phase time-point. Lanes: 0, attack-phase *fliC1* merodiploid; 1, attack-phase wild-type HD100; 2, attack-phase *motAB1* mutant; 3, attack-phase *motAB2* mutant; 4, attack-phase *motAB3* mutant; 5, no template; 6, wild-type HD100 genomic DNA positive control. There is a noted change in expression of the *mot* gene transcripts in the different *motAB* mutant backgrounds. *fliC1* expression showed there is equal amounts of RNA present in sample. From **Paper 4.** (Morehouse et al., 2011).

2.2.1 Conclusions of the Paper

As with the characterisation of the flagellin filaments publication (Lambert et al., 2006), our paper further cements the idea that *B. bacteriovorus* is dedicated to flagellar mediated swimming to be a successful predator in liquid environments. Not only showing that the HD100 genome contains three separate *motAB* motor proteins genes, but also that neither one is absolutely essential as they can be disrupted allowing *B. bacteriovorus* to have backups that are expressed throughout the lifecycle ready to be used for motility.

Before the publication of this paper, one other had shown that a *B. bacteriovorus* 109J strain containing a plasmid encoding a *motA1* antisense RNA fragment showed that it had a reduction in predation efficiency as it could not escape from the bdelloplast structure (Flannagan et al., 2004). The paper also claims there is an associated change in swimming speed and tumbling behaviour of the mutant. This is partially supported by the work above, as a *motAB1* mutant does have a delayed escape time, but is still predatory; however we have not seen any alteration in swimming speed or tumbling characteristics. Such phenotypes could

be due to either strain differences between HD100 and 109J or a segregation defect of the plasmid containing the antisense RNA as proposed in the Flannagan study. Given that there is currently no published complete 109J genome, it is not possible to speculate the number of *motAB* genes or the interplay between them and the knockdown.

As there is a delayed escape of the *motAB1* mutant it could be possible that the MotAB1 proteins are the initial sodium channel for newly released *B. bacteriovorus,* this hypothesis fits with the increased expression of the *motA1* and *motB1* genes seen by RT-PCR analysis. We have visualised *B. bacteriovorus* swimming in the bdelloplast for each of the *motAB* mutants, though having a mutation in *motAB1* that produces lower percentage motile cell populations may lead to these late lysing bdelloplasts, as the non-motile cells remain inside longer. The difference in expression of the *motAB* genes may be due to altering concentrations of protons at the varying times in the bdelloplast structure due to the breakdown of the prey cell. It would be expected that inside a prey cell there are different concentration of protons to that outside and as such the difference in proteins encoded by the *motAB* genes may reflect this by having altered allowance for proton passage into the *B. bacteriovorus* cell.

The only evidence of the MotA and MotB proteins having a separate role as a PomA or PomB came from sequence analysis as inhibiting the *motAB* mutants strains with the sodium channel blocker phenamil had no effect. It is possible that the MotA1 and MotA2 homology to the PomA sequence from *V. alginolyticus* could be due to lateral gene transfer from a halophilic ancestor of *B. bacteriovorus* and over time ameliorated to become proton driven or it could indicate the adaption of a halophilic ancestor to a less sodium rich environment. If

this is the case, the similarity between each of the MotA proteins to the PomA proteins could indicate the order of transfer, perhaps *motA3* which is most similar to a *motA* gene, was acquired or existed first for proton conduction, and *motA1* followed by *motA2* were transferred after it from halophilic ancestors. Certainly this fits with the result that mutation of the most *motA*-like gene; *motA3* has the biggest effect on motility.

2.2.2 Since the Publication and Impact

Our paper has been cited 11 times since publication in 2011, the citations include not only by several *B. bacteriovorus* laboratories but also in work by those that work on bacteria which have more than one MotAB gene set (e.g. *Shewanella* and *Bacteriovorax,* a relative of *B. bacteriovorus*).

Since the publication of the paper, further work on motility has been undertaken by the Sockett lab. The finding of gliding motility in *B. bacteriovorus* gave a separate option for cells to move who are either without flagella or on solid surfaces was found (Lambert et al., 2011). It was also found that escape from the bdelloplast was possible without the need of flagella and neither was predation occurring on a solid surface. Escape time from the bdelloplast was also not different between wild-type cells and a *fliC3* deletion mutant that lacked flagella. This perhaps enforces the phenotype seen for mutation of *motA1* in HD100 (Morehouse et al., 2011), and down regulation of *motA1* in 109J (Flannagan et al., 2004) as when predation occurs in liquid, it is the flagellum that has a role in escaping the bdelloplast structure that is powered by the Mot proteins, but not the case when predation occurs on solid surfaces, where gliding motility takes over.

In more recent findings the nature of the Mot proteins appears to be dynamic, and the bacterium will 'switch out' different Mot proteins constantly from the flagellum mechanism to control rotation of the flagellar filament and its rotation in *Rhodobacter* (Pilizota et al., 2009). This may show how dynamic the function of the flagellum is and possibly why there is no null motility phenotype in the case of *B. bacteriovorus* when each *motAB* pair is mutated by insertional activatation, as a substitute (although less-fit) is incorporated into the mechanism to promote motility.

Further work by the Sockett lab to build on the work in this paper and relate it to the early flagella paper has focused on the role cyclic di-GMP signalling has on the motility of *B. bacteriovorus.* Cyclic di-GMP is a secondary messenger in bacteria, and the upregulation of the genes; *dgcABC* have been shown to impede flagella mediated motility (Ko and Park, 2000) (Ryjenkov et al., 2006) (Pilizota et al., 2009). Cyclic di-GMP signalling has also shown to be involved in the 'braking' of the flagella in *E. coli,* as the protein YcgR binds cyclic-di-GMP and interacts with FliG and FliM to causing 'breaking' by rotating the flagella the opposite way, however no such homologue is present in the *B. bacteriovorus* HD100 genome. The Sockett lab further built on this by analysing the function of the genes *dgcABCD* in *B. bacteriovorus* and generated markless deletion mutants for each of them (Hobley et al., 2012a). They found that mutation of *dgcA* caused the abolition of motility (mentioned previously in this chapter) and that mutation in *dgcB* increased motility in HI isolates compared to wild-type. Although no *ycgR* 'break' homologue was found, perhaps DgcB is the 'accelerator' accounting for the increase in motility.

2.3 Motility Conclusions

B. bacteriovorus dedicates a lot of its genome to motility for searching for prey, in the case of host dependent isolates, to be an effective hunter of Gram-negative cells. This is apparent from the evidence presented in these two papers as mutation of the genes encoding either *fliC3-5* or *motAB1-3* had a negative effect on the rate of predation, despite the presence of multiple homologues of both the *fliC* and *motAB* genes in the same genome. Though there was a reduction in the rate of predation none of the mutations in any of the *motAB* or *fliC* genes were predatorily null, and this challenged the previous belief that predation was flagella and motility dependent and prey cell entry came from the force of collision (Stolp and Starr, 1963) (Varon and Shilo, 1969b) (Burnham et al., 1968).The papers presented here represent a significant milestone in the understanding of the importance motility has in the lifecycles of *B. bacteriovorus. B. bacteriovorus* has quite a novel duplicated gene set with regards to motility, no other organism has 6 flagellin genes and 3 sets of *motAB* genes both of which are a high number for a bacterium with a single flagellum (for *motAB* comparison, see list: (Thormann and Paulick, 2010). Only *Caulobacter* has a higher number of flagellin genes (7, and 2 *motAB* genes), and only some members of the *Desulfovibrio* sp. and a couple of gamma-proteobacteria have more *motAB* genes. Interestingly the *Desulfovibrio* belong to the delta-proteobacteria group along with *B. bacteriovorus* as such possible ancient lateral gene transfer or divergence may have occurred.

B. bacteriovorus motility gene number and the fact that it has a sheathed flagellum, something not common in the bacterial world, portray *B. bacteriovorus*

as quite a novel 'motorist'. The complexity that this picture builds and the work present in this chapter really only scratches the surface to the workings of the *B. bacteriovorus*'s flagellum.

The papers have not only investigated motility and the genes involved in them but also introduced new techniques and methodologies to the field of *B. bacteriovorus* research which today remain a fundamental part on working with the organism.

Future Work

Since the publication of the first paper in this chapter, the Sockett lab have moved onto using the sequenced *B. bacteriovorus* HD100 strain as their background for all their work hence there is a change between the flagellin mutants in the 109J strain and those of the *motAB* mutants which are in the HD100 background. It is worth noting that what can be assessed by inference of mutants made in the 109J background maybe limited and the phenotypes do not necessarily hold true across strains. With this in mind since this publication, HD100 versions of the flagellin mutants have been made and found to have similar phenotypes.

The field of *B. bacteriovorus* in general has advanced. The main way it has is through better gene mutation techniques, such as using markerless mutations. This makes it possible to have multiple deletions in the same *B. bacteriovorus* cell without relying on different antibiotic resistances, in this case kanamycin resistance, or the antibiotics having an altering effect on the growth and behaviour of the mutant cells. With this in mind it would be possible to work out

the order in which the flagellum is assembled rather than relying on the mass spectrophotometry. It would also be possible to combine the *motAB* mutants with those of the flagellar mutants to observe swimming speed in the presence of altered flagella filaments, e.g. the *fliC4* mutant.

The sequencing of the second *B. bacteriovorus* genome from strain Tiberius has shown that the flagellin and *motAB* genes are conserved in HD100 (100% in the case of *fliC3*) (Hobley et al., 2012b). This further underpins the role of flagellar motility in the lifecycle of *B. bacteriovorus;* however it would be interesting to investigate the difference in motility of the two strains, as one is isolated from soil (HD100) and the other from a river (Tiberius). In lab conditions Tiberius is a slower swimmer (personal observations) though that is not to say if its natural environmental conditions were replicated its speed would not increase.

3.0 The Trial Application of *B. bacteriovorus* as Therapeutics

Perhaps the ultimate goal of *B. bacteriovorus* research is to develop the bacterium, or its enzymes, for use as a therapeutic. To achieve this, firstly a greater understanding of the biology of *B. bacteriovorus* is needed to assess whether the bacterium can be used for the intended purpose. Secondly, for it to be eventually used in humans or to have a direct impact on humans, it has to be tested and proven to work on higher-order animals.

Before my thesis work the only studies into the use of *B. bacteriovorus* as therapeutics are their use in fish, amphibians, or an isolated rabbit-ileum (Westergaard and Kramer, 1977) (Lu and Cai, 2010), though limited and not *in vivo* studies, they represent the first steps towards an application of *B. bacteriovorus* to treat Gram-negative infections. There has also been a study into the antigenicity of *B. bacteriovorus* (Kramer and Westergaard, 1977); this is something which needs to be addressed for *B. bacteriovorus* to be used in higher organisms, as the effect of the host's immune system needs to be known. It was shown that there is an antigenic response in guinea pig and sheep sera, so should a *B. bacteriovorus* treatment enter the blood stream there would likely be an immune response, which is ideally what is wanted so that the *B. bacteriovorus* do not colonise the blood system. Though this study was not in humans, recent unpublished work by a joint venture between the Sockett and the Pleass lab has shown there is a human immune response in isolate sera infected with *B.*

bacteriovorus, and that people who have a longer exposure to *B. bacteriovorus* do not have a reduced immune response (Sockett unpublished data).

The use and development of *B. bacteriovorus* as a therapeutic is still in its infancy, though a lot of work has been done towards understanding the biology of the bacterium, there has been only one *in vivo* trial of animals to prove that predation by *B. bacteriovorus* has an effect on lowering pathogenic or harmful bacteria, and it is the paper featured in this chapter.

3.1 Paper 3. Effects of Orally Administered *Bdellovibrio bacteriovorus* on the Well-Being and *Salmonella* Colonization of Young Chicks

Robert J. Atterbury, Laura Hobley, Robert Till, Carey Lambert, Michael J. **Capeness**, Thomas R. Lerner, Andrew K. Fenton, Paul Barrow, R. Elizabeth Sockett

My Contribution

I was involved the process of experimental preparation as well as handling the bird caeca/faeca samples for dilutions and their subsequent plating for the generation of colony counts of *Salmonella* and other gut flora. I also took samples for overlay plates on prey lawns for *B. bacteriovorus* PFU counts. These numbers were used to assess the effect the addition of *B. bacteriovorus* was having on the birds.

3.1.1 Conclusions of the Paper

The main aims of this paper were to investigate the use of *B. bacteriovorus* as potential therapeutic in chickens, firstly by testing whether dosing a bird with *B. bacteriovorus* has any ill-effects on the behaviour, growth or gut-flora of the birds, and secondly whether *B. bacteriovorus* could be used to reduce an infection of potential prey *in vivo*.

In Vivo **Predation**

Our paper showed that the administration of predatory *B. bacteriovorus* to young chicks significantly reduced the number of the colonising *Salmonella* isolates compared to those that were untreated with *B. bacteriovorus* or with a predatory-null mutant (*ΔpilA*) (**Fig 19**). There were a 0.76, 1.09, and 0.64 $\log_{10}$ CFU g^{-1} drop for days 1, 2, and 3 of the trial respectively in *Salmonella* CFU in those birds treated with predatory *B. bacteriovorus* and no drop in the *ΔpilA*-treated birds, the drop in CFU was assessed to be the fault of the *B. bacteriovorus*'s predation upon the *Salmonella*.

The young chicks were first dosed with *Salmonella* enterica serovar *Enteritidis* phage type 4 strain, a known zoonotic pathogen, and allowed to be colonised over the course of 10 days. The birds where then subsequently treated with *B. bacteriovorus* and over the course of three days, their ceaca/faeca were isolated and analysed for the presence of Salmonella CFU.
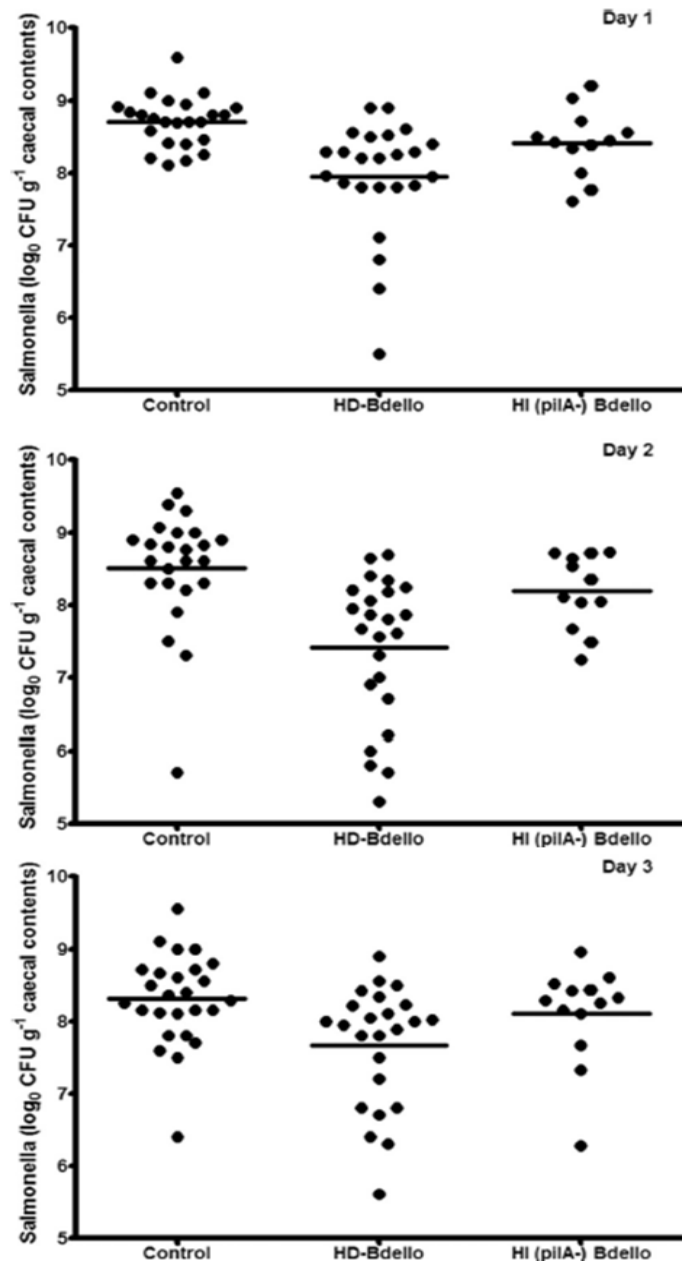
Fig 19. Scatter plots showing the effect of *B. bacteriovorus* treatment on chicken caeca colonised with *S. enteritidis*. Birds were dosed 3×10^7 *S. enteritidis* at 2 days of age and subsequently were dosed with buffer solution, 9.8×10^7 *B. bacteriovorus* HD100 or the non-predatory HI Δ*pilA B. bacteriovorus* mutant at 6 days of age.
Three days after *B. bacteriovorus* treatment, six birds from each group were sacrificed, their ceca isolated and the number of *S. enteritidis* determined by spread-plating onto brilliant green agar.
The results from four independent biological repeats were pooled from each day. Each data point represents the number of *Salmonella* colonies isolated and the horizontal line represents the means for each group. From **Paper 6.** (Atterbury et al., 2011).

Well-being Experiment

The paper also showed that administration of predatory *B. bacteriovorus* alone at had no effect on the wellbeing of the chicks with regards to lethargy, hunched posture, ruffled feathers, drooping wings, weight loss/gain, abnormal excreta, and pasty vent. This was continued over the course of 4 weeks. None of these factors differed significantly compared to the non-treated chicks (**Fig 20**). After 4 weeks, the bird's caeca were harvested and changes in gut flora were observed for those birds treated with *B. bacteriovorus,* though this did not seem to have an adverse effect on the wellbeing of the chicks with them all having no behaviour problems or morphological changes. There was an increase in the Streptococcal counts from those birds treated with *B. bacteriovorus*, suggesting that perhaps the *B. bacteriovorus* have made a window for the growth of Gram-positive bacteria by way of reducing the Gram-negative gut flora and alleviated their use of material available for growth to create new niches for more Gram-positive bacteria.

In general the morphology and appearance of the isolated caeca was improved by the administration of *B. bacteriovorus*, producing less-pale, heavier caeca isolated from those birds dosed only with *Salmonella*, which were pale, hard, and their contents difficult to solubilise, possibly due to white blood cell infiltration due the presence of the *Salmonella* (**Fig 21**) (**Table 3**).
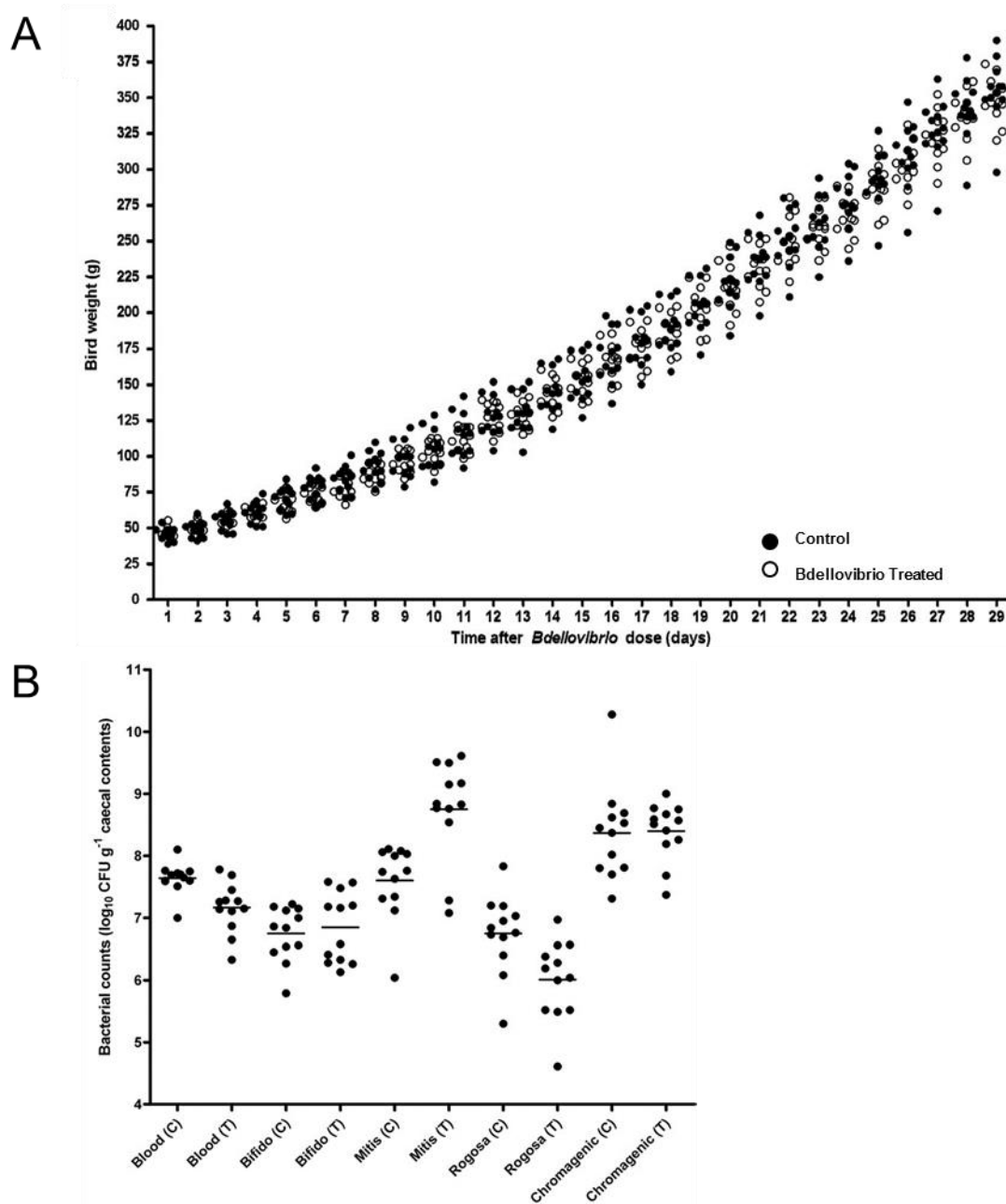
Fig 20. A. Weight gain of the non-treated birds compared to those treated with *B. bacteriovorus* at 2 days of age. **B.** Scatter plot showing CFU of bacterial populations from the isolated cecal contents from either the control (C) or the *Bdellovibrio*-treated chickens (T). The horizontal lines represent the means for each group. From **Paper 6.** (Atterbury et al., 2011).

During the experiment *B. bacteriovorus* were attempted to be isolated from both the bird's ceaca/feaca samples and the drinking water. The samples were filtered and subsequently plated to a lawn of Salmonella to produce plaques, but in all instances no *B. bacteriovorus* were re-isolated. To prove that there were no adverse effects for the *B. bacteriovorus* in micro-aerophilic conditions or higher temperatures, as those found in a chickens gut, an *in vitro* experiment was set up and showed that the *B. bacteriovorus* can survive at least 48 hours. Reasons for which no *B. bacteriovorus* were isolated could possibly be due to the short living of the *B. bacteriovorus* once administered through unfavourable conditions e.g. anaerobic etc., as the cells need to get through the gut to the caeca, or perhaps the insolubility of the samples taken made isolation of *B. bacteriovorus* more difficult.
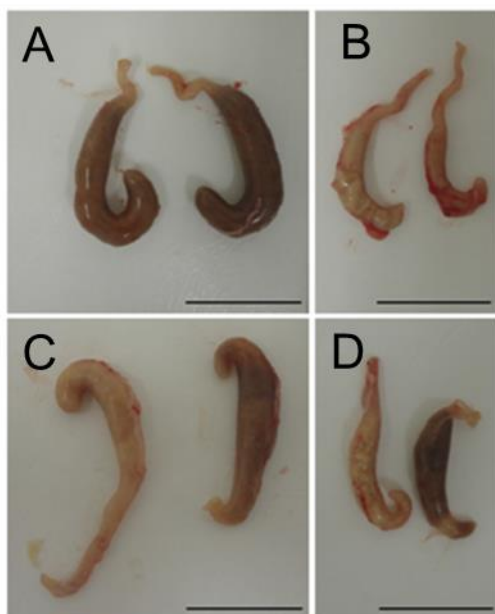

Fig 21. Typical images of ceca from the birds in this study. **A**. Caeca from birds treated with predatory HD *Bdellovibrio*; B. treated with non-predatory HI Δ*pilA Bdellovibrio*; **C**. and **D**. control birds (buffer only). The pale colour of the ceaca is indicative of *Salmonella* infection. Scale bars = 5 cm. From **Paper 6.** (Atterbury et al., 2011).

Treatment	Cecal content wt (means ± SD) on day:		
	1	2	3
Control buffer	0.49 ± 0.28	0.48 ± 0.37	0.77 ± 0.46
HD *Bdellovibrio*	0.75 ± 0.30 (*P* < 0.05)	0.87 ± 0.51 (*P* < 0.01)	0.94 ± 0.49 (NS)
HI *Bdellovibrio* nonpredatory Δ*pilA*	0.84 ± 0.48 (NS)	0.72 ± 0.50 (NS)	1.08 ± 0.54 (NS)

Table 3. Weights of the caecal content from each group of birds. A nonparametric Kruskal-Wallis with Dunn's post-test was carried on each set and compared to the control. SD; standard deviation, NS; nonsignificant *P* >0.05. From **Paper 6.** (Atterbury et al., 2011).

Suggested in the paper is also a delivery mechanism of the *B. bacteriovorus* inoculum, by the use of calcium carbonate, which aims to neutralise the stomach acid of the birds allowing the *B. bacteriovorus* to reach the area of infection, the caeca, in numbers that proved to be an effective dose. This is something not done in commercially available probiotics.

3.1.2 Since the Publication and Impact

Our paper has been cited 12 times since its publication in 2011. The citations include those looking at the dual therapy of using *B. bacteriovorus* and standard antimicrobials (Dwidar et al., 2012), as well as in a paper looking at novel antimicrobials to treat *Pseudomonas* infections (Fothergill et al., 2012). The paper present in this chapter is the first application of *B. bacteriovorus* in an *in vivo* system and represents the first steps in using *B. bacteriovorus* as a therapeutic and especially to impact the human race and as such will potentially open the flood gates for numerous other trials and research into the use of *B. bacteriovorus* as a therapeutic.

Despite *B. bacteriovorus* research being around for over 50 years there has only been talk about the use of *B. bacteriovorus* as a novel therapeutic in the last decade and before my thesis work the only papers are on its use in fish, amphibians, or an isolated rabbit-ileum (Westergaard and Kramer, 1977) (Lu and

Cai, 2010) The aforementioned work does not relate directly linked to humans, however with the work presented in this chapter could have a great impact on the first commercial use of *B. bacteriovorus* as a therapeutic or preventative measure. It is tempting to speculate that in the future, pre-dosing chickens bred for egg laying or meat production could occur, not only against *Salmonella* but also *Campylobacter*, a Gram-negative bacterium, responsible for illness and found as a natural commensal of chickens.

Very recently a study has shown that *B. bacteriovorus* is readily isolated and prevalent in healthy human guts (Iebba et al., 2013), but not in those suffering from Crohn's disease, suggesting a possible target audience for the use of *B. bacteriovorus* as a pro-biotic and actual evidence for a beneficial effect of humans having *B. bacteriovorus*.

Outside of the therapeutic use of *B. bacteriovorus* there has also been a very recent publication from the Sockett lab. In the paper Saxon and co-workers use *B. bacteriovorus* as a treatment against *P. tolaasii* on the surface of mushrooms post-harvest (Saxon et al., 2014). This presents a great potential for *B. bacteriovorus*'s use as a food security agent to lower the amount of spoilage in post-harvested crops, and could perhaps be adapted to use in other crop systems both pre and post-harvest.

3.2 *B. bacteriovorus* as a Therapeutic Conclusions

The work presented here is a large step towards the use of *B. bacteriovorus* as a therapeutic and has shown that in an *in vivo* experiment *B. bacteriovorus* can be used to reduce an infection of *Salmonella*. Also there was no evidence to show that administration of the *B. bacteriovorus* treatment had any negative effects on the birds, whereas those untreated and only infected with Salmonella had abnormal caeca.

The experiments were costly and with a lot of preparation needed before to hand to achieve the results, with a large number of support staff required as well as ethical approval. Another problem of note is the sample size vs. efficacy issue. Ideally when an experiment is carried out the sample size (n) is as high as possible, working with higher order animals however requires that the sample size to be reduced to the minimal number to limit the inclusion of birds in the study. So there is something of a paradox between statistically significance data and animal wellbeing but hopefully this paper has alleviated some unknown results and a lot of work to come will form a basis from the work presented here.

Future Work

There is no shortage of future work to be done on the use of *B. bacteriovorus* as a therapeutic.

Follow on work from this paper could explore the reduction of *Salmonella* numbers in the eggs from the same chicken strain used in the experiments. Colonisation of eggs usually occurs by way of infection the ovaries of the mother

hen, this results in 1 in 290 boxes of eggs to be contaminated (Source: food.gov.uk, 2004). A preventative treatment with *B. bacteriovorus* in a water supply could abolish or severely reduce this number.

Something which could be investigated in the future scope of this work is the chicken's immune response to the administration of the *B. bacteriovorus* and the *Salmonella* and the combined therapy. It would be expected that the *Salmonella* would cause and immune response, but nothing is known of the immune response attributed to *B. bacteriovorus*. This would be an important experiment because the LPS layer of Gram-negatives in particular are attributed to the immune response; one would think that the subsequent lysis of the *Salmonella* by the *B. bacteriovorus* (or even the 'natural' lysis of the *B. bacteriovorus*) would cause a response, the more lysis, the bigger the response. Measuring this could assess whether the *B. bacteriovorus* would make a successful therapeutic as ideally the treatment should not have a worst effect than the cause.

As mentioned previously *B. bacteriovorus* can be isolated from mammalian species including humans. Human isolates of *B. bacteriovorus* in particularly represent an ideal target for further research because they are natural flora; this means that rather than rather than giving the 'patient' environmental isolates such a *B. bacteriovorus* HD100 which is alien to the human body, a natural 'familiar' isolate can be given in its place. This has many benefits as ideally the commensal strain is adapted for growth within isolated organism so predation will occur readily and also the strain should have low immune response.

In the paper using an isolate of *B. bacteriovorus* from the chickens gut could

have alleviated the disparity between the killing efficiency by *B. bacteriovorus* on *E. coli* compared to that of the *Salmonella* strain used.

There are several questions that should be addressed during the future research with regards to the use of *B. bacteriovorus* as a therapeutic. One question is what types of infection *B. bacteriovorus* may be used to treat? It is possible that *B. bacteriovorus* may be used as a probiotic? Should it be orally administered by its self (like Yakult[®]) or in a co-culture with other probiotics (like Activia[®]) akin to what is commercially available today? Or would it be applied topically to Gram-negative skin infections? Should it be used as a preventative rather than a treatment? Should it be used as a treatment in plants or animals that are to enter the food chain, rather than in humans? The list is quite substantial, and for each question an array of different experiments need to be undertaken first.

4.0 Regulation of Predation by *B. bacteriovorus*

Predation by *B. bacteriovorus* on Gram-negative prey is perhaps the most studied area in *B. bacteriovorus* research and as such it is the understanding process that leads to their application as a 'living antibiotic'. A vast amount of early work was carried out during the 1960's and 1970's but until the publication of the *B. bacteriovorus* HD100 genome the genes responsible for predation remained undefined.

A large bacterial prey range has been characterised for certain *B. bacteriovorus* strains, such as 109J (Shilo, 1969) (Jurkevitch et al., 2000) and is ever increasing. It has been known since their isolation that *B. bacteriovorus* use flagella to swim towards prey and the actual physical impact was thought to be the reason for the entry into the prey cell. Flagella were then found to be unessential for predation (Lambert et al., 2006) (see chapter 2) (Evans et al., 2007). More recently flagella-mediated motility was thought to be the only method of movement used by *B. bacteriovorus* until gliding motility was found (Lambert et al., 2011). *B. bacteriovorus* were found to have pili early in the research history (Shilo, 1969) (Abram and Davis, 1970) and it was hypothesised that these may be necessary for prey cell entry, with this only being proved only relatively recently (Evans et al., 2007). Prey modification has been studied with early chemical analysis of the prey cell peptidoglycan analysed and found to be degraded by the invaded *B. bacteriovorus* (Thomashow and Rittenberg, 1978), this has been further characterised with the genes pinpointed (Lerner et al., 2012). The prey's DNA degradation by the invaded *B. bacteriovorus* has also

been studied with up to 80% of the prey cell DNA being incorporated into *B. bacteriovorus* progeny (Matin and Rittenberg, 1972) (Rosson and Rittenberg, 1979) with further characterisation more recently (Lambert et al., 2012).

Transportation of proteins and macromolecules features heavily in all bacteria, but much more in the relatively short predatory lifecycle of *B. bacteriovorus*, and as such the HD100 genome contains a large, almost disproportionate amount of genes for the translocation of macromolecules. This is due to not only having to invade and degrade the prey cell during predatory growth once invaded but also, *B. bacteriovorus* can grow Host Independent (HI). In total 406 proteins are thought to encode 172 different transporter pathways, with an estimated 161 proteins having hypothetical roles (reviewed in (Barabote et al., 2007)).

The research into predation by *B. bacteriovorus* is one of two halves, the early years where observational and chemical studies were undertaken and the current era where genetic engineering is used to assay for those genes that play a part in the predation cycle.

To study predation various methodologies have been devised and used. A common feature in the Sockett lab is to look at the expression of genes throughout the predatory lifecycle using RT-PCR (Lambert et al., 2006) (Evans et al., 2007). This highlights whether the expression of the gene of interest is increased upon predation commencing. Further on from this micro/macroarrays and RNAseq have been used to measure RNA levels of both the invaded prey, and the predatory *B. bacteriovorus* to give a genome wide idea of gene expression under predation (Lambert et al., 2010b) (Lambert et al., 2010a) (Karunker et al., 2013). Insertionally inactivated and markerless deletion mutants

are developed in genes thought to have a role in predation, often generating a *B. bacteriovorus* mutant that is less good at predation or completely predatory null (Lambert et al., 2003) (Evans et al., 2007)

Predation itself is a complex and highly regulated process that can be characterised by a series of different steps: location, attachment and invasion, establish, growth phase, division and finally prey cell lysis and escape (**Fig 1**). In the case of *B. bacteriovorus* HD100 at 15 minutes incubation with a susceptible prey strain (e.g. *E. coli*) attachment occurs and invasion begins. Were the *B. bacteriovorus* cell releases different enzymes to make a hole in the outer membrane of the prey cell and uses its pilus apparatus to pull itself into the prey cell's periplasm rounding up the prey cell in the process (Evans et al., 2007) (Lerner et al., 2012). At thirty minutes the *B. bacteriovorus* establishes itself in the periplasm of the prey cell and begins a hierarchical degradation of the prey cell content to which it uses to grow as a filament, which eventually synchronously septates to produce multiple progeny. By the fourth hour of infection these progeny then escape the now nutrient-spent bdelloplast to repeat the predation cycle once again.

The initial steps of this process are important to characterise to understand the regulatory steps that the predatory *B. bacteriovorus* cell undergoes to begin replication. It stands to reason that if the conditions are not right, e.g. the prey is not susceptible, initiating replication or trying to enter a cell with no periplasm would prove disastrous to the *B. bacteriovorus*. Once the *B. bacteriovorus* cell is inside it also must go through further checkpoints to determine whether there is enough prey cell content to initiate replication. If there isn't, the invaded *B.*

bacteriovorus cell produces no progeny and leaves the bdelloplast structure at the same time point the progeny would (4-5 hours) (Fenton et al., 2010a).

There is a lot of data and evidence chemically looking at predation, but less so genetically but with the advent of the HD100 genome sequence areas and pathways have formed to make clearer boundaries for targeted research into the field, many of which are pinpointed in the review paper.

4.1 Paper 4. A predatory patchwork: membrane and surface structures of *Bdellovibrio bacteriovorus*.

Lambert C, Hobley L, Chang CY, Fenton A, Capeness M, Sockett L.

My Contribution

For this review book chapter I wrote and collated the data for the chapter entitled: The role of type IV pili in predation. It investigates how typeIV pili are involved and necessary for successful predation by *B. bacteriovorus*. In particular it looks at the predicted function and regulation of the type IVa pilus and the possibility that a separate pilus system is present; the flp (fimbriae-like protein) pilus.
I also contributed work to the sec system chapter in the form of RT-PCRs of the genes *secA* and *gspD*, investigating their expression across the predatory time course.

Conclusions of the Chapter

As well as my section on the role of pili in *B. bacteriovorus* the chapter also reviews what was currently know about predatory lifecycle. Such topics highlighted include the role of flagella motility and chemotaxis in predation, prediction of outer membrane proteins and autotransporters as well as the sec system. Finally the chapter looks at the peptidoglycan synthesis in *B. bacteriovorus* and also the degradation of the prey cell's peptidoglycan. The review itself used the current bioinformatics at the time to delve deeper into the genetic function of the genes found in the HD00 genome, highlighting many research areas that have since become under investigation.

The role of flagellar motility and chemotaxis in predation subchapters

This part mainly focusses on work regarding the role of flagella in predation previously mentioned in the motility chapter of this thesis. The subchapter also includes data published previously found in the chemotaxis paper and knockout studies of the *mcp2* gene in *B. bacteriovorus* 109J (Lambert et al., 2003). Additionally analysis by bioinformatics of sequence data after the publication of the sequence of *B. bacteriovorus* HD100 in 2004 (Rendulic et al., 2004) gives a comprehensive list of the chemotaxis genes and their homologues for potential future studies. (Paper 4 Table 2; see paper).

The role of type IV pili in predation subchapter

The pilus subchapter which I wrote features a list of pilus related genes and their potential homologues in *B. bacteriovorus* HD100. It highlights the fact there is no predicted genes encoding a protein for the 'tip' of the pilus (PilC1/Y1) or minor pilins (PilE, V, W, X and FimU) as is normally found in *Mxyococcus* (a close relative to *B. bacteriovorus*) and *Neisseria*. However it is also suggested that like *Pseudomonas*, cysteine residues at the C-terminus of pilA negate the need for a specific surface adherence protein. This may be why *B. bacteriovorus* recognise a plethora of different potential prey strains. The data is summarised in cartoon form showing the differences in the consensus formation and structure of a type IV pilus against the predicted structure of the type IV pilus in *B. bacteriovorus* (**Fig 22**) and is based on predicted gene function from the HD100 genome (**Table 4**).
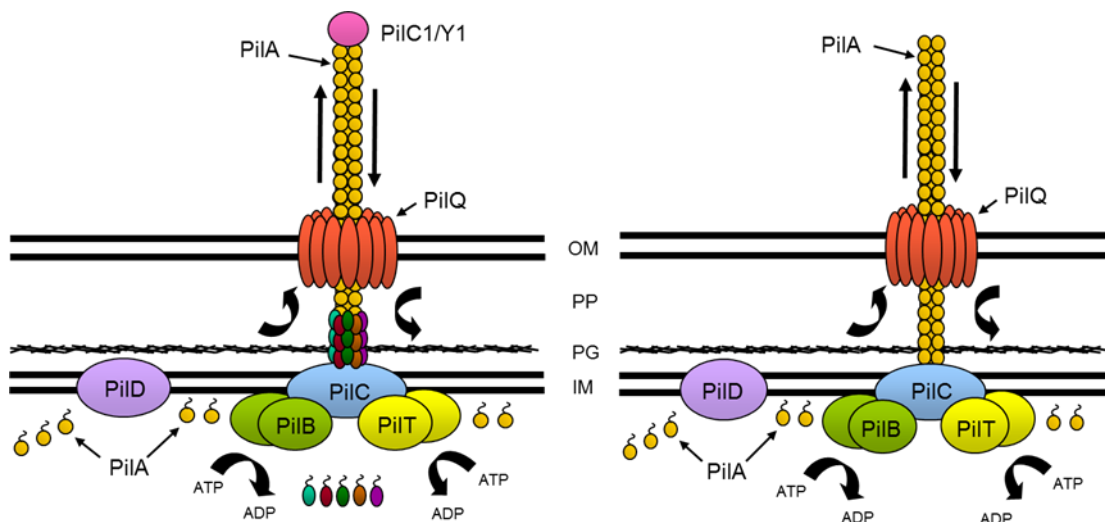
Protein	Function	Diagram Representation
PilA	(left) Immature pilin subunit found in cytoplasm, (right) functional pilin subunit making up the pilus	
PilB	ATPase used for pilus extrusion	
PilC	Polytopic pilus protein required for pilus biogenesis	
PilD	Pre-pilin peptidase	
PilE, V, W, X, FimU	Minor Pilins	
PilT	ATPase used for pilus retraction	
PilQ	Outermembrane multimer complex through which the pilus is extruded	
PilC1/Y1	Pilus tip protein used for adhesion/invasion	

Fig 22. A coloured version of figure 4. from the predatory patchwork. **A.** General structure of a Type IVa pillus in bacteria. **B.** The predicted structure of the type IVA pillus in *B. bacteriovorus* based on the genes present in the HD100 genome. From **Paper 2.** (Lambert et al., 2009).

The presence of a flp pilus was also assessed and a table different homologues

in the *B. bacteriovorus* genome given (**Table 5**). Interestingly it was found that

there is an absence of the genes *flp-1* (the flp pilus subunit), *rcpB* and *rcpC*

(secretins), *tadE* and *tadF* (pseudopilins), *tadG* (an anchor protein), *tadV* and

tadZ all of which have been reported to be essential for the synthesis of flp pili.

The subchapter concludes that pili are completely essential for predation for *B.*

bacteriovorus and that the bacterium does not express flp pili, though possible

this may have done at some point in its ancestry.

Gene	Function	HD100 gene number
pilA	Pilus fiber protein	Bd1290
pilB	ATPase pilus extrusion	Bd1509
pilC	Pilus biogenesis	Bd1511
pilD	Pre-pilin peptidase and methylase	Bd0862
pilF	Required for PilQ multimer formation	Bd3829
pilG	Part of ABC transporter required for pilus biogenesis with pilHI	Bd1291
pilH	Part of ABC transporter required for pilus biogenesis with pilGI	Bd0860
pilI	Part of ABC transporter required for pilus biogenesis with pilGH	Bd0861
pilM	Required for pilus biogenesis	Bd0863 Bd1585
pilN	Pilus biogenesis	Bd0864
pilO	Pilus biogenesis	Bd0865
pilP	Stabilizes PilQ multimer complex in outer membrane	Bd0866
pilQ	Outer membrane multimer complex through which PilA fiber is extruded	Bd0867
pilS	Negative regulator via two component system with PilR	BD1512
pilR	regulates *pilA* expression via two component system with PilS	Bd1513
pilT	(Best homolog) ATPase pilus retraction	Bd3852
pilE, V, W, X, fimU	Minor Pilins	No homologs

Table 4. Type IVa associated pili genes, their function and their predicted
homologues in the *B. bacteriovorus* HD100 genome. From **Paper 2.** (Lambert et al.,
2009).

Gene	Function	Bdellovibrio homolog
flp-1	Pilus subunit	N/A
flp2	Duplication of *flp1*	N/A
tadA	ATPase	*tadA* (*Bd0111*)
		cpaF (*Bd0793*)
tadB	Pilus anchor	*tadB* (Bd0110)
tadC	Pilus adherence protein (similar to *pilC1/Y1*)	*tadC* (Bd0470)
tadD	Production of fimbriae	*aglT* (Bd0833)
		Bd0225
tadE	Pseudopilin	N/A
tadF	Pseudopilin	N/A
tadG	Pilus anchor related protein	N/A
tadZ	Possible extracellular secretion vesicles	N/A
rpcA	Secretin similar to *pilQ*	*gspD*
		pilQ
rpcC	Function unknown	N/A

Table 5. Type IVb associated pilus genes, their function and their predicted homologues in the *B. bacteriovorus* HD100 genome. From **Paper 2.** (Lambert et al., 2009).

Outer membrane proteins, Autotransporters and the role of the Sec system subchapters

This subchapter focusses on the bioinformatics and RT-PCR data on different transporters and outer membrane proteins that are predicted to have a role in predation by *B. bacteriovorus*. The *B. bacteriovorus* genome is predicted to encode 100 outer membrane proteins >60% of which have no known function. Included in this set are proteins involved in nutrient uptake such as nucleoside, sugar and siderophore transport. Others have a role in transport and potentially surface motility. RT-PCR analysis of the genes *ompF*, *ompA1* and *ompA2* show that they are more expressed towards the end of the predation lifecycle (3-4 hours) as the *B. bacteriovorus* begins to septate and produces multiple progeny (**Fig 23**). Interestingly *lamB* expression is highest between 45 minutes and 2 hour post infection time point suggesting it is involved in maltose and oligosaccharide uptake from the prey or possibly a structural role for the now filamentously growing *B. bacteriovorus*. Its small expression window also suggests that the established *B. bacteriovorus* cell limits oligosaccharide to this specific time rather

than having the transporters present throughout the lifecycle.

Bioinformatic studies of the *B. bacteriovorus* HD100 genome also found there are 16 putative auto-transporter genes, all of which contain a Sec-secretion signal at the N-terminus allowing their translocation to the outer membrane via type II secretion (Table 4, Table 5, see paper).

Of the 16 identified proteins, 8 belong to YapH-like proteins, identified to have possible roles in adhesion of the *B. bacteriovorus* to either surfaces or other cells, such as prey cells. The other 8 proteins belong to RTX-like family of proteins, a family of diverse exoproteins that would be translocated outside the cell, possibly into the prey cell's membrane once the predatory *B. bacteriovorus* cell has established itself within.
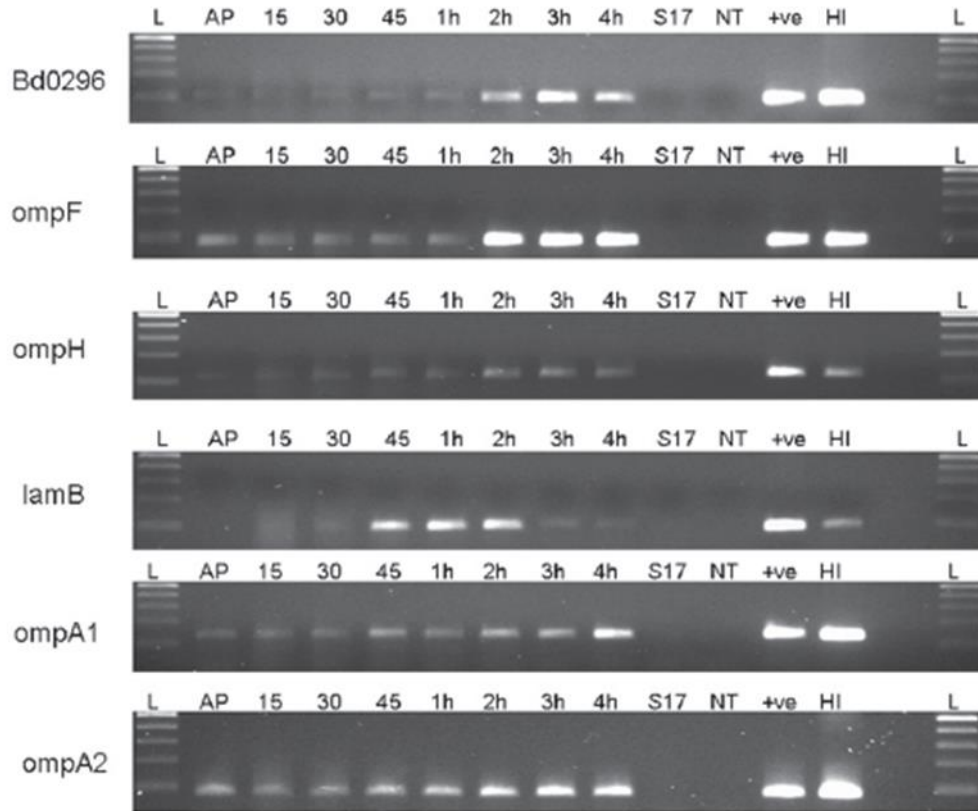
Fig 23. RT-PCR products of different predicted outer membrane protein genes and their expression over the predatory time-course of b. bacteriovorus HD100. Lanes: L , NEB 100 bp ladder; AP - attack-phase; 15, 15 min; 30, 30 min; 45, 45 min; 1 h – 4 h, 1 hour – 4 hours post-infection; S17, *E. coli* S17-1 prey only; NT, no template; +ve, positive control of B. bacteriovorus HD100 genomic DNA; HI, host-independent. From **Paper 2.** (Lambert et al., 2009).

Fig 24. RT-PCR of genes for predicted sec proteins the predatory lifecycle. Lanes: L , NEB 100 bp ladder; AP - attack-phase; 15, 15 min; 30, 30 min; 45, 45 min; 1 h – 4 h, 1 hour – 4 hours post-infection; S17, *E. coli* S17-1 prey only; NT, no template; +ve, positive control of *B. bacteriovorus* HD100 genomic DNA; HI, host-independent. From **Paper 2.** (Lambert et al., 2009).
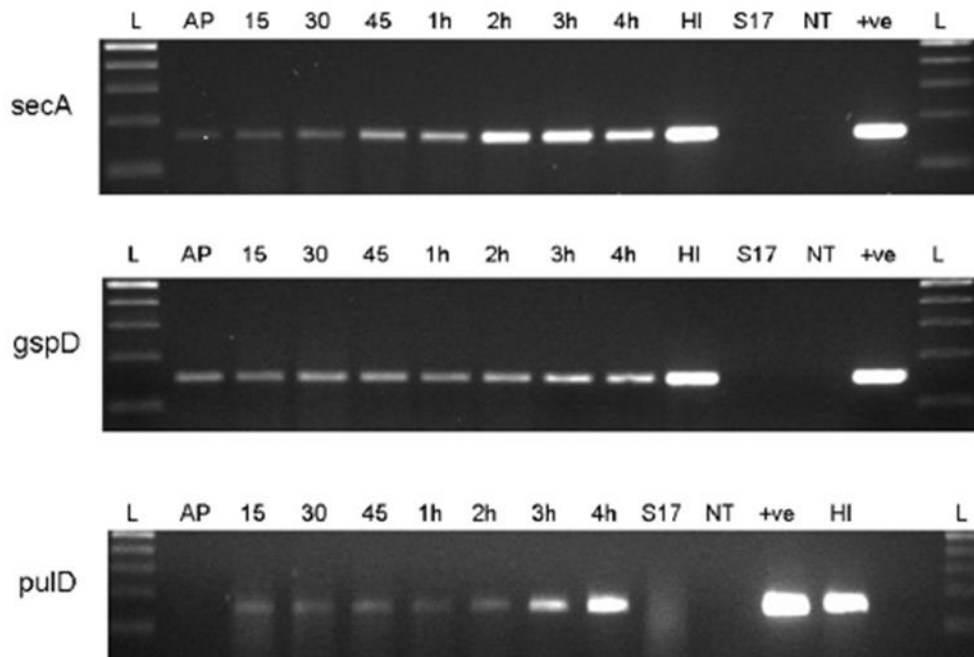
In the *B. bacteriovorus* HD100 genome 808 genes were predicted to encode proteins that contain a Sec-signal for translocation by the Sec system (type II secretion). The expression the core gene components of the Sec system, *secA*, *gspD* and *pulD*, show that it increases throughout the predatory cycle and that there is a constant level of these proteins (**Fig 24**).

Membrane chemistry and Peptidoglycan chemistry and metabolism subchapter

In the *B. bacteriovorus* HD100 genome it was predicted there are 23 genes encoding proteins that have functions as putative lipases and that some have predicted type II secretion signals that possibly are exported into the prey cell during predation (Paper 4 Table 7, see paper).

The *B. bacteriovorus* HD100 genome was found to contain a complete set of genes for *de novo* peptidoglycan synthesis implying that it does not depend on the prey cell supplying the precursor molecules. Additionally the genome also has multiple copies of the transglycosylase genes and DD-carboxypeptidases/endopeptidases, suggesting they are important to the predatory lifecycle (Paper 4 Table 8, see paper). These potentially would not only be involved in housekeeping but also modification of the prey's cell wall, perhaps forming the bdelloplast structure upon entry.

4.1.1 Since the Publication and Impact

Since its publication in 2008, the Predatory Patchwork book chapter has been cited 7 times in research involved in other predatory delta-proteobacteria such as *Micavibrio aeruginosavorus* as well as in the *B. bacteriovorus* research field itself.

The book chapter represents a 'snapshot' of the current understandings of the predatory cycle in *B. bacteriovorus* at the time of publication by reviewing previous work such as the involvement of pili, and flagella in predation. The chapter also proposes other fields of interest that were eventually studied in more depth and form a basis for forth-coming publications. Two such areas are peptidoglycan synthesis and the role of pili in predation.

The role of flagellar motility and chemotaxis in predation subchapters

The understanding of motility in *B. bacteriovorus* has also been developed further in a paper investigating the motor proteins (Mots) in *B. bacteriovorus* HD100 (Morehouse et al., 2011) (see chapter 2.2). This paper is mentioned in the motility chapter of this thesis. There is also further development with regards to the composition of the flagellum in published in 2009 (Iida et al., 2009). This paper showed that there is hierarchical order to the assembly of the flagellar filament, and that they are arranged in the following order: FliC3, FliC5, FliC1, FliC2 and FliC6.

Perhaps the biggest change since this book chapter was published is that we now know that flagella mediated motility is not the only method of motility that *B.*

bacteriovorus uses; in a paper published by the Sockett lab it was found that *B. bacteriovorus* can use gliding motility on surfaces to move around and locate prey (Lambert et al., 2011).

The role of type IV pili in predation subchapter

Since the book chapter was published there has been further work to characterise the role of pili in the predatory lifecycle of *B. bacteriovorus* (Mahmoud and Koval, 2010). It mainly confirms, by immunofluorescence and protein purification, that the pilus fibre present on the surface of an attack-phase *B. bacteriovorus* is that encoded by the gene *pilA,* this further confirmed the findings that were previously published by the Sockett lab (Evans et al., 2007).

Another paper investigating the role of pili in predation is one that I am first co-author of and features in the housekeeping and HI growth chapter of this thesis. The paper claims that the presence of pilus fibres on the surface of *B. bacteriovorus* cells is correlated to the mutation in *bd0108*, a gene known to be involved in Host Dependent to Host Independent switching. Those cells having a markerless deletion in *bd0108* (of all but 5 codons) are significantly decreased for the presence of pili on the cell surface (Capeness et al., 2013) (see chapter 5.3). This not only implies the role for pilus presence in HI cells but also that *bd0108* is involved in regulation of pili.

Outer membrane proteins, Autotransporters and the role of the Sec system subchapters

The Sec system in particular was found to be up-regulated in micro-arrays undertaken by the Sockett lab (Lambert et al., 2010a) (this paper is analysed in

further detail later is this chapter). The Expression increased at 30 minutes post-infection in predatory *B. bacteriovorus*, and also in Host Independent (HI) isolates compared to free-swimming 'attack-phase' predatory cells in the absence of prey bacteria. Also in this dataset the majority of outer membrane proteins and autotransporter genes were found not to be up-regulated, suggesting they are expressed later in the predatory cycle. In another paper by the Sockett lab the Twin-Arginine Transporter (TAT) pathway has been further characterised showing it is involved in the secretion of proteins into the prey cell once the invading *B. bacteriovorus* has been established (Chang et al., 2011) (see chapter 5.1).

Membrane chemistry and Peptidoglycan chemistry and metabolism subchapter

The up-regulation of peptidoglycan synthesis genes was confirmed to be an early event in the lifecycle of the predatory *B. bacteriovorus* and at 30 minutes post-infection was significantly increased in micro-array datasets (Lambert et al., 2010a) (This paper is covered in more depth later in this chapter).

In a paper by the Sockett lab, two of the the DD-endo/carboxypeptidases genes of *B. bacteriovorus* HD100 (*bd0816* and *bd3459*), that are predicted to be involved in the synthesis of peptidoglycan, were shown to be expressed upon attachment of the *B. bacteriovorus* cell to the prey cell and had a role in the degradation and de-crosslinking of the prey's peptidoglycan to cause the rounding up to form the bdelloplast structure. While another DD-endo/carboxypeptidase was shown to be a housekeeping gene as it was constitutively expressed (Lerner et al., 2012).

Finally another paper from the Sockett lab touches on all these areas, and features as the main paper in the next chapter of this thesis. It focuses on the use of microarrays to establish a 'predatosome' (a proteome of predatory proteins/genes) and as such includes candidate genes found in this book chapter's topics.

4.2 Paper 5. The First Bite— Profiling the Predatosome in the Bacterial Pathogen *Bdellovibrio*

Carey Lambert, Chien-Yi Chang, Michael J. **Capeness**, R. Elizabeth Sockett

My Contribution

I was mainly involved in RNA preparations from the samples of taken from the synchronous *B. bacteriovorus* time course as well as testing their synchronicity and verifying they were DNA free, these were later used for both the array experiments and the RT-PCRs of the genes (*narL*, *bd1640*, *dnaK*, *bd0881*, *bd2462, bd2439, bd0487, bd2289* and *bd1904*) to confirm some of the findings and to show there is no difference in expression in varying HI strains. I also contributed to the writing of the manuscript and helped to interpret some of the array data.

What the Paper Claims

The main aim of this research was to find which genes are associated with which growth phase and get further insight into the differences between HI cells and HD cells and whether a newly invaded *B. bacteriovorus* is growing akin to a growing HI cells or *vice versa*. It was also hoped that the genes identified would infer some future targets for mutation into biochemical pathways.

By growing *B. bacteriovorus* in both lifecycles, predatory and host-independently, it was possible to have contrasting expression patterns of genes (measured by micro-array), the comparison of which gives great insight into the different genes responsible for growth in the two different phases but also a common gene set.

Experimental

Initially predatory *B. bacteriovorus* HD100 cells were subcultured for three days for synchronous expression of genes; these were then used in a concentrated predatory culture as well as a no-prey culture (attack-phase). Samples were taken samples at 30 minutes post-infection, a time point where the *B. bacteriovorus* has become established within the prey cell (confirmed by microscopy). RNA was then isolated from the samples.

Host-Independent cells were grown to an OD_{600} of 0.6 (±0.1) and samples taken for RNA isolation, three HI isolates were used; HID2, HID13 and HID22 all of which have different/mutant *bd0108* genes. Control samples of the prey alone in buffer were also taken, as there would be confounding levels of prey-transcription and RNA present in the infection samples (**Fig 26** – though only HID13 is shown).

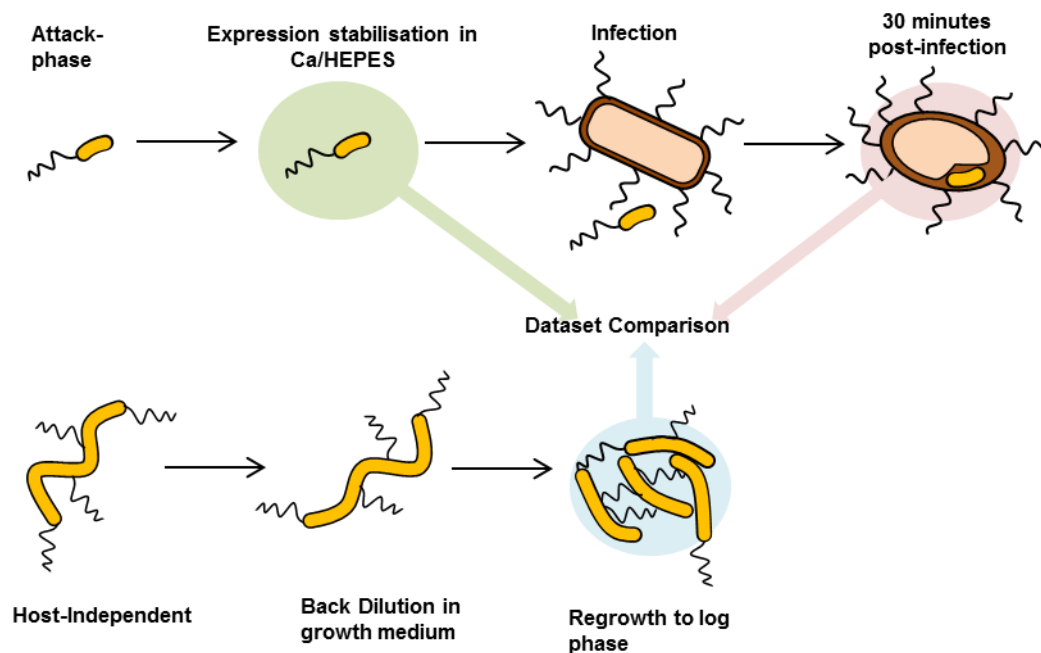**Fig 25.** Outline for the method of RNA isolation for the First Bite paper and where the each of the datasets comes from. From this it is possible to find the genes that have/do not have a role in their respective point of each of the lifecycles.

Comparison of gene expression between attack-phase and 30 minutes post-infection show those genes responsible for *B. bacteriovorus* establishment, prey cell killing, and also the initial wave of expression as the invaded *B. bacteriovorus* begins to breakdown the prey cell. Comparison of the Host-independent samples with the attack-phases and 30 minutes post infection show those genes responsible for each of the growth phases, with similar genes expression between the attack-phase and HI samples corresponding to house-keeping functions.

A select sample of gene expression differences were also verified by RT-PCR and QPCR, the former of which was undertaken by me. RT-PCR analysis showed that there were no differences in expression of the genes *narL*, *bd1640*, *dnaK*, *bd088*, *bd2462* and *bd2439* in the HI strains HID13, HID2, HID22 and HID26 though the micro-array data is carried out on HID13 it was important to test whether assumptions about gene expression could be made about other strains. Q-PCR of some of the genes and others was also used to see that the expression profile of certain genes matches that of the micro-array but was only carried out on HID13 strain (**Fig 26**) (**Fig 27**).
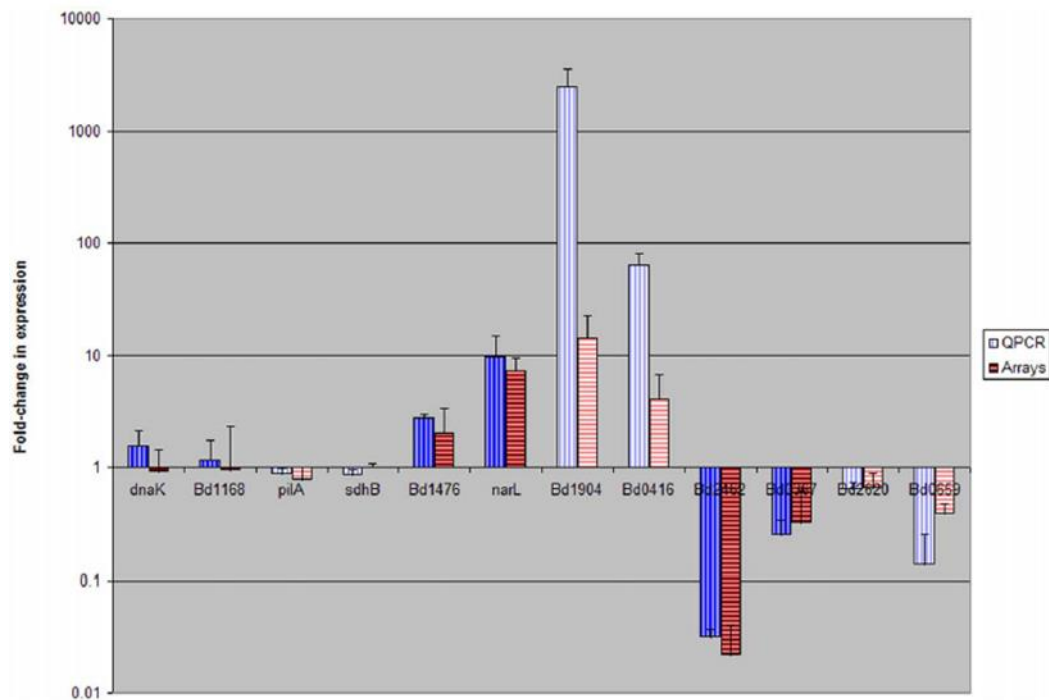
Fig 26. Q-PCR results confirming the micro-array results and showing that *sdhb* and *pilA* were constitutively expressed between attack-phase and 30 minutes post-infection nor were *dnaK* and bd1168 between attack-phase and HI growth. bd1904, bd0416, bd0659, and Bd2620 were significantly differentially regulated between attack-phase and 30 mintues post-infection while *narL*, *bd1476*, *bd2462* and *bd0367* were significantly differentially regulated between attack-phase and HI growth. Darker colours show that the genes confirmed the HI array data while the lighter colours are to show that the genes in the 30 minutes post-infection also confirm the micro-array data. From **Paper 3.** (Lambert et al., 2010a).
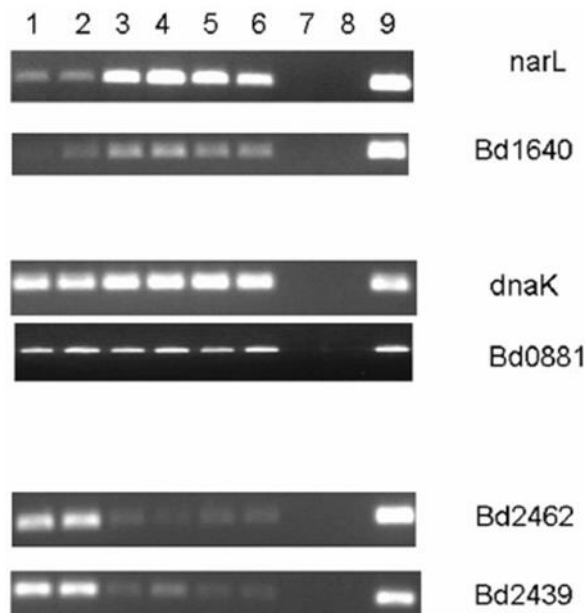
Fig 27. RT-PCR analysis of expression genes from different HI isolates on matched amounts of total RNA. Lanes: 1, attack-phase from HD *B. bacteriovorus*; 2, 4 hours post-infection HD *B. bacteriovorus;* 3, HID13; 4, HID2; 5, HID22; 6, HID26; 7, *E. coli* S17-1 (prey) RNA; 8, no template; 9, HD100 genomic DNA. RT-PCR was used to verify the micro-array dataset from the HI growth phase. The two up-regulation genes were *narL* and *bd1640*, the two constitutively expressed genes were *dnaK* and *bd00881* and the two down-regulated genes were *bd2462* and *bd2439*. Each gene showed similar expression in the different HI strains. From **Paper 3.** (Lambert et al., 2010a).

The Dataset

The research found that between attack-phase and 30 minutes post-infection, there was an up-regulation of expression of 13% of the genome (479 genes) and 6.4% of the genome down-regulated (230 genes) by at least 1.3 fold differences. In the comparison between HI growth and attack-phase there were 37.2% (1333 genes) and 36.7% (1317 genes) of the genome up- and down-regulated respectively (**Fig 28**).
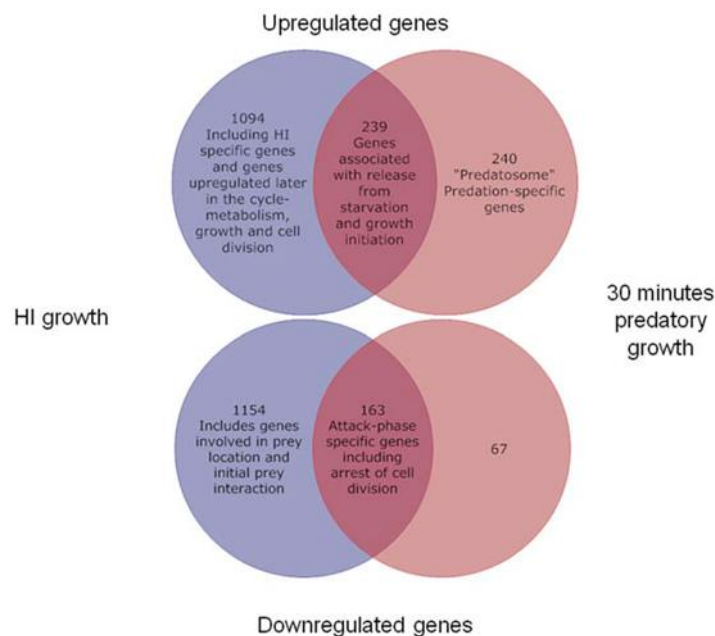
Fig 28. A Venn diagram of the datasets from the micro-arrays showing the number of genes up/down-regulated in the different time-points and growth phases. From **Paper 3.** (Lambert et al., 2010a).

Comparisons between those up-regulated genes at 30 minutes of predation and those in HI growth, show a gene set for HI growth specifically (1094 genes, up-regulated in HI only), those genes responsible for growth/metabolism initiation (239 genes, up-regulated in both) and those genes used for prey degradation (240 genes, up-regulated in 30 minutes post infection). Conversely down-regulation of genes between the two datasets also show genes responsible for prey-location/interaction (1154 genes, down-regulated in HI only), genes

responsible for attack-phase processes (163 genes down regulated in both), and genes not required for either growth initiation of early prey-degradation (67 genes, down-regulated in both) (**Fig 29**).
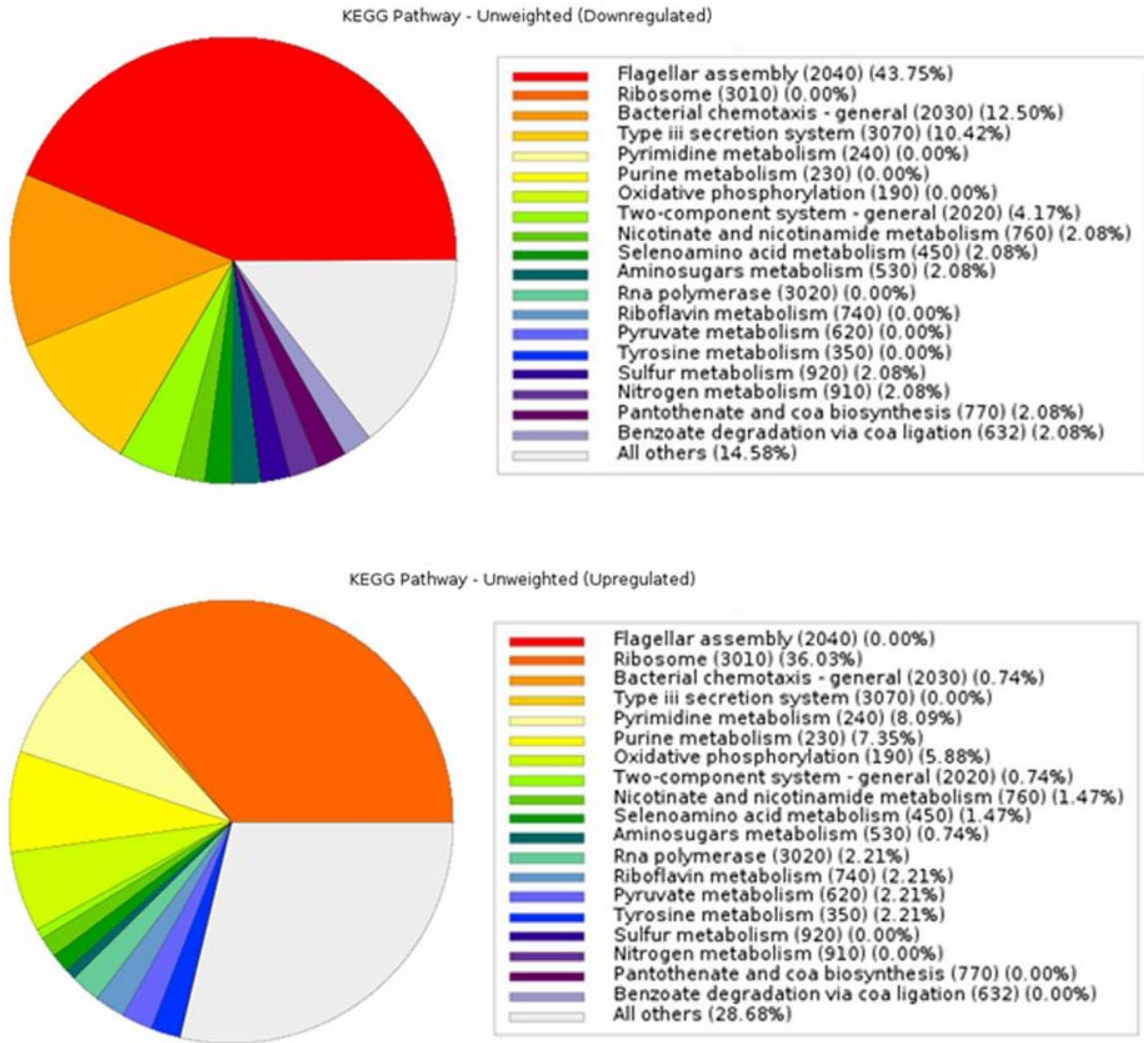
Fig 29. Pie charts showing the vast different in protein families and pathways **u**p/down-regulated from the shift from attack-phase to predatory (30 minutes post-infection). From **Paper 3.** (Lambert et al., 2010a).

Genes up-regulated in both HI and Predatory datasets: 239 genes

Going from a non-replicative attack-phase to either a HI or predatory phase involves the up-regulation of genes responsible for RNA polymerisation, RNA polymerase sigma factors and other transcription and translation related genes.

All of which will have a role in the increased expression of enzymes used for cell elongation and prey-degradation.

Upon this entrance into growth phase there is also a set of ATP synthase genes up-regulated as well as genes involved in electron transport used to power transportation of degraded prey contents such as the Sec and TAT system and ABC transporters, whose components are also up-regulated.

Genes up-regulated in Predatory but not HI datasets: 240 genes

It is worth noting that the genes in this data set are up regulated at 30 minutes post-infection and the invaded *B. bacteriovorus* has not begun its lytic breakdown of the prey's content and as such this time-point is one of 'establishment' and preparation for the subsequent lysis. As such the vast majority of the lytic enzymes the HD100 genome encodes have not begun to be expressed. Those that are up-regulated include 11 proteases (of which HD100 has 150), two endonucelases and two hydrolases as well as some genes thought to protect against oxidative stress in the prey's periplasm.

This gene set features those whose functions have a role specifically in predation rather than the more 'house-keeping' and growth orientated HI phase or the motility/chemotaxis orientated attack-phase stage of the *B. bacteriovorus* lifecycle. The vast majority (174) of the genes are hypothetical and have no known homologues in other bacteria which make them the key to the novel lifestyle of an intra-periplasmic predator. The major gene cluster it contains is the *bd0417-0420* operon with a predicted some of which have homology to TonB-like systems found in *Myxobacteria* and had a role in adventurous gliding motility and surface sensing. In *B. bacteriovorus* they potentially have a role in establishment

and sensing the intra-periplasmic environment of the newly invaded prey. Other genes include those that have a role in transcription regulation and sensory kinases possibly involved in the up-regulation of the genes for the impending lytic onslaught the invading *B. bacteriovorus* will use to break down the prey cell content. Also featured are peptidoglycan modification and mobilisation genes thought to provide more space for the invaded *B. bacteriovorus* by adapting the host's cell wall and perhaps getting ready for the *B. bacteriovorus's* own subsequent elongation. Also specific for predation are genes involved in protein synthesis, protein folding and ATP synthesis, all of which will be used as the *B. bacteriovorus* grows and replicates.

Genes that are down-regulated in predation from attack-phase: 163 genes

Though attack-phase is thought to be a 'transcriptionally mute' phase of the *B. bacteriovorus* lifecycle there is a gene set that are likely to encode proteins involved in environmental response such as chemotaxis and motility and as such these genes make up the majority of the content. Also found in this dataset are 100 genes thought to be outer membrane proteins, with 60 of them having unknown functions. It is thought that these genes have a role in surface sensing other either natural environments or even prey cells. Clearly the attack-phase is more complex than previously thought.

Attack-phase genes down regulated upon predation but not in HI growth: 67 genes

This dataset represents those genes that are down-regulated only on the initiation of predation, but are still present in the HI dataset. By comparing these two datasets it was hoped to show genes whose expression is early in the predatory cycle, such as those involved in prey attachment or even invasion. Such genes include those encoding the aforementioned TonB-like proteins, cell wall hydrolases, lipases, two proteases and transcriptional regulators, many of which are involved in starvation signalling, and the cell-septation preventer DnaK and DivIVA. The gene cluster *bd0108-0121* is also down regulated from attack-phase predatory growth but not in HI growth emphasizing its importance in these growth phases.

The dataset also highlighted genes specific to *B. bacteriovorus* (and *B. Marinus*) with no known homologues in other bacteria and that are up-regulated in predation, and are therefore predatory specific. From this I carried out RT-PCR analysis of the genes Bd0487, Bd1904 and Bd2298 across the lifecyle of the predatory HD100 (**Fig 30**). It was found that not only are they initially up-regulated at the 30 minute time-point, thus confirming what is seen in the array, but also that they are at their highest expression at the 45 minute to 1 hour mark, indicating a possible function in late establishment.

Bd0487

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Bd2298

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Bd1904

1 2 3 4 5 6 7 8 9 10 11 12 13 14
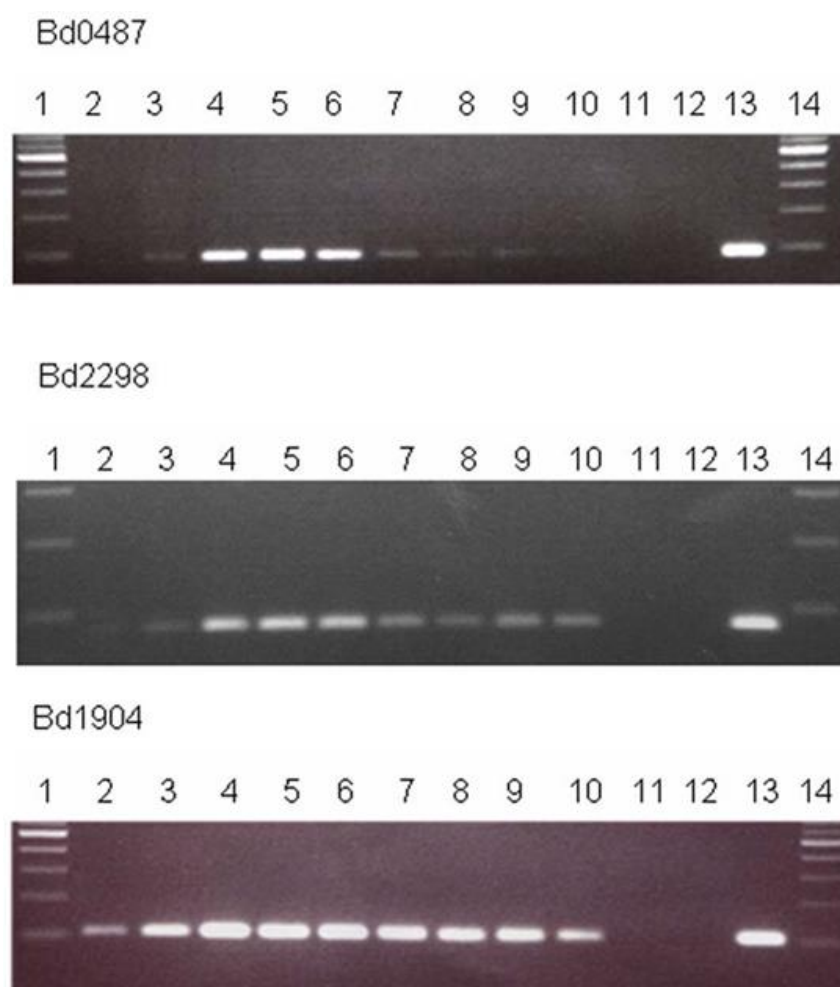
Fig 30. RT-PCR Expression of bd0487, bd2298 and bd1904 across the lifecycle of predatory *B. bacteriovorus.* These genes were found to be significantly upregulated from attack phase to 30 minutes post-infection. Lanes 1 & 14 NEB 100bp ladder, 2: attack-phase 3: 15 minutes predation, 4: 30 minutes predation, 5: 45 minutes predation 6–9: 1,2,3,4 hours predation respectively 10: HI growth, 11: negative control ,12: E. coli S17-1 only RNA control, 13: HD100 genomic DNA control.

4.2.1 Conclusions of the Paper

The most important conclusion of this paper is that there are separate gene sets involved in the different lifecycles of *B. bacteriovorus* and that a *B. bacteriovorus* enter one phase of growth from another undergoes a massive change in gene expression. The regulation of this phase switching, is more complex than previously thought with many of the genes involved being hypothetical, particularly in the predatory growth phase. This underlines not only that *B. bacteriovorus* is a novel organism with unique metabolic pathways but also that research into the bacterium is still in its infancy.

We now have gene sets for predatory, attack-phase and HI growth phases and that the *B. bacteriovorus* must undergo complex expressions cues to switch from one phase to the other in most cases it is not just a case of up-regulation but also down-regulation of genes corresponding to the previous phase. Such examples include how necessary flagellar biosynthesis is important in attack-phase cells but not inside the bdelloplast or the HI growth phase. Similarly peptidoglycan synthesis and genes involved in ATP synthesis are massively up-regulated by the *B. bacteriovorus* in the early bdelloplast. Until the publication of this dataset these genes would be thought to be up/down-regulated at their specific times, but previously we would have to of carried out individual RT-PCRs on each gene to confirm this. Thus the dataset supplies us with a large amount of information.

Interestingly on upon entry and establishment into the prey cell the *B. bacteriovorus* does not show up-regulation of all of its proteases (11 out of 150),

which shows further evidence of a hierarchical degradation of the prey cell but does show expression of nucleases. This was previously thought to be the case based on chemical evidence (Starr and Baigent, 1966) (Matin and Rittenberg, 1972).

The difference between the predatory gene set and the HI growth set also highlights that these are two different growth phases, and a HI cell is not just a predatory *B. bacteriovorus* growing without a prey but a complete separate cell line in itself. This can be seen by how few genes (relatively) that the HI growing cells and the predatory ones have in common, this has been previously noted in 2D gel analysis of differently grown *B. bacteriovorus* samples, with many genes overlapping in this study (Dori-Bachash et al., 2008).

4.2.2 Since the Publication and Impact

Though *B. bacteriovorus* is difficult to work with due to confounding factors such as having the *E. coli* present in the background to the RNA isolations and subsequent array analysis, it has been possible to undertake RNA expression studies. This is the first time in which micro-arrays has been carried out for *B. bacteriovorus* and as such this paper has really opened up a large field of interest in which to do further experiments, with an indication for where phenotypes may present themselves. It represents an enormous amount of data that is freely available to the science community in hope that it helps to advance the study of *B. bacteriovorus*.

At the time of this thesis the paper received 18 citations since its publication in

2010, not only being cited by the *B. bacteriovorus* community but also by reviews looking at the genes involved in predatory bacteria (Pasternak et al., 2012) and studies focused on a specific gene, *pif1*, that is not normally found in prokaryotes.

One paper from the *B. bacteriovorus* research community uses the dataset to assign function to genes from a newly sequenced member of the delta-proteobacteria class; *Bacteriovorax marinus* SJ. It used the data to compare where the differences between *B. bacteriovorus* and *B. marinus* differ both genetically and in their respective lifecycles.

There is a multitude of follow on publications from this paper from the Sockett lab all of which cite this piece of research as evidence for the work being carried. Specifically genes involved in gliding motility, Twin-Argenine Transportation (see chapter 5.1), flagellar motility, sigma-factors, peptidoglycan synthesis/degradation, cyclic-di-GMP signalling and growth phase switching have all been worked on as a result of this paper. The dataset also features majorly in the genome comparison between the lab strain of *B. bacteriovorus*, HD100 and the newly sequence environmental isolate *B. bacteriovorus* Tiberius, which is the subject of the next paper in this chapter.

Another and more recent major continuations of this paper is one that I feature on as first author and investigates growth phase switching of *B. bacteriovorus,* it uses the microarray data in this study to compare it to RNA sequencing data and finds that there is much overlapped between the two techniques and therefore verifies each of the datasets (Capeness et al., 2013). This paper features later in

this thesis. The work present in this paper will also continue to be referenced in future as a basis of experimental research and by the wider research community.

4.3 Paper 6. Genome analysis of a simultaneously predatory and prey-independent, novel *Bdellovibrio bacteriovorus* from the River Tiber, supports in silico predictions of both ancient and recent lateral gene transfer from diverse bacteria.

Hobley L, Lerner TR, Williams LE, Lambert C, Till R, Milner DS, Basford SM, **<u>Capeness</u>** MJ, Fenton AK, Atterbury RJ, Harris MA, Sockett RE.

My Contribution

For this paper I was involved in the bioinformatic analysis of the newly isolated and sequenced *B. bacteriovorus* Tiberius. This involved finding genes not conserved between this strain and the HD100 lab-strain and using the sequence of these genes to find homologues and predict their functions. In total I looked at nearly 250 Kb of the Tiberius genomic sequence and analysed it against HD100, I also helped to streamline the process of annotation of this new genome. I also grew the Tiberius and HD100 strains for comparisons of the carotenoid content to show that they differ.

What the Paper Claims

The main aim of this paper was to sequence the genomic DNA of a newly isolated aquatically sourced *B. bacteriovorus* Tiberius strain and compare it to the HD100 lab-strain that is terrestrially sourced, to give an insight to the differences in genetic makeup, growth phenotypes/morphologies and evolutionary backgrounds of both the strains. As such the paper represents the first full

comparative genome analysis in *B. bacteriovorus* research and Tiberius is the second member of the *B. bacteriovorus* genus to have its genome fully sequenced and made available.

Growth and morphology

B. bacteriovorus Tiberius was isolated from a freshwater sample of the river Tiber in Rome, Italy. Pure cultures of strain were obtained the main finding of the research shows that Tiberius can grow simultaneously host-independently and host-dependently forming both colonies of PY medium and plaques on a lawn of *E. coli* cells respectively but also grow HI-like in minimal media. The HD100 strain however requires a large number of cells (10^7) to do this and often requires the mutation in the *bd0108* gene; suggesting that Tiberius is in some way can grow both HI and HD without mutation.

The plaques it formed on lawns of prey were reminiscent of those seen in earlier works of HI isolates growing in a similar manner in that they both formed a clearing region with a colony like growth in the centre. The morphologies of the cell population also showed that in liquid culture, set up from a single colony, there was a mix of short vibriod cells and long, filamentous cells akin to a mixed culture of attack-phase cells and HI cells of HD100 (**Fig 31**).

As Tiberius is a new isolate of the *B. bacteriovorus* species an experiment into auto-predation was also carried out, with both strains expressing differently-tagged fluorescent proteins. The protein chosen to tag was the very-abundant nuclear binding protein HuaA and was tagged with either mCherry, or TFP, both of which have discrete wavelengths for excitation and emission excitation (TFP:

excitation 550-600 nm; emission: 610-665 nm and mCherry: excitation: 458-500nm; emission420-454). Any incidence of one colour inside a bdelloplast coloured by the other, would show that one/both strains can prey on one another. This was also to address the presence of the round bdelloplast-like structures occasionally found in liquid cultures of HI cells. However auto-predation did not occur, suggesting that the bdelloplast-like structures are more certainly sphaeroplasted HI cells and that *B. bacteriovorus* are not susceptible to auto-predation, and that the outer surface of the B. bacteriovorus is suitably different from the surface of potential prey cells.

The Tiberius strain was also observed to use gliding motility similar to the reports published previously by the Sockett lab (Lambert et al., 2011). Even though this is an aquatic strain surface gliding must still be of importance as functionality is still maintained and maybe as required by Tiberius as it is with a soil *B. bacteriovorus* such as HD100. There are many possible reasons that gliding motility is still maintained, perhaps because it allows Tiberius to be an efficient predator of biofilms or it helps with the gliding across the bed of a river.

a(i)   a(ii)   b(i)   b(ii)

c(i)   c(ii)   c(iii)   c(iv)   c(v)

d   T=0   T=38   T=45   T=111   h

e   T=0   T=23   T=78   T=253   T=275   T=300

f   T=0   T=13.5   T=35   T=55   T=75   T=87.5   T=100

g   HD100   High Nutrients   Low Nutrients   OR

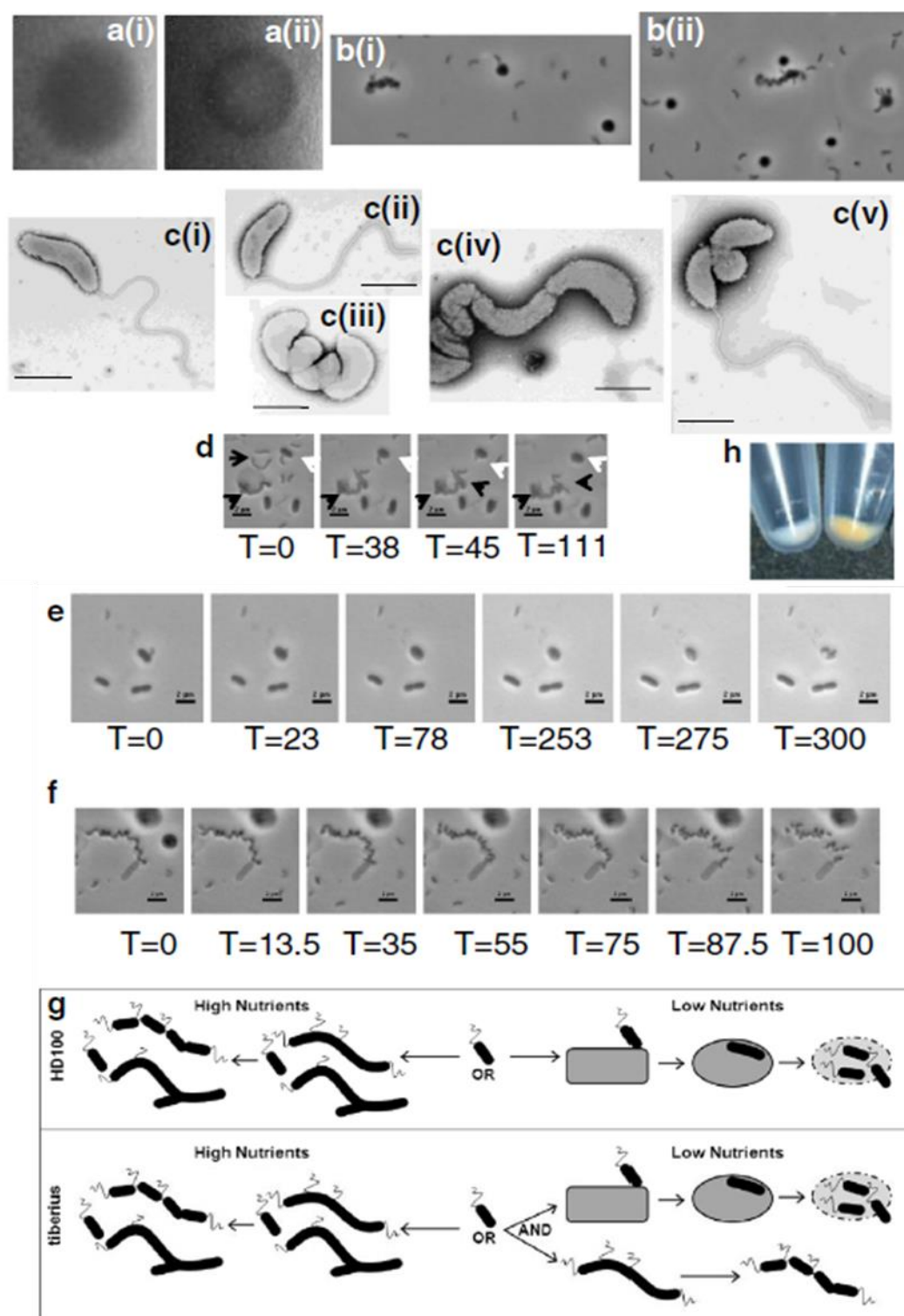tiberius   High Nutrients   Low Nutrients   OR   AND

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Fig 31. Simultaneous predatory and prey-independent growth by *B. bacteriovorus* Tiberius in comparison to HD100. (**a**) Plaques on overlay plates containing *E. coli* (i) a HD100 clear plaque and (ii) a Tiberius plaque with central colony growth by the Tiberius. (**b**) Bright-field microscopy images of serpentine HI-like growing Tiberius cells alongside free-swimming HD-like cells and bdelloplasts. (**c**) Electron micrographs of Tiberius cells (i and ii) attack phase, predatory cells (iii-v) filamentously growing cells, HI cells growing free of prey cells. (**d – f**) stills from time-lapse bright-field microscopy showing invasion of E. coli cells (white arrows) by Tiberius while at the same time a HI-like cell divides (black arrows) (**d**). (**e**) Invasion of the *E. coli* results in multiple progeny akin to HD-like predation over the course of 300 minutes. (**f**) Septation of a long filamentous cell by synchronous fission outside of a prey cell. (**g**) Comparison of the different predatory phases of HD100 and Tiberius under different nutrient availability. (**h**) Cell pellets of predatory cells of both Tiberius (pale) and HD100 (yellow) showing how the carotenoids levels differ. From **Paper 8.** (Hobley et al., 2012b).

Genome Comparison

With the advent of the sequencing of the Tiberius strain it was possible to do a comparative analysis between it and the HD100 lab-strain (**Table 6**). The comparison shows that between the Tiberius and the HD100 lab-strain there was great genome synteny between the isolates; this is despite the strains being isolated far apart and from different environments (terrestrial vs. aquatic). However there were differences, mainly the genome of Tiberius contained an extra 205 Kb encoding some 201 protein-coding regions, Tiberius contained 312 different genes not found in HD100, and HD100 had 256 genes not found in Tiberius. Reciprocal Smallest Distant (RSD) analysis; carried out by Dr. Williams, added to these numbers further by bringing the estimate to 535 genes found only in Tiberius and 384 genes only found in HD100. The majority of these genes were attributed to LGT rather than being inherited from the last common ancestor and then being selected for in each strain. The additional genes in Tiberius were thought to be the result of an increased number of IS (Insertion Sequence) elements and how some of the additional genes are found at tRNA genes, indicating potential ICE-like (Integrative Conjugative Elements) sequences having a role in gene acquisition, it was therefore hypothesised that these factors are

driving LGT in Tiberius.

B. bacteriovorus strain.	Size	%GC	Predicted Genes	Predicted Proteins
HD100	3.78	49.9	3628	3586
Tiberius	3.99	50.6	3779	3738

Table 6. A comparison between the genomes of the HD100 and Tiberius strains of *B. bacteriovorus.*

Of those genes that were acquired by Tiberius, many of them have predicted functions that potentially maybe involved in HI growth, while others had predicted functions that may be involved in resistance to pollutants found in the river Tiber such as genes encoding efflux pumps. Other genes were predicted to be part of mobile DNA events such as nucleases and DNA-modifying genes, while others had more of a role in growth pathways such as sigma factors, methylation, lytic transglycosylases and transferases. Some genes showed adaption to the environment from which the Tiberius strain was isolated; these include surface adhesion, LPS modification and pilus fibre modification. These further imply that the Tiberius is an adapted predator to fit its niche environment.

From the list of additional genes in Tiberius further clues suggesting LGT were found, as a number of genes highest homology were to marine or freshwater bacteria, namely; *Anabaena*, *Nostoc*, *Roseiflexus*, *Rhodospirillum* among others. The proteins made by the genes present in HD100 and not Tiberius have higher homology to those from more terrestrial bacteria indicating large amounts of LGT from co-inhabiting bacteria and that they are of ancient origin. There were also plant and soil species found to have transferred genes to Tiberius including *Rhizobium* and *Pseudomonas* as well as species known to contaminate the river

Tiber such as *Clostridium*, *Vibrio* and *Shewanella* (Marcheggiani et al., 2008). Interestingly no *E. coli* or *Salmonella* genes were found in the gene set despite them being found in the Tiber.

Bd0108 and Bdt0101 comparison

Given the noted morphological phenotype seen in the liquid culture of the Tiberius; a comparison of the HD100 *bd0108* gene to that of the Tiberius *bdt0101* was carried out (**Fig 32**). Previously this has been shown to be mutated in 89% of HI isolates, with 46% of the mutations being a deletion of 42 bp of the *bd0108* gene (Wurtzel et al., 2010). The region of the 42 bp deletion of in *bd0108* is flanked by 10 bp direct repeats and the same repeats are found in the Tiberius *bd0108* homologue; *bdt0101*. From an alignment at the protein level it was shown there are 3 substitutions from HD100 to Tiberius; V31A, A96T and G97S all of which appear not to be very conserved in terms of amino acid properties, however none of the mutations have been noted to exist in HIs isolated from HD100 (**Fig 32**). It may be possible that this has some bearing on the HI-like phenotype observed in liquid culture, however upon generation of HI isolates from Tiberius, using the same method for HI generation in HD100, none of the isolates contained different mutations or the common 42 bp deletion widely observed in the HD100 *bd0108* gene, despite the fact the Tiberius strain still has the direct repeats flanking the region. Given this, the method of HI growth control in Tiberius may be different to that of HD100 and involve more than just the *bdt0101* gene.
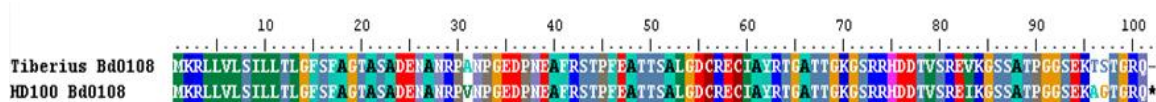
Fig 32. Protein alignment of Bd0108 protein from *B. bacteriovorus* Tiberius and *B. bacteriovorus* HD100. Identical amino acids are highlighted in similar colours. Compared to HD100 the Tiberius has three substitutions V31A, A96T and G97S.

Other Gene comparisons

Both the flagellin gene number (6) and *mot* gene pair number (3) are conserved between HD100 and Tiberius, and the gene *fliC3*, previously mentioned the motility chapter that gave a non-motile phenotype, is 100% identical at the amino acid level, indicating its importance. Chemotaxis genes are also highly conserved, though HD100 contains 4 genes not found in Tiberius, but Tiberius has 4 MCP genes not found in HD100, this possibly indicates environmental adaption. As expected the Type IVa genes pili are also conserved between the strains and no alternate adherence pathways appear to be present.

From the publication mentioned previously in this thesis regarding *B. bacteriovorus* microarrays (Lambert et al., 2010a), the genes present in the HD100 dataset for phases of growth were compared to those present in the Tiberius genome. Around 60% of the genes not conserved in Tiberius were found to be in the up-regulated in attack-phase dataset highlighting the need for potential differences between the environments which the two bacteria carry out predation this is further supported as around 10% of the genes are predicted to have roles in outer/inner membrane at which predation assigned proteins would have a role.

Around 6% of the non-conserved genes in Tiberius were predicted to have roles in predation establishment, the 'predatosome', within the prey cell while, possibly indicating that the genetic queuing of the Tiberius once established varies from that of HD100. The majority of the genes however found in the 'predatosome' gene set in HD100 are conserved in Tiberius, indicating a core set of genes required for effective predation and very similar metabolic pathways and queues across the two different bacteria.

The vast majority of genes found up-regulated in the HI dataset in HD100 are also conserved in Tiberius predicting that the HI growth and replication and the metabolic 'housekeeping' of each strain is very similar, though different enough to manifest itself in different morphologies in liquid culture.

Other non-conserved genes in Tiberius fall outside of the microarray datasets and so would belong to time points later in the growth cycle, such as escaping from the bdelloplast or even regulation events to promote synchronous cell division.

4.3.1 Conclusions of the Paper

The main conclusion of this paper is that between two strains of *B. bacteriovorus* there is a large amount of gene conversation and synteny despite one being of terrestrial origin and the other being aquatic. There is also massive overlap between the genes found in the Tiberius genome and those present in a prior study into the gene up-regulation of HD100, and that a core 'predatosome' exists

The paper also investigated LGT and showed that some of the additional genes came from potential prey and other bacteria occupying the aquatic environment from which Tiberius was isolated. The work builds on research previously carried out *in silico* by the Pan and Gophna labs (Pasternak et al., 2012) (Pan et al., 2011) and shows that LGT does occur despite *B. bacteriovorus* being an organism that effectively digests foreign DNA as part of its predatory lifecycle. The majority of the genes highlighted to have ancient LGT in the study by the Gophna lab are conserved in Tiberius showing not only their study to be correct but also that Tiberius and HD100 share common ancestry.

The failure of either the Tiberius or the HD100 to auto-predate also lends itself to the idea of LGT, as potentially conjugation between two strains of *B. bacteriovorus* is preferable to predation, this may pose a similar reason for the lack of LGT from *E. coli* or *Salmonella* is seen, as it is more preferable to carry out predation upon them than to transfer genetic material. The large amount of donors of LGT in Tiberius could be a result of the HI/predatory mixed culture and perhaps the HI cells, which are generally less efficient at predation and less motile, may be the receptacle for the conjugation of the DNA.

The mode of HI formation in Tiberius is something that will require much greater experimentation, it was hoped that exploration into the sequence divergences of the *hit* locus between Tiberius and HD100 would shed greater light onto the nature of mutation and how it promotes HI growth. This however was not found to be the case, as mutations were observed that differ from those reported in the literature and seen by the Sockett lab which adds further complexity to the mode

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of action.

4.3.2 Since the Publication and Impact

Since the publication of the paper in late 2012, the paper has not been cited and so has yet to have its full impact. The Tiberius strain is an aside from the main work carried out on HD100 both by the Sockett lab and generally the *B. bacteriovorus* community as a whole and represents an end point in terms of genome comparison until a new sequence is made available to build upon the story.

A paper was more recently published comparing and contrasting the *B. bacteriovorus* HD100 genome and that of *Bacteriovorus Marinus* SJ, another delta-proteobacterium, which has a similar invasive lifecycle to *B. bacteriovorus* but lives in salt water. *B. marinus* was originally designated a *B. bacteriovorus*, though due to genomic divergences and phenotypic distances was reclassified. The comparison found that only 34% of the genome had homology to *B. bacteriovorus* and 291 of those genes were unique to both strains, suggesting a core prey-interaction/invasion gene set. Over 34% of the *B. marinus* genome also had no homology to anything else in the databases, and as such there was no gene synteny observed between the two strains

With the arrival of the newly sequenced Tiberius strain future experiments carried on HD100 will also carried out on it and slowly a better understanding of the differences between the two strains will emerge. This is quite an exciting prospect as no cross complementation between *B. bacteriovorus* strains has

been carried out before so it could significantly improve our knowledge on the lifecycle of *B. bacteriovorus.*

Perhaps one follow on publication from this work is that further investigation of the roles of Bd0108 and Bd0109 and their roles in HI growth switching and pili production (see chapter 5.3). Though there is no direct link between this and the Tiberius work being published it does begin to explore the nature of the predatory to HI growth switching seen in HD100 and finds that a result of this are variations in pili length (Capeness et al., 2013). The gene *bd0109*, which is next to and co-transcribed with *bd0108* in HD100 (the proteins of which interact) is 99% identical at the protein level and still contains the RHS elements that may give Bd0109 a role in pilus function in *B. bacteriovorus*. The later paper hypothesises that the differences in the sequence of Bd0108 interact differently with Bd0109 resulting in alternate pilus presence which leads to HI growth. It is possible that Bdt0101 interacts in this way with Bdt0102 (the Bd0109 homologue in Tiberius) to regulate the somewhat simultaneously HI and HD nature of Tiberius. The Bd0108 and Bd0109 interaction is something greatly expanded on later in this thesis (see chapter 5.3).

4.4 Regulation of Predation Conclusions

It is clear that predation by *B. bacteriovorus* is very complex and we are only just beginning to understand the basics of the vast amount of regulation involved. The shear amount of data from the micro-arrays alone asks more questions than it answers, this coupled with a newly isolated *B. bacteriovorus* strain means that there is even more scope for new insights into predation and a plethora of experiments to come based on this findings. The newly isolated *B. bacteriovorus* has given further insight into the evolution of *B. bacteriovorus* and the essentiality of some of the gene sets between this and HD100.

Future Work

The research has come a long way from purely biochemical experiments of 1990's and before to having an abundance of data. Though some of the micro-array data has been further explored there still remains many questions. There is a large number of genes that are *B. bacteriovorus* specific, many of which appear to be predation specific also, it is this gene set that more understanding of predation will come.

Perhaps one way in which the data could be further explored is by continuing micro-array (or RNAseq) experiments on different time points of *B. bacteriovorus* predation to underpin not only what genes are up-regulated, but relating this to the previous study show how long they are up-regulated for, giving further insight into the role of the gene in question. RNAseq in particular can be used to show which genes are in operons, as well as non-coding RNA in the genome possibly involved in regulation/expression. Micro-array or RNAseq on the Tiberius strain

compared to the HD100 can further identify possible genes that are fundamental to regulation events, as the current comparison between the micro-array of HD100 and the genome of Tiberius only shows absence/presence.

Another way to explore the regulation of predation is to carry out ChIP-seq to identify the proteins involved in repression/expression of those genes highlighted in the micro-arrays/RNAseq experiments hypothesised above. This is has not been carried out in *B. bacteriovorus* before so would present a significant advancement to the understanding of DNA-binding proteins.

Biofilm predation by *B. bacteriovorus* is something that has long been noted, the discovery of gliding motility and a new environmental isolate puts forth the potential for it to be used (perhaps more effectively) to study this. By the above methods it could be possible to find the genes involved surface motility, particularly at the 1-2 hour period on a surface shortly before the *B. bacteriovorus* initiate surface gliding, this potential has a large impact on predating within a biofilm. It could also be possible to establish if there are any differences in the early, pre-invasion time point when predating on a biofilm compared to 'free' prey in liquid culture.

With a newly isolated strain of *B. bacteriovorus* it is now possible to do cross species complementation with the potential to look at genes such as *bd0108*/*bdt0101* that is involved in growth switching and given the HI/HD phenotype in liquid cultures of the Tiberius, could complementation of a *bd0108* deletion with the Tiberius lead to a similar phenotype in HD100.

5.0 Secretion Events and Signalling in HI and HD Growth Phases

B. bacteriovorus is a complex organism that can carry out predation of Gram-negative bacteria, but also grow prey-independently in rich media; because of this bi-phasic lifestyle its genome must contain a large array of genes whose products suit the different molecular secretion environments and growth phases. With regards to predatory growth, specific cues such as pili retraction, must occur at the right time to ensure efficient growth i.e. after prey invasion, and to bring on appropriate waves of secretion events as it would be futile to release DNA degradation enzymes when outside of a prey cell, or increase chemotaxic protein production to regulate motility when trapped inside a bdelloplast. The issue is made more complex by the inclusion of HI cells and the transition between prey-independency and sessile growth modes (and vice versa).

Work reported in this thesis using micro-arrays (see chapter 4.2) to look at gene expression at different growth stages of the two *B. bacteriovorus* HD100 lifecycles has shown that gene expression is quite distinct (Lambert et al., 2010a) and there is a core 'predatosome' responsible for initial degradation and establishment inside a prey cell, and this differs from the gene set being expressed by HI cells in rich media.

Secretion

As *B. bacteriovorus* can grow in two distinct ways, its genome encodes all the

genes for the uptake of nutrients whether they are from the surrounding media or from the enzyme-created 'soup' made from an invaded prey cell's content found inside an bdelloplast. From the HD100 genome paper the list of secretion/transporter pathways is quite vast, 244 predicted proteins (6.8% of the total genome) encode products implicated in membrane transport mechanisms (Rendulic et al., 2004). Of these 147 are predicted to be ABC-type transporters and 97 encode permeases making up the type I and type II transporters that can transport substrates across the inner and outer membranes, there is however no evidence for type III or IV secretion found (Rendulic et al., 2004). It was also noted the genome encodes the TAT apparatus that actively facilitate the secretion of proteins encoding a twin-arginine at the N-terminus (consensus S/TRRxFLK), it is with this the next publication in this thesis concerns (Rendulic et al., 2004) (Barabote et al., 2007).

The TAT pathway

The TAT pathway is made of generally three proteins, TatABC forming the protein recognition and shuttle complex (TatBC) that holds protein for signal sequence cleavage, checks it is folded correctly and passes it to the transporter made of multi-meric TatA protein for eventual translocation across the inner membrane to the periplasm (**Fig 33**). The pathway is found in both Gram-negative and Gram-positive bacteria though it lacks the TatB protein in the latter, and has roles in outer membrane integrity, metal acquisition and toxin production in *Yersnia*, *Pseudomonas* and *E. coli* (Berks et al., 2005) (Palmer et al., 2005) (Sargent, 2007) (Stevenson et al., 2007). As the roles of the TAT system are

quite diverse we wished to know what its role is in both the *B. bacteriovorus* lifecycle and which part of the lifecycles they are involved in.
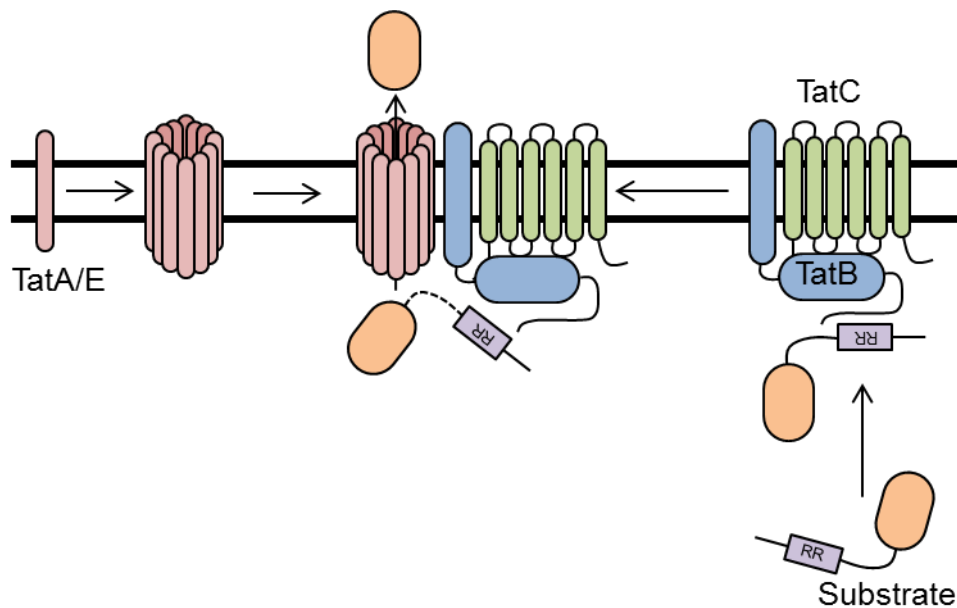

Fig 33. A schematic of the TAT system. The substrate of the TAT system; a protein containing the twin arginine repeat is carried to the TatBC complex (usually by a chaperone) and is recognised as a TAT specific substrate. TatA/E multimerises forming a pore and is incorporated into the TatBC complex. The substrate has its RR-signal sequence removed and is transported into the periplasm. Adapted (Lee et al., 2006)

Signalling

Due to the complexity of *B. bacteriovorus* and it requiring complex queues it stands to reason there needs to be a system in place to regulate it to produce effective growth and predation. This features a diverse group made up of 134 signal transduction proteins including those signals responsible for chemotaxis and aerotaxis of which there are (20 and 1 resp.) (Rendulic et al., 2004). Signalling can also have a role, not only internally to the cell but also externally between cells such as quorum sensing in a biofilm or in social gliding. One relatively new signalling pathway that has gained considerable interest over the last few years is that of cyclic-di-GMP signalling. Though originally identified 25 years ago interest and understanding of the pathway has greatly improved in the last 10 years, with the pathways in proteobacteria being the most studied. Cyclic-

di-GMP signalling is a secondary messenger RNA system that is involved in the bacterium cell response mechanism as the molecule binds to proteins containing a GGDEF, EAL or HD-GYP motif, synthesis or degradation of this molecule is then carried out to elicit a response (**Fig 34**). In *B. bacteriovorus* there are some 28 genes for cyclic-di-GMP signalling including 5 GGDEF containing proteins, 15 PilZ containing proteins, 6 HD-GYP containing proteins, 1 EAL protein and 1 riboswitch, This signalling system in other bacteria has been shown to be involved in a wide range of bacterial pathways including motility, biofilm formation, pathogenicity and the cell cycle itself (Reviewed in (Romling et al., 2013)). Despite the vast amount of research being undertaken by the scientific community, cyclic-di-GMP signalling in *B. bacteriovorus* remained unstudied until the Sockett lab published the first paper on the topic which is discussed here.
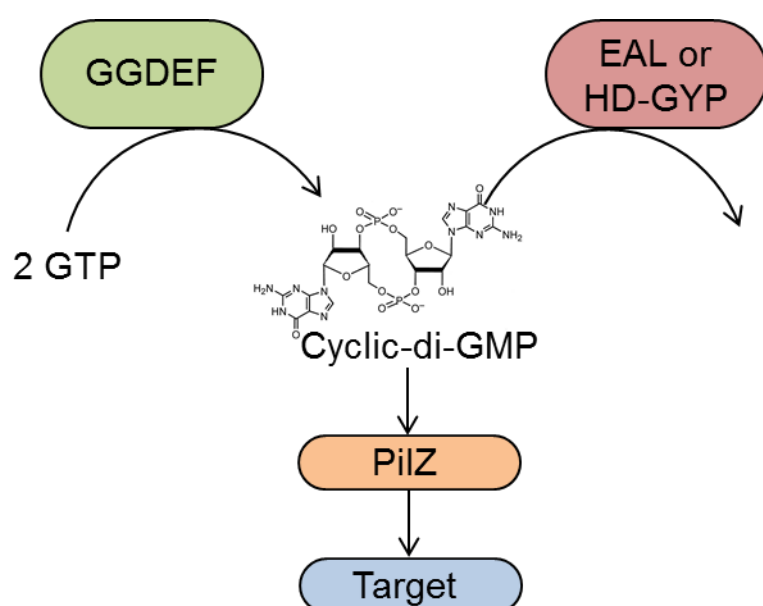

Fig 34. Pathway of the cyclic-di-GMP. Cyclic-di-GMP is synthesised from GTP by proteins containing the GGDEF consensus or degraded by proteins containing the EAL or HD-GYP consensus to pGpG. Cyclic-di-GMP is bound by proteins such as those containing the PilZ motif which target other proteins to illicit responses in various bacterial pathways.

HI Growth switching

Perhaps one of the biggest and oldest questions in the *B. bacteriovorus* research community is that of how a predatory *B. bacteriovorus* switches its growth cycle from a predation dependent (HD) one to that of a host-independent (HI) one. Though first noted in 1963 by Stolp and Starr (Stolp and Starr, 1963) it wasn't until 1992 that this was linked to be as the result of mutation/s in the *hit* locus gene *bd0108* (Cotter and Thomashow, 1992) though this was not linked to a pathway only that of a growth phenotype. Further complicating the issue is that not every HI isolate has a mutation at this point suggesting that the *hit* locus itself is not alone in the triggering of this growth phase switch, and other genetic mutations must play a role.

5.1 Paper 7. The *Bdellovibrio bacteriovorus* twin-arginine transport system has roles in predatory and prey-independent growth.

Chang CY, Hobley L, Till R, **Capeness** M, Kanna M, Burtt W, Jagtap P, Aizawa S, Sockett RE.

Microbiology. 2011 Nov;157(Pt 11):3079-93

My Contribution

For this paper I was involved in the cloning and subsequent mutation by insertion of a kanamycin cassette of the *tatC1* gene. I also carried out the initial RT-PCR expression analysis of the TAT genes that were repeated (and thus confirmed) by Dr. Chang. I also carried out the initial mobilisation of the a*praR* cassette into pUC19 to make the pUC19:apraR construct. This provided another useful antibiotic resistance marker for use in *B. bacterivorus* that could be used in conjunction with the kanamycin one to carry out double insertion mutations at a time when a method for markerless deletions did not exist.

What the Paper Claims

Insertional activation of each of the TAT genes, *tatA1*, *tatA2*, and *tatC* was attempted with the *tatA2* and *tatC* mutations proving lethal in both HD and HI growth phases of *B. bacteriovorus*. Inactivation of the *tatA1* gene resulted in a mutant that had a decreased predation rate when compared to the wild-type *B. bacteriovorus.* This showed the predation time of the mutant was lengthened leading to bdelloplast structures still being present in the infection mixture at an

equivalent time to the wild-type *B. bacteriovorus* finishing the predation cycle and the progeny being released (**Fig 35**). Growth rate of the *tatA1* HI mutant was 3.2 fold lower than that of multiple 'wild-type' HI isolates.
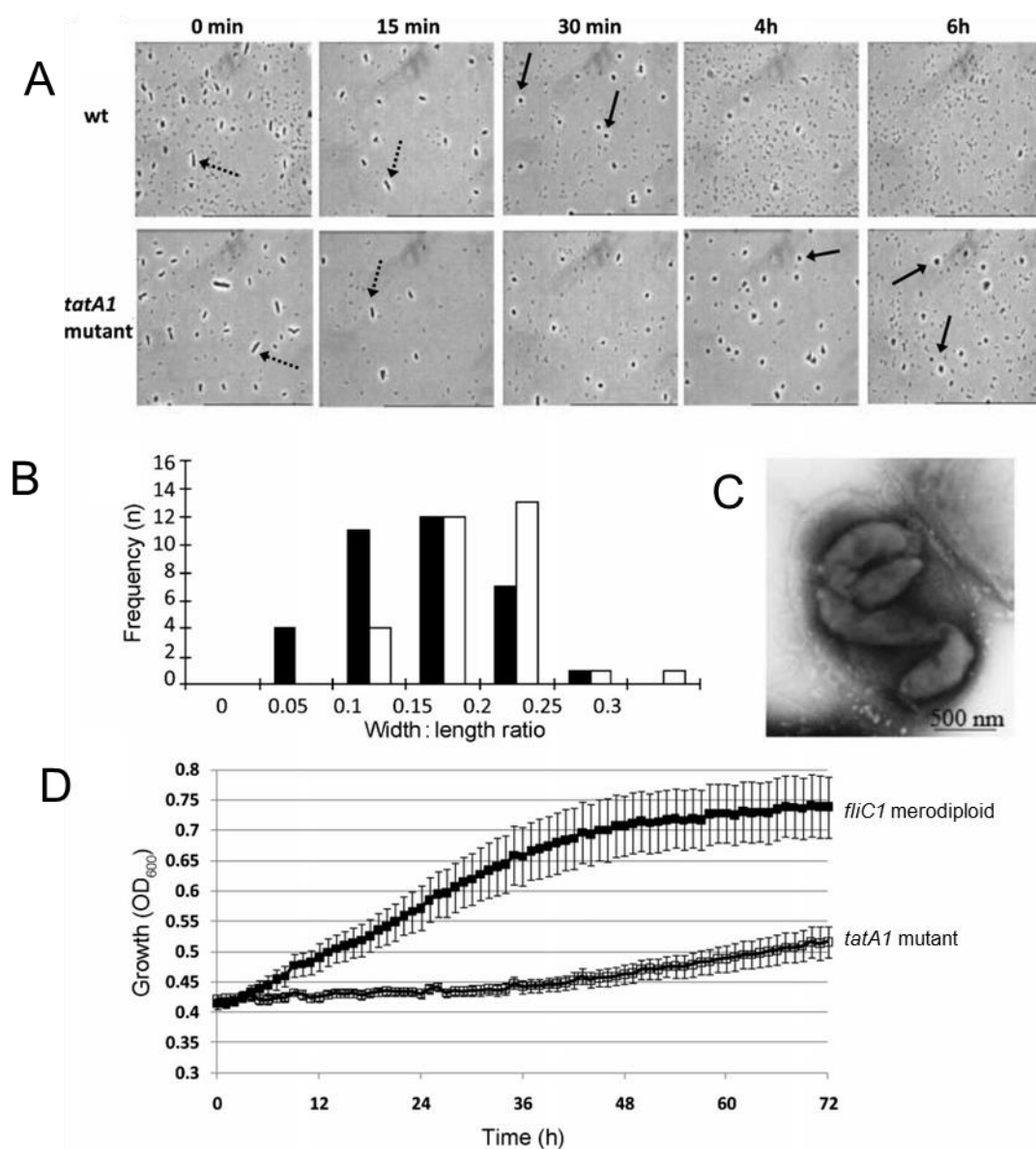

Fig 35. Phenotype of the *B. bacteriovorus tatA1* mutant. **A.** Bdelloplast persistence assay during predation of *E. coli* S17-1 (dashed arrows) resultant in bdelloplasts (solid arrows) once invaded by with wild-type HD100 or the *tatA1* mutant. **B.** Width : Length ratios of the *tatA1* mutant (white) versus the HD100 wild-type (black). **C.** Electron micrograph of the *tatA1* mutant during the 3-4 hour time point of the predatory cycle. **D.** Growth of HI isolates of the *tatA1* mutant and the *fliC1* merodiploid (wild-type) over the course of 72 hours from a matched OD600. Error bars show the 95% confidence interval around the mean. From **Paper 5.** (Chang et al., 2011).

Expression analysis of the TAT genes in wild-type *B. bacteriovorus* showed that

tatA1 was highly expressed at the 15 minute time-point, suggesting that it has a

role in attachment and invasion in the predatory lifecycle. The *tatA2* mutant

however showed constitutive expression across the lifecycle (**Fig 36**). Expression

of both *tatB* and *tatC* remained constant throughout wild-type lifecycle suggesting

it is the initiating of the *tatA* genes that would start translocation in a predatorily

specific role. Expression of the *tatB* and *tatC* genes from *B. bacteriovorus* altered

in the *tatA1* mutant background, showing a peak at the 15 minute and 2 hour

time-point possibly to compensate for the lack of *tatA1*.
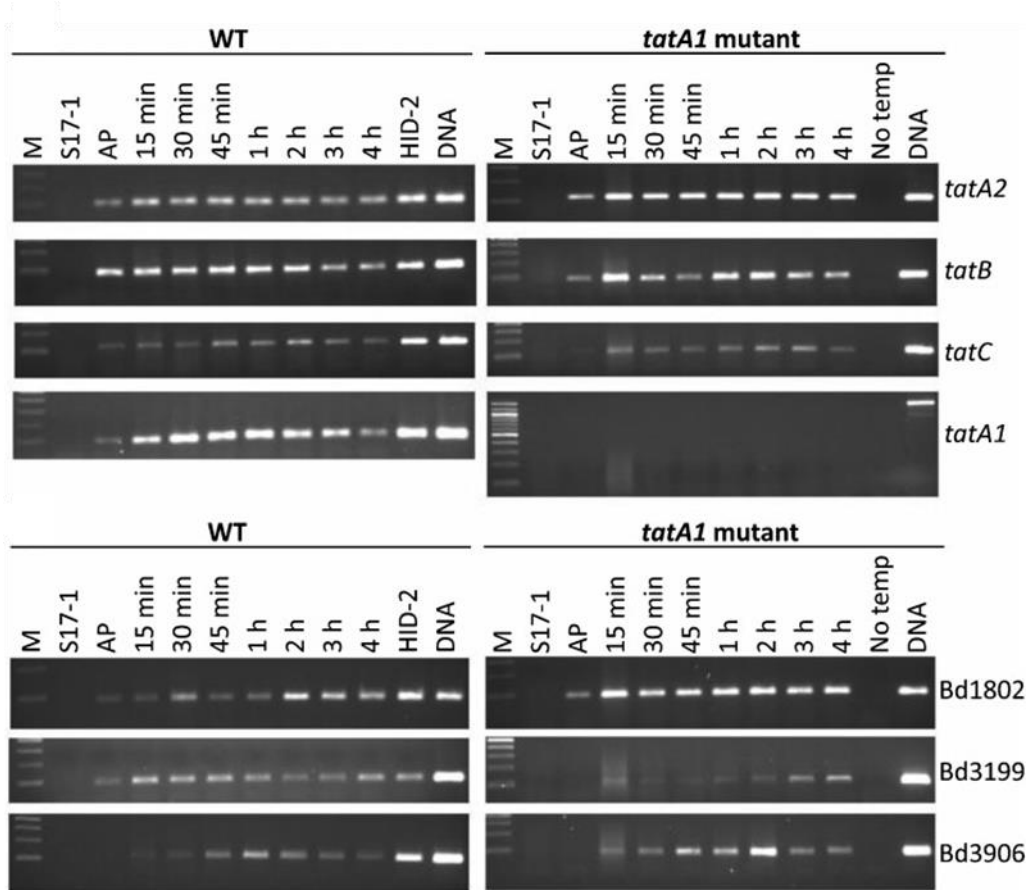
Fig 36. RT-PCR analysis of *B. bacteriovorus* wild-type HD100 and the *tatA1* mutant across the predatory lifecycle. **A.** The various TAT genes from the HD100 genome. **B.** Three TAT substrates. Lanes: M – 100 bp ladder; S17-1, *E .coli* alone; AP – attack-phase; 15 min-4 hours, time points post-infection; HID-2 a HI isolate of HD100; DNA – HD100 genomic DNA template; No temp – no template added. From **Paper 5.** (Chang et al., 2011).

Using the online TAT substrate predictor TatP (Bendtsen et al., 2005) and PRED-TAT (Bagos et al., 2010), 21 potential TAT substrates were predicted to be encoded from the HD100 genome based on the presence of the twin-arginine motif and potential TAT cleavage signals. Seven of the genes have no known function, 6 are predicted to have redox functions, 1 phosphodiesterase and 1 membrane degradation function.

The expression of the predicted TAT substrates genes by RT-PCR analysis showed constitutive expression across the *B. bacteriovorus* predation lifecycle, though three genes; *bd1802*, *bd3199*, and *bd3906* showed different expression patterns. In the wild-type background *bd1802* had peaks at 30 minutes and 2 hours, *bd3199* peaked at 15 minutes, and *bd3906* showed a peak at 1 hour (**Fig 36**). These however changed when the expression was looked at in the *tatA1* mutant background with *bd1802* highest expression seen at 15 minutes, *bd3199* highest expression was seen highest later in the lifecycle at 3-4 hours and *bd3906* highest expression points expanded to 2-4 hours. This suggests that *tatA1* potentially has a role in regulation of TAT substrate expression, and perhaps the build-up of substrate due to failures in exportation (due to the lack of TatA) may feedback to alter the expression of the substrate genes itself.

Fluorescent tagging of TAT substrates

To further explore the TAT substrate, one protein, Bd1802; which is predicted to encode a ferrodoxin like protein involved in redox pathways and a potential iron-

sulphur protein, was fluorescently tagged with eGFP and put in the *B. bacteriovorus* wild-type background via a single cross-over. Fluorescence of the protein showed that it was exported to the bdelloplast structure at the 3-4 hour mark, which corresponded with the gene expression seen in the lifecycle at the 2-4 hour mark. Insertion mutation of the *bd1802* in the *B. bacteriovorus* genome did give viable isolates that were not reduced in predation efficiency.

The TAT substrate protein Bd3906 was also tagged fluorescently; it is predicted to encode a ParA-like protein involved in chromosome partitioning, this substrate however was not seen by fluorescent microscopy in the bdelloplast and only inside the *B. bacteriovorus* itself at the 2-4 hour mark. Fluorescence of both the tagged Bd3906 and Bd1802 proteins was also looked for in the *tatA1* mutant background, though was not observed. Though *bd3906* and *bd1802* are still expressed in the *tatA1* mutant (**Fig 37**), expression of the tagged genes was not seen and this may possibly be why there is no fluorescence seen. Alternatively the expression of tagged gene may still occur though due to misfolding of the RNA, due to the extra eGFP sequence, may not be translated.
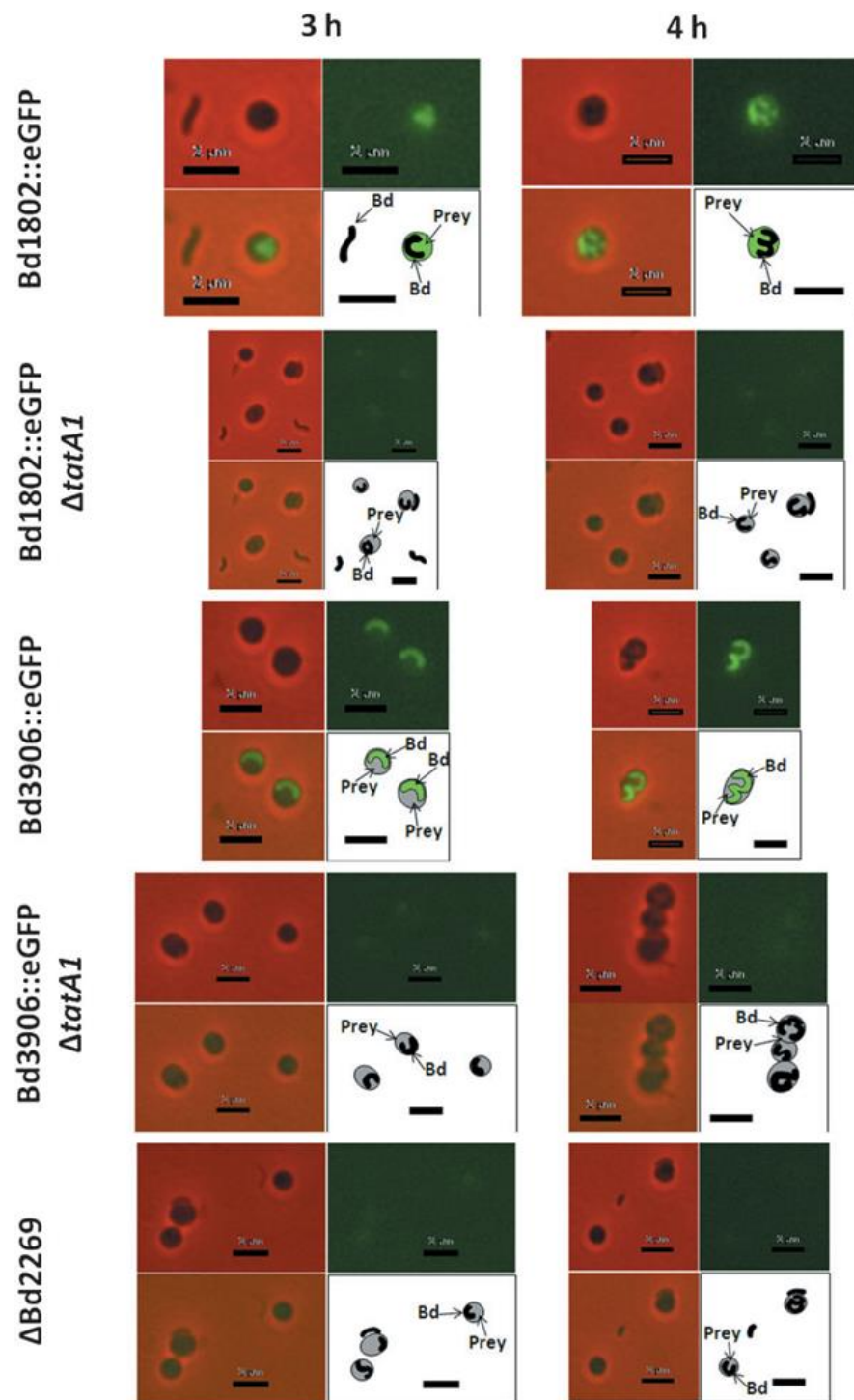
Fig 37. Fluorescent microscopy images of the predatory growth of *B. bacteriovorus* with predicted TAT substrate-eGFP fusions. The predation was carried out on non-fluoresence *E. coli* S17-1 and imaged at 3 and 4 hours post-infection. The Bd1802::eGFP was shown to be exported into the bdelloplast, while the Bd3906::eGFP was located within the *Bdellovibrio* itself. The HD100 *Δbd2269* mutant was used a wild-type. From **Paper 5.** (Chang et al., 2011).

Complementation of *E. coli* TAT mutants with *B. bacteriovorus* TAT genes

The *E. coli* TAT mutants: JARV16-P (*tatAE* deletion), BØD-P (*tatB* deletion) and B1LK0-P (*tatC* deletion) which were kindly supplied by the Tracy Palmer laboratory (Sargent et al., 1999) (Buchanan et al., 2002) were used as recipients for *B. bacteriovorus* TAT genes to test if the *B. bacteriovorus* equivalent genes could be used to complement them in an SDS plate assay (**Fig 37**). By cloning the *B. bacteriovorus* TAT genes into a plasmid where they were inducible by IPTG and transforming them into *E. coli* it was hoped that successful complementation would increase the resistance of the *E. coli* to SDS treatment by maintaining the cellular envelope and thus improving the viability of the cells. It was found that the *B. bacteriovorus tatA2* could complement the JARV16-P mutant once induced showing that the *B. bacteriovorus TatA2* functions in the TAT pathway of *E. coli*, the *tatA1* gene could not do this however. B1KL0-P could also be complemented with the *B. bacteriovorus tatC* gene, however not when expression was induced suggesting that large amounts of *B. bacteriovorus* TatC protein are toxic to the *E. coli tatC* mutant cell. *B. bacteriovorus tatB* gene could not complement the *E. coli tatB* mutant (BØD-P) and inhibited the growth of the MC4100 wild-type *E. coli*. Inhbition of MC4100 was also seen in the *E. coli* expressing the *B. bacteriovorus tatC* gene.
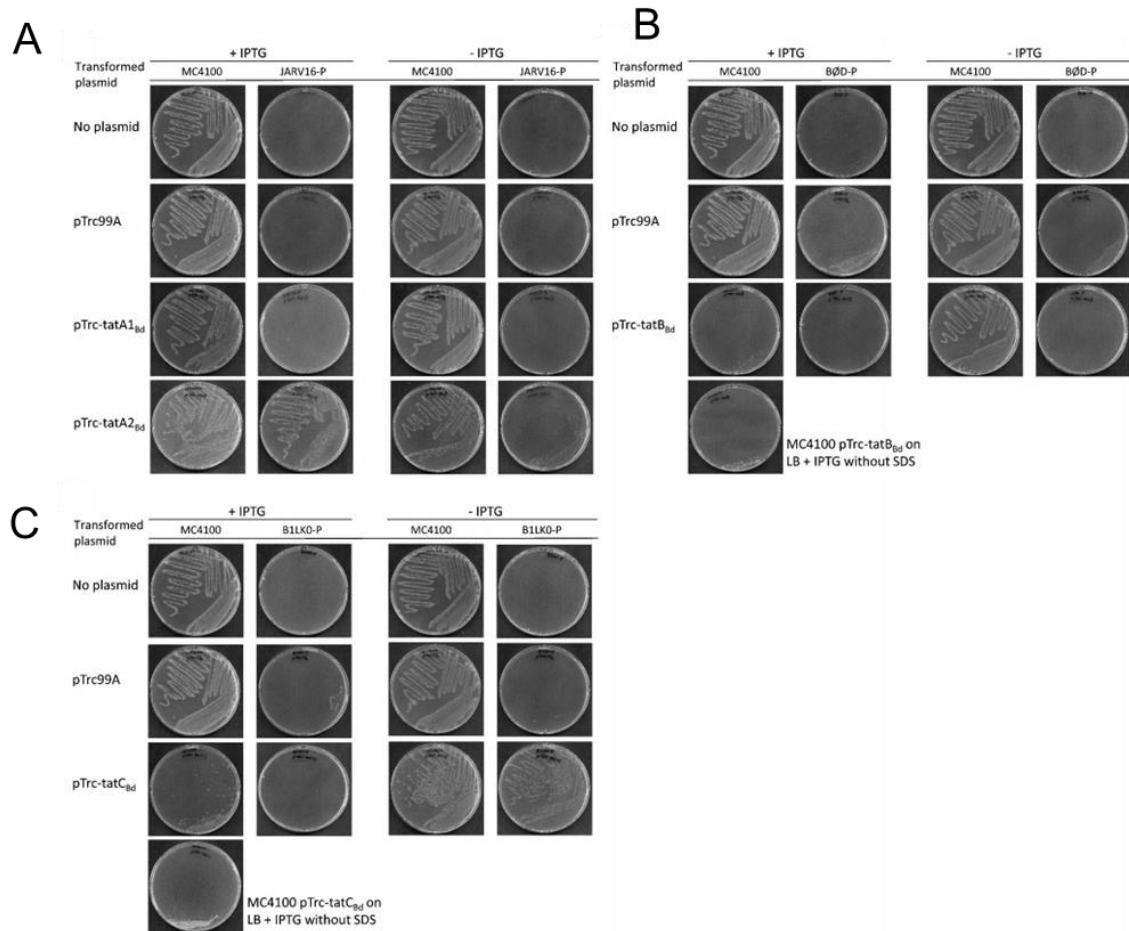
Fig 38. SDS resistance assay to test cross-complementation of the *B. bacteriovorus* TAT genes in various TAT mutants of *E. coli*. **A.** Complementation assay of the *tatAE* mutant of *E. coli* (JARV16-P) with the *B. bacteriovorus* HD100 *tatA1* and *tatA2*. **B.** Complementation assay of the E. coli *tatB* mutant (BØD-P) with the *B. bacteriovorus tatB*. C. Complementation assay of the E. coli *tatC* mutant (BILK0-P) with the *B. bacteriovorus tatB* gene. All plates contained 2% SDS and expression of the *B. bacteriovorus* genes was under IPTG (100 mM) induction. From **Paper 5.** (Chang et al., 2011).

5.1.1 Conclusions of the Paper

The main aims of this research was to look at the TAT pathway in *B. bacteriovorus* and testing how and if it fits into its bi-phasic lifecycle and to test its essentiality in both phases by doing mutational studies of the TAT pathway genes and also by looking at the expression of the genes across the lifecycles.

The main conclusion of the paper is that the TAT pathway is essential for viability of *B. bacteriovorus* in both the HD and HI growth phases. This is something that is not common among bacteria or archea, with only a few organisms requiring it for life with only *Mycobacterium tuberculosis*, *Sinorhizobium meliloti* and some haloarchea being reported (Saint-Joanis et al., 2006) (Pickering and Oresnik, 2010) (Thomas and Bolhuis, 2006).

Interestingly the *B. bacteriovorus* HD100 genome encodes two *tatA* genes, which is also uncommon in most bacteria. Although there is a large similarity between *tatA1* and *tatA2*, there was no redundancy in the *B. bacteriovorus* TAT system as deletion of one gene (*tatA1*) reduced predation and growth rates and deletion of the other (*tatA2*) gave a non-viable phentotype, suggesting they both have evolved separate functions that the other cannot carry out despite their similarity in sequence. This is in contrast to *E. coli* where deletion of one of the *tatA* genes (*tatE*) present no phenotype, while deletion of the other (*tatA*) did not affect viability (Sargent et al 1999).

Clearly *B. bacteriovorus* requires the TAT pathway where most other bacteria do not, this could be a result of its niche lifestyle, it is possible that a bacterium that translocates its proteins to a hostile, lytic environment such as that found on the inside of a bdelloplast needs to ensure prior protein folding occurs (via the TAT pathway) to stop the degradation by *B. bacteriovorus*'s own proteases. It would be an interesting set of experiments to find which *B. bacteriovorus* TAT substrates where essential in which growth phase of the *B. bacteriovorus*, with the hypothesis that those essential in predation were not essential in HI growth.

Successful fluorescent tagging of the TAT substrate Bd1802 has also showed that the at least one of the predicted TAT substrates has a role in predation as it is exported into the bdelloplast at the later stages of predation, even though the mutation of this gene resulted in a viable isolate (possible due to the presence of other redox-related proteins in the genome) it is interesting to speculate whether those other predicted TAT proteins have a role in *B. bacteriovorus* cell maintenance or prey degradation.

Changes in expression of TAT and TAT substrates genes between the wild-type and *tatA1* mutant background also show a more complex story and feedback mechanisms having a role in the *B. bacteriovorus* TAT pathway, this is something which has not been well characterised in other bacteria though has been seen in *E. coli* (Ize et al., 2004) and would require further study.

5.1.2 Since the Publication and Impact

Since the paper was first published in 2011 it has received 5 citations. Among them are a review into the TAT pathway highlight presence/absence and the effect of TAT mutagenesis in different bacteria (Palmer and Berks, 2012) and as an example into how the TAT pathway can be essential to bacteria (Luo et al., 2013). The paper is also cited in the newly isolated strain of *B. bacteriovorus;* Tiberius which encodes the same complement of TAT related genes as HD100 (Hobley et al., 2012b), this further adds weight to the requirement for each of these genes to be an effective predator.

Since this publication no further work on the TAT pathway or its substrates has been published by the Sockett lab or elsewhere, and so it remains the only comprehensive and encompassing work on the TAT pathway in *B. bacteriovorus*. However there are a number of potential publications that follow on from this work in terms of methodology. For the first time ever *B. bacteriovorus* genes were used in complementation studies to alleviate mutant effects in other organisms so this research gave the Sockett lab a chance to work on pathways already characterised in another bacterium to learn more about *B. bacteriovorus* itself. This is not very common as seldom do genes worked on in *B. bacteriovorus* have homologues in other bacteria and mutants in that pathway readily available. A lot of the genes worked on are also deleterious to others such as *E. coli*, as they normally encode lytic enzymes or proteins which are very specific to *B. bacteriovorus*. It is hoped more of this comparative work can be carried out in the future particular in between species of *B. bacteriovorus* now there is a second genome sequenced (Hobley et al., 2012b).

5.2 Paper 8. The Structure of an Unconventional HD-GYP Protein from *Bdellovibrio* Reveals the Roles of Conserved Residues in this Class of Cyclic-di-GMP Phosphodiesterases

Andrew L. Lovering, Michael J. **Capeness**, Carey Lambert, Laura Hobley, R. Elizabeth Sockett

My Contribution

I markerlessly deleted the *bd1817* gene from the *B. bacteriovorus* HD100 genome and phenotyped the resultant mutant by carrying out electron microscopy and growth/predation assays of the HD and HI isolate. I also tagged the Bd1817 protein with a C-terminal fluorescent mCherry tag and carried out the fluorescent microscopy. I also carried out the RT-PCR expression analysis of the Bd1817 gene confirming Dr. Lambert's preliminary results.

What the Paper Claims

The main aim of this paper was to obtain the first ever structure of a HD-GYP protein and characterise the novel HD-GYP protein found in the *B. bacteriovorus* genome, by making a mutant for the gene, tagging it fluorescently and observing any phenotypes. Production of pure Bd1817 protein and the solving of its crystal structure were also carried out to shed greater insight into its function and to be the first of its kind to be solved and is hoped to enhance the understanding of the role of the protein not only in *B. bacteriovorus* but also the wider field of cyclic-di-GMP signalling. The *B. bacteriovorus* Bd1817 proteins differs from the consensus HD-GYP as it lacks the tyrosine of the 'GYP' region, and in the wider

sequence has an associated glutamate instead of a histidine. It was hoped because of this it would lead to a more stable protein for crystallisation.

Markerless deletion of *bd1817* mutation and its expression

The gene *bd1817* was deleted in HD100 and was found not to be essential in either the HD or HI growth phases and had no significant effect on the predation rate or the growth rate when compared to wild-type. If a phenotype is present it does not manifest itself as a growth related one. Expression the *bd1817* gene was also looked at by RT-PCR and showed that it was most highly expressed at the 3-4 hour time points, corresponding to the end of the predation cycle, and that it is expressed in the HI growth phase (**Fig 39**). Bd1817 was also tagged with a fluorescent mCherry protein and put into HD100 wild-type by single crossover. Fluorescent microscopy showed that the protein is expressed and is fluorescent, with a modest amount of fluorescence seen in the cytoplasm (**Fig 40**). Though there was a limited phenotype the expression and mutational analysis did conclude that the *bd1817* gene is not a pseudogene as expression is seen, so further in depth analysis of its structure is valid.

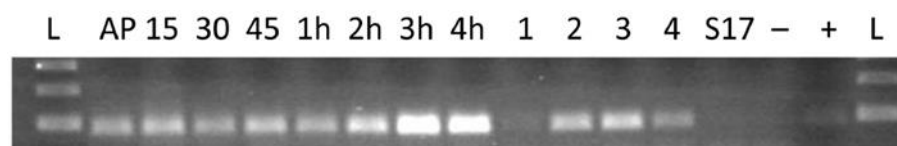**Fig 39**. RT-PCR of *bd1817* from RNA isolated from the predatory cycle of *B. bacteriovorus* HD100 predating upon *E. coli* S17-1. Lanes: L – 100 bp ladder; AP – attack-phase; 15-4h time points post-infection; 1-4 expression of *bd1817* in HI isolates HD100 (HD), HID2, HID13 and HID26 respectively using matched amounts of RNA; S17-1, E. coli RNA alone; -, no template; +, HD100 genomic DNA. From **Paper 7.** (Lovering et al., 2011).
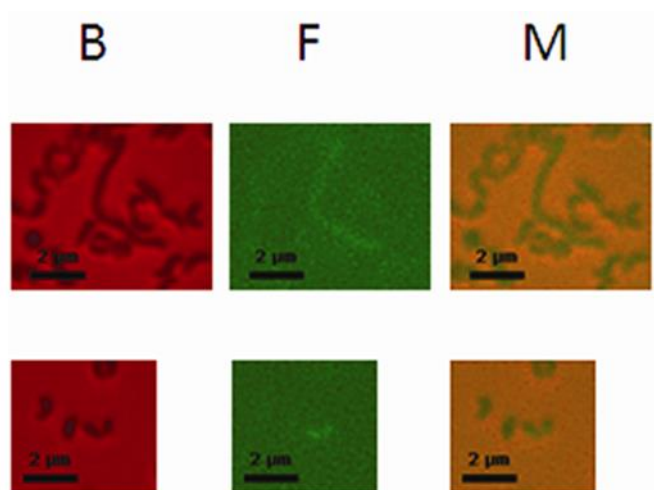
Fig 40. Fluorescence microscopy of 1817-mCherry fusion protein in a HI isolate of *B. bacteriovorus* HD100. The construct was integrated into the genome via single crossover to utilise the genes natural promoter. The fluorescence was seen throughout the *Bdellovibrio* HI cell. B – brightfield; F – fluorsecence; M – merged. From **Paper 7.** (Lovering et al., 2011).

Protein Structure

The Bd1817 was successfully engineered with an N-terminal polyhistidine tag by Dr. Lovering for purification and a thrombin tag to cleave the polyhistidine tag away allowing for a more native structure determination. The structure of Bd1817 was solved with a resolution of 1.28 Å and from it showed two clear domains for each part of the protein, with the N-terminus separated from the HD-GYP domain (**Fig 41**). The linker region connecting these two domains is reminiscent of the one found in RpfG of *Xanthomonas* which has been shown to be involved in HD-GYP turnover (Ryan et al., 2006).

Interestingly the structure of the N-terminal domain of Bd1817 was found to have slight structural similarity to the protein pleckstrin, which contains a RalA binding domain and has GTPase activity, suggesting that the Bd1817 has a role in protein-protein interactions and cellular signal responses, this is fitting with it's role in the cyclic-di-GMP signalling.

The structure also suggests that Bd1817 contains a cyclic-di-GMP binding inhibitory site, similar to that found in GGDEF proteins, while the overall structure of the HD-GYP domain mimics the multi-helical structure of other HD superfamily proteins and also that it appears to be monomeric in the crystallised form.
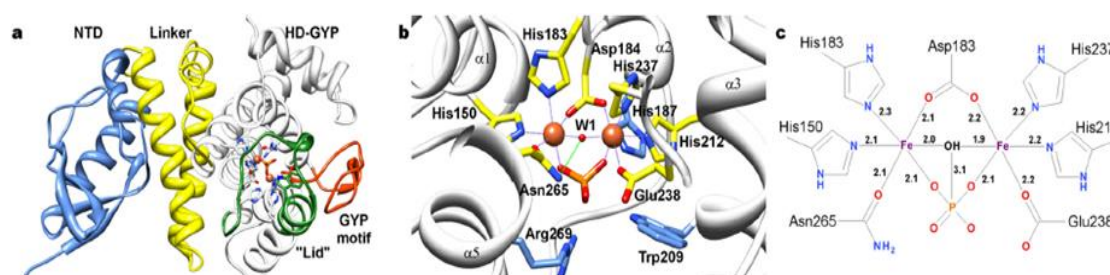

Fig 41. Crystal structure of the Bd1817 protein from *B. bacterivorus* HD100. **A**. The different modules of the Bd1817 protein, the NTD (1-78 amino acids), the linker helices (79-146), the HD-GYP domain (147-308), the 'lid' (188-211), the GYP domain (239-255). **B**. The active site containing the binuclear metal sites (tan spheres) **C**. Schematic of the active site showing the interactions of the metal ions on the surround amino acids. From **Paper 7.** (Lovering et al., 2011).

The active site however is different to the HD superfamily previously reported, as it contains a bi-nuclear metal 'pocket' containing many acidic residues, though this is quite novel, it is reported to be present in a sub-family of the HD proteins. Due to the arrangement of the residues forming around the 'pocket' it was thought that the metal it binds could be iron, though this is still yet to be confirmed. From the structure of Bd1817 and the structure of cyclic-di-GMP, it was also thought that this pocket would be occupied by the phosphodiester found on the substrate molecule with the metal ions carrying out a hydroxide attack leading to polarisation of the substrate and eventual catalysis.

The 'GYP' motif of Bd1817 was found to be very ordered and to have a novel conformation, forming two U-shaped turns that are exposed, but slightly covered

by a 'lid'. This lid would require a conformational change to warrant a molecule of cyclic-di-GMP to have access to the active site and also physically fit inside. Once in the active site interaction with the metal ions would form a hydroxide attack leading to polarisation of the substrate and eventual catalysis (**Fig 41**).

How Bd1817 HD-GYP contains an extra glutamate, an aberrant histidine and a missing tyrosine, it was thought that this has evolved in tandem to stabilise the active site and keep its conformation and is quite possible that the glutamate binds GGDEF domains found in receptor proteins in place of the consensus tyrosine, albeit maybe not as well.

Ultimately due to the deviation from the consensus for HD-GYP proteins, there was no determinable phosphodiesterase activity of the Bd1817 protein.

5.2.1 Conclusions of the Paper

The most important conclusion of this paper is that for the first time a HD-GYP protein has been crystallised and its structure has been solved and it is hoped from this further understanding of the mechanism of action of cyclic-di-GMP signalling can be gained.

Mutagenesis of the *bd1817* in *B. bacterivorus* gave a completely viable isolate and was not defective in either predation as a HD isolate, or in growth as a HI isolate, this coupled with the lack of phosphodiesterase activity seen leads to the thought that the deviation from the consensual of its HD-GYP domain has led to

the protein being of limited activity in *B. bacteriovorus*. Though as it is still expressed is not a pseudogene.

High resolution mapping of the active site of the Bd1817 by Dr. Lovering has also put forward a model for the hydrolysis of the cyclic-di-GMP molecule, showing potential important amino acid residues and their interaction with the substrate molecule and also how they lead to a stable active site.

Due to the deviation of the HD-GYP consensus the Bd1817 not only became more stable allow Dr. Lovering to solve its crystal structure it also lacked any phosphodiesterase activity, though this is somewhat ironic, the structure put forward will undoubtedly continue to aid in the mapping of other HD-GYP proteins. Potential co-crystallisation with metal irons for stability could potentially be one way.

5.2.2 Since the Publication and Impact

Since the paper was first published in 2011 it has been cited by 5 times, all of the citations are not from *B. bacteriovorus* community and are from labs who work on cyclic-di-GMP signalling and two component signalling. This is what was really set out to be achieved, as the Bd1817 structure is the first of its kind to be solved and has helped other groups to solve their HD-GYP structures by mapping them against the one from *B. bacteriovorus.*

In one publication they purified a HD-GYP containing protein from *Thermotoga maritime* and found that it did contain iron adding further evidence for the Bd1817 metal ions to be iron (Plate and Marletta, 2012).

A more recent citation showed the solving of the structure of a HD-GYP containing protein from *Xanthamonas* which showed the active site relies on a tri-iron centre. Their structure also proved to be very similar to Bd1817 as it contains the double-U bends forming the active site (Bellini et al., 2014). A further step the group has taken is co-crystallisation with cyclic-di-GMP and GMP to show how the substrate and product bind and have an effect on the conformation of the protein.

Perhaps a direct follow on for this work has come from the Sockett lab and focussed on the other part of cyclic-di-GMP signalling, the GGDEF proteins that function as diguanylyl cyclases to make cyclic-di-GMP to initiate a response (Hobley et al., 2012a). Interestingly they found that deletion of different genes containing the GGDEF domain resulted in *B. bacteriovorus* with different phenotypes, for example Δ*dgcB* could not grow predatory, while Δ*dgcC* could not grow as a HI. So it appears that *B. bacteriovorus* use cyclic-di-GMP signalling as a way to control different growth phases.

The paper also represented the first collaboration between the Sockett lab and Dr. Andrew Lovering at the University of Birmingham. From this 2 more (so far) papers have been a result of this initial collaboration, the latest of which is featured next in this chapter. This partnership opens multitude of genetic and biochemical research avenues to explore for *B. bacteriovorus* researchers; currently the Bd1817 protein is only the third *B. bacteriovorus* protein to have its structure solved.

5.3 Paper 9. Activity of *Bdellovibrio hit* Locus Proteins, Bd0108 and Bd0109, Links Type IVa Pilus Extrusion/Retraction Status to Prey-Independent Growth Signalling.

Michael J. **Capeness**, Carey Lambert, Andrew L. Lovering, Kaoru Uchida, Roy Chaudhuri, L.J. Alderwick, David J Lee, David Swarbreck, Rob Till, Shin-Ichi Aizawa and R. Elizabeth Sockett

My Contribution

Along with Dr. Lambert I was the main author of the manuscript and along with him designed (with Prof. Sockett) and carried out all the experiments. I deleted the *bd0108* gene in HD100 and fluorescently tagged both the *bd0108* and *bd0109* genes. I also made the complementation plasmids and with Dr. Lambert carried out the HI growth and HD predation assays and looked at CFU:PFU ratios. I also looked at the expression of *bd0108* and the surrounding genes by RT-PCR in various HI backgrounds. I also measured all the pili lengths and carried out the statistical analysis on the data; to test whether there were any differences between the pilus lengths of the various HI isolates.

What the Paper Claims

The main aim of this paper was to explore the role *bd0108* has in the bi-phasic lifecycle of *B. bacteriovorus* by mutagenesis and subsequent phenotyping by growth/predation assays and electron microscopy, as well as expression studies of the mutant and other HI cells of *bd0108* and its gene neighbours. Protein interaction was also used to find a possible role for the Bd0108 protein and how this may be involved in the lifecycles of *B. bacteriovorus*.

Deletion of *bd0108* and growth phenotypes

Markerless deletion of the *bd0108*, encoding the *hit* locus in *B. bacteriovorus* was successfully accomplished and was found to result in HI cells that were still predatory. Interestingly *bd0108* could only be deleted from HI cells and could not be deleted directly as a HD isolate, possibly due to the nature of the mutation resulting HI cells that are out-competed by single crossovers or revertants that would grow host dependently better than the deletion. Deletion of the neighbouring gene, *bd0109* was also attempted but was found to be lethal in both growth phases despite the screening of many isolates (**Table 7**). Deletion mutants of *bd0108* were observed by fluorescent microscopy entering the fluorescent prey *E. coli* in a similar time to wild-type though in liquid culture complete lysis of the *E. coli* prey occurred slower than wild-type possible due to the nature of the deletion in populations (**Fig 42**).

	Δbd0108		*Δbd0109*	
Number of deletion strains	HD = 0	HI = 2	HD = 0	HI = 0
Number of revertants	HD =343	HI = 46	HD =207	HI = 217
Number of Conjugations	4		8	

Table 7. The number of *bd0108* deletion strains obtained versus the number of screened isolates. The Δ*bd0108* was only isolated as a HI and not a HD, however the Δ*bd0109* could not be isolated in either growth phase. From **Paper 9.** (Capeness et al., 2013)

HI Isolate	Mutation in bd0108	Resultant Amino Acid Sequence
HD100	Wild-type	MKRLLVLSILLTLGFSFAGTASADENANRPVNPGEDPNEAFRSTPFEATTSALGDCRECI AYRTGATTGKGSRRHDDTVSREIKGSSATPGGSEKAGTGRQ*
HID2	Wild-type	MKRLLVLSILLTLGFSFAGTASADENANRPVNPGEDPNEAFRSTPFEATTSALGDCRECI AYRTGATTGKGSRRHDDTVSREIKGSSATPGGSEKAGTGRQ*
HID6	CC insertion after base 217	MKRLLVLSILLTLGFSFAGTASADENANRPVNPGEDPNEAFRSTPFEATTSALGDCRECI AYRTGATTGKGSPVAMTTPFPEKSKAPARRLVDLRKPGLEDSNPICLET*
HID13	G->A substitution at base 3	IKRLLVLSILLTLGFSFAGTASADENANRPVNPGEDPNEAFRSTPFEATTSALGDCRECI AYRTGATTGKGSRRHDDTVSREIKGSSATPGGSEKAGTGRQ*
HID18	Deletion of the base G at base 211	MKRLLVLSILLTLGFSFAGTASADENANRPVNPGEDPNEAFRSTPFEATTSALGDCRECI AYRTGATTGKAPVAMTTPFPEKSKAPARRLVDLRKPGLEDSNPICLET*
HID22	42 bp deletion from base 210 to base 252	MKRLLVLSILLTLGFSFAGTASADENANRPVNPGEDPNEAFRSTPFEATTSALGDCRECI AYRTGATTGKGS--------------------------SATPGGSEKAGTGRQ*
HID26	Wild-type	MKRLLVLSILLTLGFSFAGTASADENANRPVNPGEDPNEAFRSTPFEATTSALGDCRECI AYRTGATTGKGSRRHDDTVSREIKGSSATPGGSEKAGTGRQ*
HD100 Δbd0108	Complete deletion of all but 5 codons	MKGRQ*

Table 8. Mutations in the *bd0108* gene of the various *B. bacteriovorus* strains in this paper and their resultant changes to the amino acid. Amino acids sequence in blue corresponds to the wild-type sequence of Bd0108; amino acids sequences in red are the resultant change to the sequence due to the mutation. Note that despite the 42 bp deletion in the HID22 strain, the reading frame is still maintained. Also of note is the similarity between the altered amino acid sequence (in red) between HID6 and HID18. From **Paper 9.** (Capeness et al., 2013)
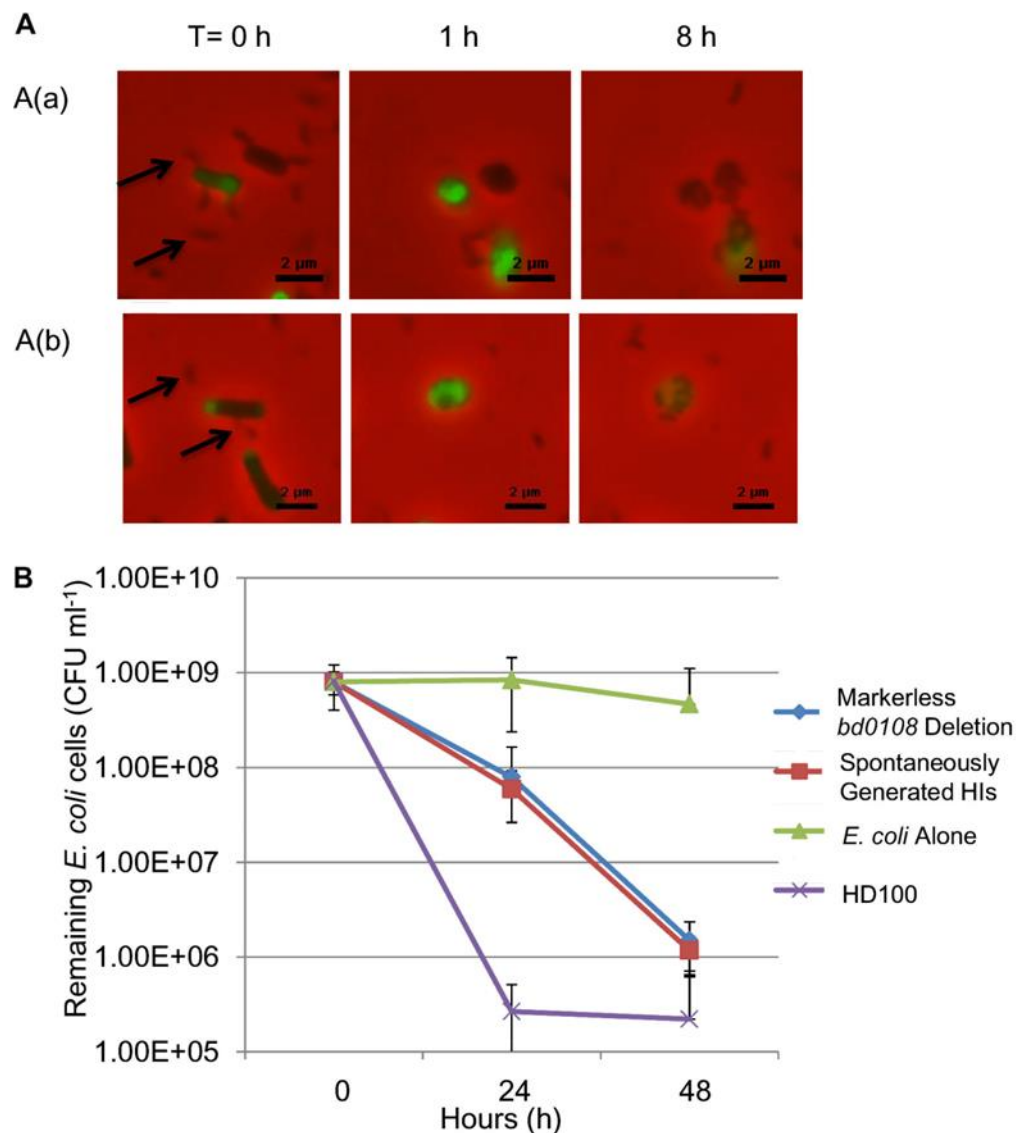
Fig 42. Fluorescence microscopy and predatory rate of HI strains versus HD wild-type. A. Time-lapse fluorescence of predation by the HD wild-type (HD100) and the Δ*bd0108* mutant on *E. coli* carrying the plasmid pMAL-p2_mCherry. T=0, attachment and invasion of the prey cells by the *Bdellovibrio* (arrows). T=4, 4 hours post-infection, establishment and growth of *Bdellovibrio* inside the bdelloplast. T=8, 8 hours post-infection, the Bdellovibrio have septated and are about to burst free from the now empty bdelloplast. Predation was carried out on a 1% agarose pad, with images being taken every 2.5 minutes. **B.** Drop in *E. coli* CFU over the course of a 48 hour predation using the Δ*bd0108* mutant (HI), spontaneously generated HI isolates and HD100 (HD). Error bars are at the 95% confidence interval around mean. From **Paper 9.** (Capeness et al., 2013).

For growth assays a number of other 'wild-type' HI isolates were used alongside the *bd0108* deletion mutant, they had been isolated through the standard lab method from HD cultures of HD100 (Lambert et al., 2006) (**Table 8**). They

contain different mutations (or lack of) in the *bd0108* gene; all of them were slower at predation than wild-type but still could complete the predation lifecycle.

By using the plasmid pSUP404.2 that replicates in *B. bacteriovorus* (Roschanski and Strauch, 2011) it was possible to carryout complementation studies of the *bd0108* mutant, and other HI isolates by attempting to complement them with empty plasmid, one encoding the *bd0108* gene, or one encoding a common variant of the *bd0108* gene that has a deletion of 42 bp. By the addition of a luminescent strain of *E. coli* to liquid cultures containing the HI cells, decrease in luminescence (and therefore *E. coli* cells) indicates the efficiency and rate of predation. It was shown that complementing the *bd0108* deletion mutant and other HI isolates with wild-type *bd0108* gene increased predation (**Fig 43**). It was also possible to observe this complementation on overlays plates where the different cultures of *B. bacteriovorus* were spotted onto lawns of *E. coli* to show there is restoration of the mutated *bd0108* phenotype (**Fig 44**).

Complementation was also seen by measuring the ratio of CFU to PFU, showing a significant drop in the CFU number when the HI cells were complemented with the wild-type *bd0108* gene indicating that complementation was happening and was restoring the HI cells back to 'wild-type' predation dependent (**Fig 45**).
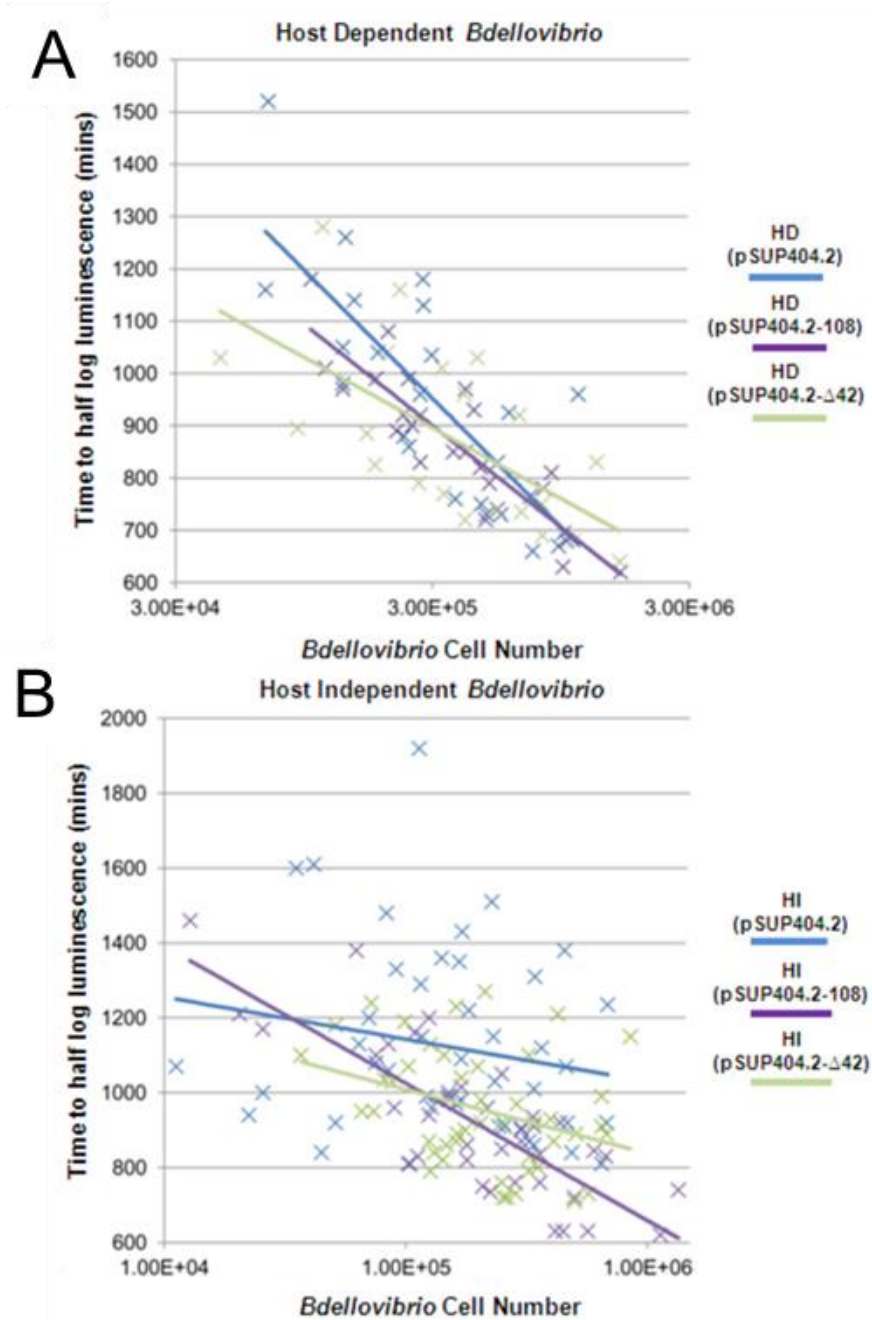
Fig 43. Predation efficiency of HD and HI isolates on luminescent *E. coli* prey. **A.** HD dependent cells containing a wild-type copy of the *bd0108* gene (genomic) and either an empty pSUP404.2 plasmid, one containing a copy of *bd0108* (pSUP404.2-108) or a plasmid encoding the 42 bp deletion variant (pSUP404.2-Δ42). There is typical logarithmic reduction in luminescence (prey-killing) with the addition of more HD *Bdellovibrio*. **B.** *Bdellovibrio* HI isolates containing the various plasmids as before, though there is not logarithmic reduction with the addition of more cells, complementation with pSUP404.2-108 is seen leading to a higher rate of prey killing. From **Paper 9.** (Capeness et al., 2013).

Fig 44. Double-layer YPSC overlay plates of *B. bacteriovorus* strains on a lawn of *E. coli* S17-1. *Bdellovibrio* cells were diluted in Ca/HEPES and 10 µl was spotted onto the overlay of each dilution. Complementation of the Δ*bd0108* mutant was not seen despite having extra copies of *bd0108*, the HID13 (which has an ATG->ATA mutation of the first codon in *bd0108*) however was complemented. From **Paper 9.** (Capeness et al., 2013).
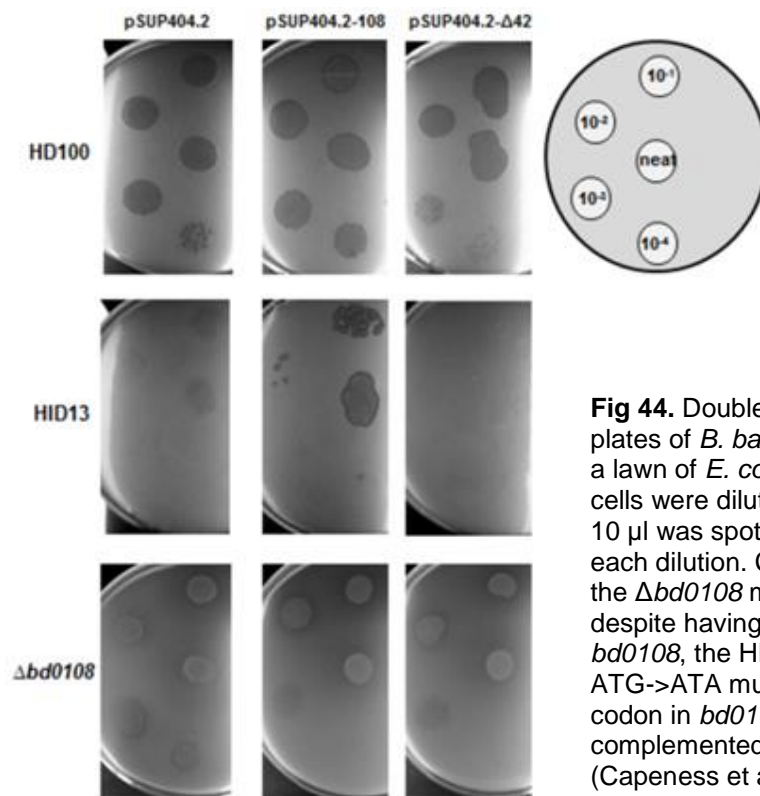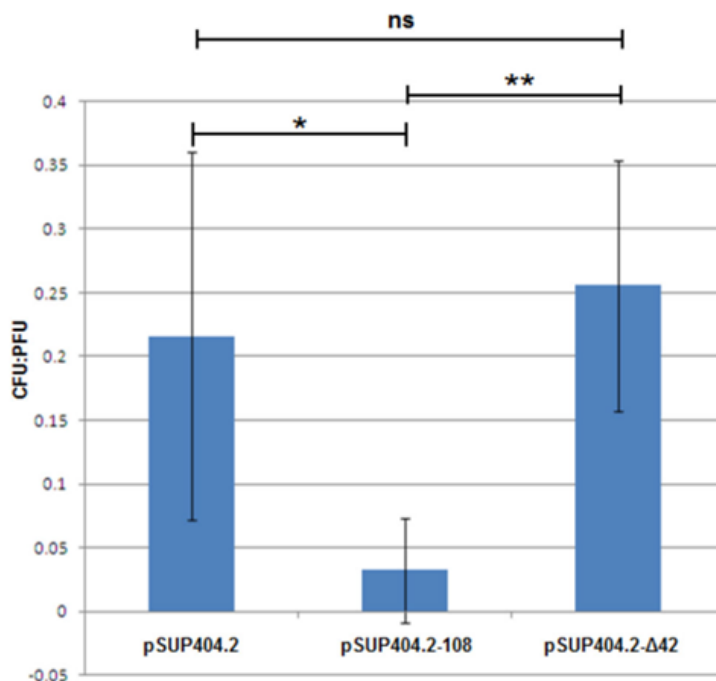

Fig 45. Ratios of PFU to CFU for HI isolates (both spontaneous and markerless Δ*bd0108* mutants - pooled). HID2, HID6, HID13, HID18, HID22 and Δ*bd0108* showed a significant reduction in the number of CFU when complemented with the pSUP404.2-108 plasmid compared to the same plasmid containing either the 42 bp deletion variant of *bd0108* or empty plasmid. The ratio of CFU:PFU reduction was from 2.16×10^{-1} (pSUP404.2) to 3.21×10^{-2} (pSUP04.2-108) (p = 0.026). There was no significant reduction in the strains containing pSUP404.2-Δ42 (2.56×10^{-1}) (p = 0.31). There was also a significant difference between strains carrying the pSUP404.2-108 and the pSUP404.2-Δ42 (P<<0.01). From **Paper 9.** (Capeness et al., 2013).

Expression of *bd0108* and the surrounding genes (*bd0109-0111*) was investigated by RT-PCR on matched amounts of total RNA, and it was found they are all expressed together on the same strand of mRNA, as intergenic priming all gave positive results, indicating they are all in an operon so may have associated function. Interestingly expression of *bd0108* in HID13 was lower in other HI isolates, and though the HID22 strains has a 42 bp deletion in *bd0108* transcription was still seen, although gave a reduce band size due to the deletion (**Fig 46**).
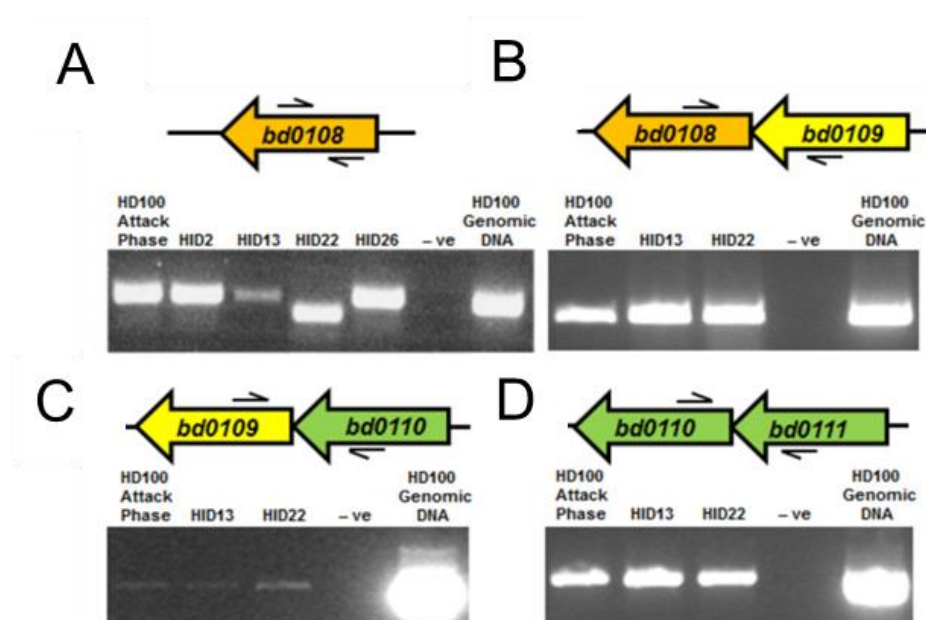
Fig 46. RT-PCR analysis of the *hit* locus genes on matched amouns of RNA. In both wild-type and spontaneously generated HI strains of *B. bacteriovorus* HD100 *bd0108-bd0111* was found to be expressed. Though there is a reduction in the size of the *bd0108* transcript In the HID22 isolates as the primers flank the region of the 42 bp deletion. There was also a noticeable reduction of the intensity of the *bd0108* transcript in HID13. It was also found that there is co-transcription of *bd0108* and *bd0109* (**B**), *bd0109* and *bd0110* (**C**), and *bd0110* and *bd0111* (**D**). Primers are indicated as arrows around the gene cartoons. From **Paper 9.** (Capeness et al., 2013).

The predicted function of Bd0108 and Bd0109

Though there is no homology of *bd0108* to other proteins outside of the *B. bacteriovorus* family, bioinformatic analysis of Bd0109 showed it had homology to RHS-elements involved in sugar-binding wall-associated protein and contained a repeating YD domain. It has also been reported that a mutant in a RHS-element in *Mxyoccocus xanthus*, a close relative of *B. bacteriovorus*, could not retract its pili. There are four (out of 6) other RHS-elements in the HD100 genome and are found next to genes involved in pilus structure and function and it was also noted that the N-terminus of the Bd0109 protein contains a potential pilin fold when analysed by the online protein prediction tool PHYRE (Kelley and Sternberg, 2009).

With all this in mind and the fact that *bd0108* and *bd0109* are co-transcribed, the proteins which they encode were purified and shown to interact by means of a protease chymotrypsin digest, showing that there is less protein degradation when the proteins are assayed together, as one would protect the other from the protease (**Fig 47**). Fluorescence quenching also showed that the two proteins interacted as the addition of Bd0108 (which is not naturally fluorescent due to the lack of tryptophan in the protein) to Bd0109 (which is fluorescent), showed a decrease in fluorescence due to the two proteins interacting.
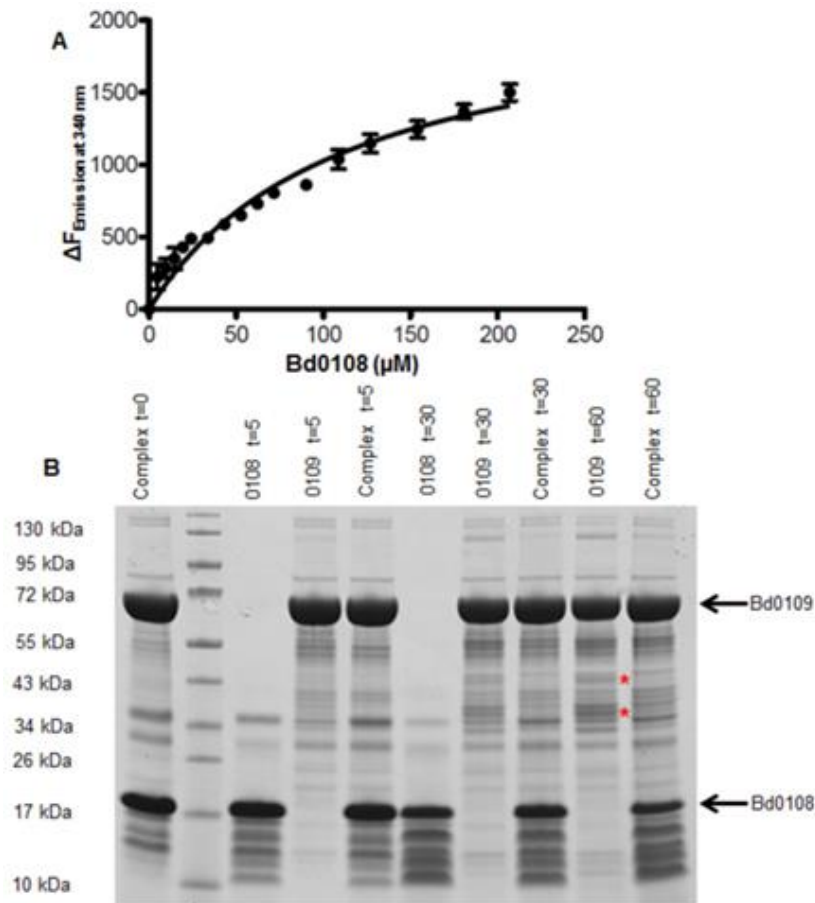
Fig 47. Evidence for the interaction between Bd0108 and Bd0109. **A.** Fluorescence quenching assay of the tryptophan-containing Bd0109 (2.41 μM) titrated against Bd0108 (1.95 μM) (non-tryptophan containing). Increasing the concentration of Bd0108 increased the fluorescent quenching, indicating there is an interaction between the two proteins. **B**. Chymotrypsin protection assay showing there is less digestion of the Bd0108-Bd0109 complex than that of each protein singularly. Extra bands (and 35 KDA and 43 kDa, indicated) appears in the singular digestion of Bd0109 over the course of 60 minutes. From **Paper 9.** (Capeness et al., 2013).

Finally to show localisation of the Bd0108 and Bd0109 proteins, both were fluorescently tagged with mCherry and mTFP (respectively). When conjugated into *B. bacteriovorus* no fluorescence was seen, however in *E. coli*, both localised to the periplasm of the cell, indicating a possible role in the periplasm indicating there is a sec-signal for transportation as predicted (**Fig 47**). Fluorescent tagging of each of the proteins in *B. bacteriovorus* may have attenuated its function and targeted it for degradation.
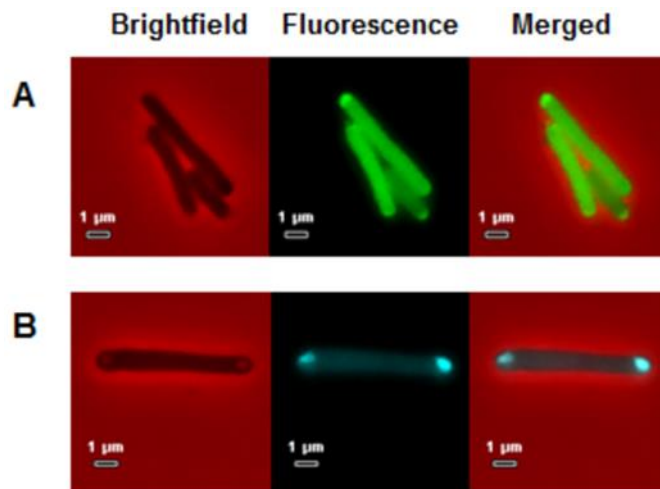
Fig 48. Expression of the fluorescently tagged Bd0108 and Bd0109 proteins in *E. coli* S17-1 *in trans*. **A.** *E. coli* expressing a C-terminal mCherry fusion of Bd0108. Fluorescence was located in the periplasm of the cells with large amounts at the poles due to plasmolysing of the cell. **B.** *E. co*li expressing a C-terminal mTFP fusion of Bd0109 with fluorescence being located at the poles. From **Paper 9.** (Capeness et al., 2013).

Electron Microscopy of the *bd0108* deletion and other HI isolates

To further characterise the *bd0108* markerless deletion mutant, the isolate, grown as HI, was analysed by electron microscopy to determine a phenotype based on its morphology, other HI isolates, with varying mutations (or lack of) in *bd0108* were also investigated. It was observed that the markerless deletion of *bd0108* isolate gave significantly less (almost none) cells with pili, while HID22, that contains the common 42 bp deletion of *bd0108* gave cells with extra-long pili compared to HID2 (which is not mutated in *bd0108*) (**Fig 48**). A pilus shearing experiment conveyed that the HID22 pili were comprised of PilA (Bd1290) as previously reported (Evans et al., 2007), as expected there were no bands in the *bd0108* markerless deletion mutant.

Strain	bd0108 genotype	Number of cells	Piliated cells (%)	Average Pili length (nm)	Percentage >1 µm (%)
HID22	Δ42bp	110	24.3	613	15.0
Δbd0108#1	deletion	127	0	n/a	0
Δbd0108#2	deletion	167	1.1	592	0
Wild-type	WT	248	13.3	469	5.5
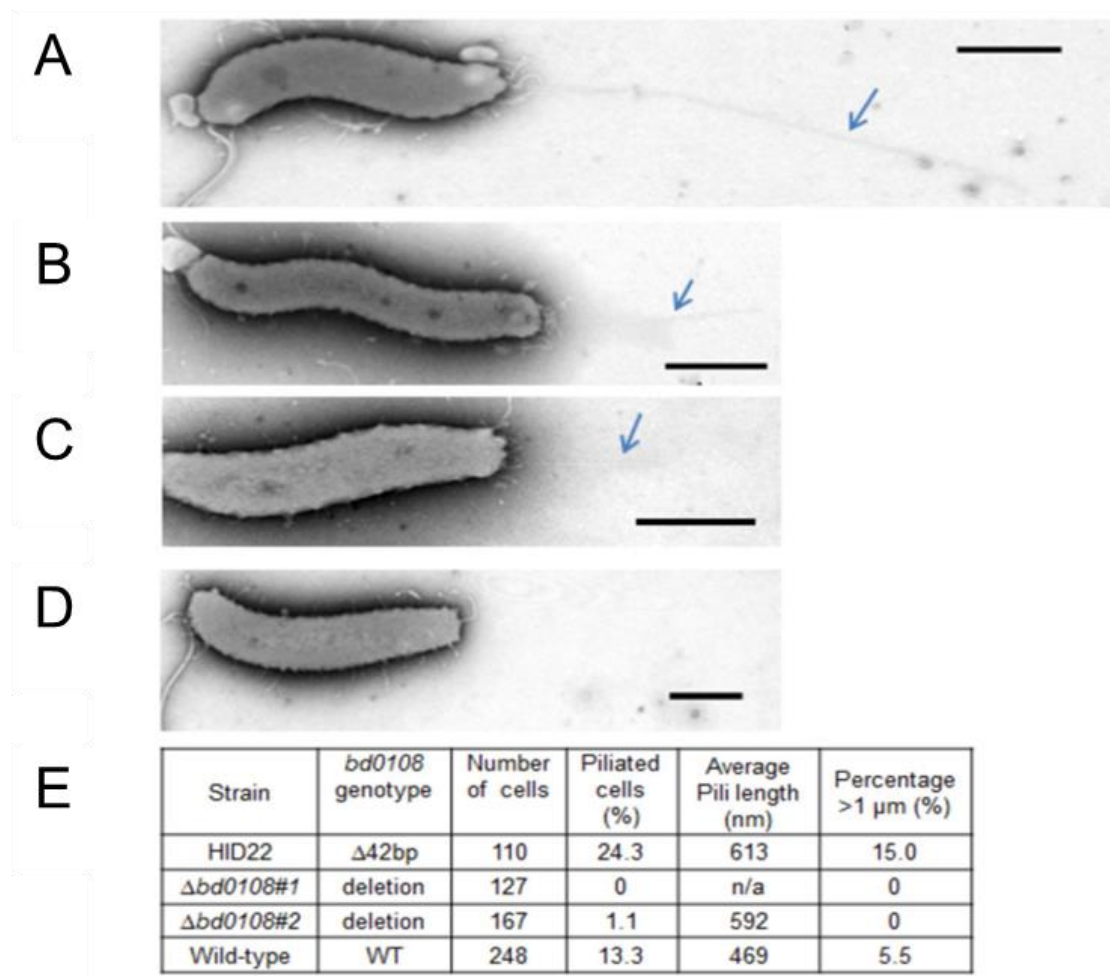
Fig 49. Representative electron micrographs of the different HI strains of *B. bacteriovorus* showing various pili lengths and even absence. **A.** HID22 containing the 42 bp deletion in *bd0108* showing an extra-long pilus which was present in 15% of the HID22 isolates. **B**. HID22 showing a standard length pilus. **C**. A HI isolate containing a wild-type copy of *bd0108.* **D.** The Δ*bd0108* deletion mutant showing no pilus-like structures. **E**. A table showing pilli length/presence of various HI isolates. All samples were stained with 2% PTA pH7.0, scale bars = 500 nm. From **Paper 9.** (Capeness et al., 2013).

HD *Bdellovibrio* Strain	Number of cells	% of cells piliated	Average length of pili (nm)	Percentage over 1 µm
Δbd0108#1 (pSUP404.2)	73	12.3	245	0
Δbd0108#2 (pSUP404.2)	91	13.2	201	0
Δbd0108#1 (pSUP404.2-108)	56	39.3	541	22.6
Δbd0108#2 (pSUP404.2-108)	69	42.0	402	8.7

The complemented strains had more pili with a longer average length showing that the paucity of pili in the mutants was complemented (P<0.01).

Table. The complementation of the Δ*bd0108* mutant with either empty plasmid (pSUP404.2) or one containing the wild-type *bd0108* gene (pSUP404.2-108). There is an increased number of piliated cells when complemented with the wild-type copy of *bd0108* and also a higher incidence of pili over 1 µm. From **Paper 9.** (Capeness et al., 2013).

In a complementation experiment the *bd0108* deletion strain was grown with the pSUP404.2 plasmid either empty or containing the wild-type *bd0108* gene. The experiment was carryout with cells growing as HD isolates as complementation of the deletion strain with the *bd0108* gene would give cells that were deficient in HI growth as mentioned previously. The *bd0108* deletion strain did possess extruded pili; however complementation with the wild-type *bd0108* did dramatically increase pilus presence and length (**Table 9**).

RNAseq analysis of *B. bacteriovorus*

The complete global transcriptome of different *bd0108* mutant isolates and wild-type HD100 was analysed by RNAseq in a hope to find a genetic switch for this bi-phasic lifecycle which has not been found in RNA array data mentioned previously in this thesis (Lambert et al., 2010a). The previous study however showed good overlap with the RNAseq data in this paper.

The RNAseq dataset showed a large dataset unique to the HI isolates and not the HD wild-type strain (1163 genes upregulated, and 1207 down regulated), this showed that although there are differences in *bd0108* mutation isolates, growth rate and cell morphology, there is a core set of genes responsible to warrant being a HI (**Table 10**). This builds on the previous RNA arrays which only features one HI isolate.

Strain	Number of genes/intergenic regions differentially regulated >2-fold			
	Upregulated vs HD100	Downregulated vs HD100	Upregulated vs HD100, unique to this strain	Downregulated vs HD100, unique to this strain
HID13	1352	1421	44	43
HID22	1333	1422	82	113
Δbd0108	1420	1463	74	55
Common to all HI strains	1163	1207	N/A	N/A

Table 10. The number of genes differently regulated between the HD100 attack-phase and the different HI isolates. Notice there is a large overlap between the HI strains compared to the attack-phase suggesting that the HI transcripts are growth mode specific rather than strain specific. From **Paper 9.** (Capeness et al., 2013).

Based on the previous finding that there is a change in pili length and presence in the various HI mutants, there was down regulation of the majority of the pilus apparatus relative to wild-type HD *B. bacteriovorus,* included in this are the *bd0108-bd0111* operon. The HID22 strain, which had a higher number of extra-long pili, was less down regulated than the other HI isolates.

The end result of the RNAseq data is the list of likely candidates to have a role in the growth and phase-switching of the HI strains, of which it is likely there are different genes involved in the different HI isolates, as although all the HI strains used for RNAseq in this study are mutated in *bd0108*, the differences in the transcription profile of both the genes and the intragenic regions infer they are altered in different pathways or different points of the same pathway.

Interestingly a non-coding RNA was identified to be up-regulated in all the *bd0108* mutant HI strains (though not the HD100 wild-type or HID2 HI isolate which has a wild-type *bd0108*) between the intragenic region between *bd0103* and *bd0108*. RT-PCR analysis of the region showed up-regulation of this region in all HI isolates compared to both HID2 and HD100, though slightly less in HID22, suggesting this region maybe up-regulated upon mutation of *bd0108* (**Fig 50**).
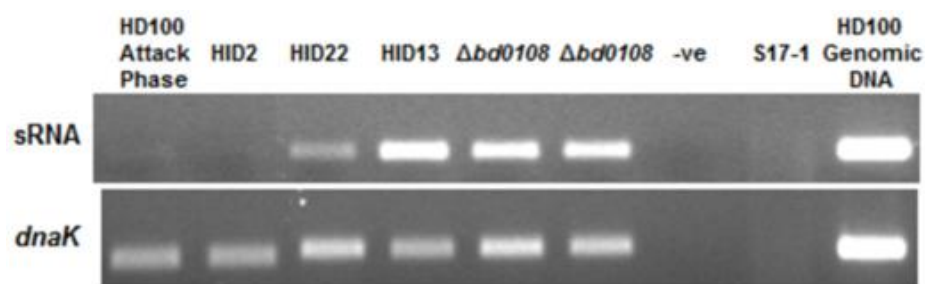
Fig 50. RT-PCR expression of the sRNA downstream of *bd0108* and its expression in different strains of *B. bacteriovorus*. All expression is carried out on matched amounts of RNA and constant expression of *dnaK* serves as proof of this. Expression of the sRNA is seen to be different in different strains and absent in those strains containing a wild-type *bd0108* (attack-phase ad HID2). From **Paper 9.** (Capeness et al., 2013).

5.3.1 Conclusions of the Paper

The biggest conclusion of the paper is that Bd0108 and Bd0109 have a role in pili function, with deletion of *bd0108* almost entirely abolishing pilus extrusion, something that is essential for predation. Despite previous *pilA* mutants only growing as HI isolates (Evans et al., 2007), the *bd0108* mutant can still carryout predation, albeit it at a reduced rate. So deletion of *bd0108* does not abolish synthesis of the pilus fibre but does limit its extrusion, creating undetectable amounts of pili on the surface of the cell leading us to hypothesise either the synthesis of or actual extrusion of the fibre is altered.

The pili extraction/retraction model

With the aforementioned evidence for the roles of Bd0108 and Bd0109 in pili function, it led to the formulation of a potential model for how each regulates the extrusion and retraction of Type IV pili in *B. bacteriovorus* (**Fig 51**). As Bd0108 interacts with Bd0109, and Bd0109 has the properties of being associated with

pilus interaction it is thought that Bd0109 is regulated by Bd0108, altering the extrusion/retraction of the pilus fibre. In the absence of Bd0108 as in the deletion strain, Bd0109 is unbound by Bd0108 and free to retract the pilus fibre unchecked, leading to no pili on the outside of the cell. Though a different phenotype is seen in the 42 bp deletion variant strain (HID22), the end result is the same as failure to extrude a pilus and failure to retract it both would trigger the cell to grow HI.

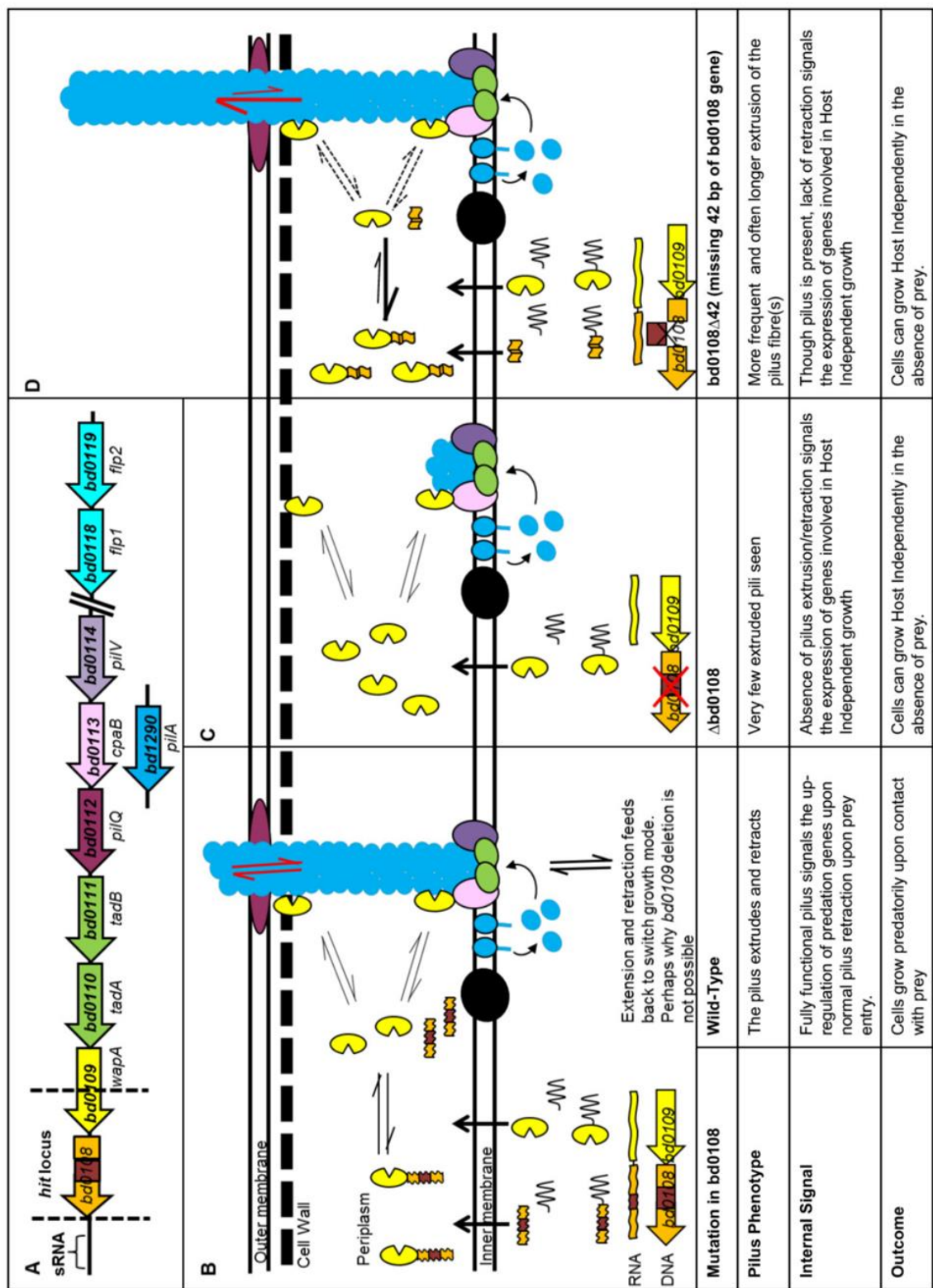| Mutation in bd0108	Wild-Type	Δbd0108	bd0108Δ42 (missing 42 bp of bd0108 gene)
Pilus Phenotype	The pilus extrudes and retracts	Very few extruded pili seen	More frequent and often longer extrusion of the pilus fibre(s)
Internal Signal	Fully functional pilus signals the up-regulation of predation genes upon normal pilus retraction upon prey entry.	Absence of pilus extrusion/retraction signals the expression of genes involved in Host Independent growth	Though pilus is present, lack of retraction signals the expression of genes involved in Host Independent growth
Outcome	Cells grow predatorily upon contact with prey	Cells can grow Host Independently in the absence of prey.	Cells can grow Host Independently in the absence of prey.

Fig 51. Proposed model for the function of Bd0108 and Bd0109 and their roles in the extrusion and retraction of pili. **A.** Structure of the *hit* locus and surrounding genes. **B.** Wild-type cells expressing *bd0108* and *bd0109* (co-expressed) and translated into protein which is then secreted via the sec-system into the periplasm. Bd0108 and interacts with Bd0109 sequestering it. When not sequestered, Bd0109 anchors at the cell wall or with the mature pilus fibre where it regulates the pilus extrusion/retraction i.e. the de-/polymerisation of the mature pilus fibres (Bd1290) held in the inner membrane by potentially the ATPases Bd0110 and Bd0111. **C.** In the absence of Bd0108 (as in the markerless deletion strain), Bd0109 is not sequestered and is free to interact with the growing pilus fibre causing less/reduced extrusion. D. In HI strains containing the 42 bp deletion of *bd0108*, the truncated form of the Bd0108 there is potentially over-sequestration of Bd0109 resulting in a longer pilus fibre. In both **C.** and **D.** the resultant pilus has the same effect of Host Independent growth. From **Paper 9.** (Capeness et al., 2013).

A future experiment to test this hypothesis would be to assay the amount of pilin monomers on the inside of the cell, with the hypothesis that the *bd0108* mutant shows a similar amount or increase amount compared to wild-type as the pilus fibre is still created (as it must be for the cells to carryout predation) but this is not extruded so remains intracellular.

This work builds on the previous work by Cotter and Thomashow (Cotter and Thomashow, 1992) as well as the work of Roschanski (Roschanski et al., 2011) further underpinning the role the cryptic *bd0108* gene has in the growth switching of *B. bacteriovorus* and assigning a function to it rather than an attributed growth phenotype previously reported. This is by no means the end of the *bd0108* story; further work is needed to establish how the lack of hyper-retraction/extrusion of the pilus fibre leads *B. bacteriovorus* to initiate the growth switch at the gene level, and what gene targets play a pivotal role in the regulation. With the RNAseq data of the various HI isolates, this is now possible, though there are a lot of genes implicated, it does somewhat narrow the potential candidates down.

Some candidates have been reported previously may function in the same pathway that Bd0108 and Bd0109 function in, and have included proteins

involved in c-di-GMP signalling (Bd2325), proteases (Bd2428, Bd3534), and amino acid and prolioprotein transferases (Bd1131 and Bd3375 respectively) (Tudor et al., 2008). Though the genes for none of these features in our potential list of regulators for HI growth switching, they may be the regulation targets.

5.3.2 Since the publication and Impact

Since the paper was published in late 2013 it is one of the newest publications from the Sockett lab and has been cited once very recently by another publication from the Sockett lab. In the paper Milner and co-workers identified that deletion of *mglA* produced cells that were HI only and also have a reduced amount of type IV pili extruded from the cells similar to the *bd0108* deletion isolate mentioned here (Milner et al., 2014). However unlike the *bd0108* deletion, the *mglA* deletion were completely predatory null suggesting lack of retraction of the pilus is the reason the strain fails to enter cells and carryout predation. In *M. xanthus* MglA regulates Type IV pili switching, involved in gliding motility and cell reversal, though *B. bacteriovorus* do not require Type IV pili to glide, MglA may have evolved to function as a regulator of prey invasion using Type IV pili.

The paper represents the first *in trans* complementation experiment using a plasmid that replicates in *B. bacteriovorus*; pSUP404.2 (Roschanski and Strauch, 2011) and is a great genetic tool with various applications such as shown above. Previously to complement any gene deletion in *B. bacteriovorus* single crossovers of plasmids encoding the wild-type gene were required, this resulted in the integration of the entirety of the plasmid into the genome which possibly

has confounding effects to transcription to neighbouring genes, especially if they operate expressed from a single promoter. Though the pSUP404.2 plasmid does have some drawbacks such as multi-copy number leading to increased gene dosage, it is a great tool that will become more used in any future work. With the advent of the newly sequenced strain of *B. bacteriovorus*: Tiberius (Hobley et al., 2012b) potentially this could be used for cross species complementation, a great target for this would be *bd0108*/*bd0109*, as the Tiberius strain does grow simultaneously HD and HI.

An important point that leads from this data is using HI isolates as wild-type controls and carefully selecting which ones to use to measure certain phenotypes. HI isolates are pleomorphic in general and often even between isolates that carry the same mutation in *bd0108* if not grown in a similar manner. This is something Milner and co-workers did in the aforementioned paper, comparing a wild-type *bd0108* HI isolate with their *mglA* deletion mutant that was not mutated in *bd0108*. This was particularly important as they were comparing pili presence in one experiment. Here we expanded the 'benchmark' for the use of HI's by going some way to phenotype growth, predation and gene expression and it is hoped that in future experiments this will help attribute a phenotype in HI isolates to the target gene deletion rather than those due to the nature of the mutation at the *hit* locus.

5.4 Secretion Events and Signalling in HI and HD Growth Phases Conclusions

This final chapter represents just how far the research has come over the past 10 years and incorporates many new techniques and methodologies previously unused in the field, but also uses some of the fundamental experiments set up in earlier papers and builds upon them.

Future Work

The future, with regards to secretion events and HI vs HD growth holds a great number of possibilities, the dataset generated from the RNAseq experiment will continue to inform on potential roles that different genes have in their respective growth phases; much like the microarrays did before it. The Sockett lab will also continue to collaborate with Dr. Lovering on matters regarding protein purification and structure determination as it opens up a vast possibility of deducing the function of not only the roles of proteins in a novel organism such as *B. bacteriovorus* but also a variety of novel proteins themselves which are not found in other bacteria.

Complementation is something that will undoubtedly feature in future work in *B. bacteriovorus* to further prove any mutants made and perhaps efforts will be made for the adaption of the pSUP404.2 plasmid to be a better genetic tool, such as modification of it to be used as an inducible vector for very regulated protein expression, potentially this could even be used in an *E. coli* host to express *Bdellovibrio* proteins to look at prey cell degradation. Complementation could also be used cross-species given the newly isolated Tiberius strain.

6.0 Conclusion

In this thesis I have reported my contribution to the field of *B. bacteriovorus* research over the past 10 years spanning every aspect of its novel lifecycle/s, from motility to invasion and prey degradation, to growth without prey as well as its use as a 'living antibiotic'. The field has advanced a lot since the early days from when we only just had the sequence of the first isolate to now having two fully sequenced strains, conducting therapeutic trials and understanding the basics that make *B. bacteriovorus* such a fascinating bacterium to work with.

There is now a plethora of techniques at the researchers disposal and I have helped develop them and they have become fundamental to the further characterisation this organism.

The future holds lots of exciting possibilities for the field to expand into and there is no shortage of questions to answer, though I hope I have answered a few of them during my time.

The work has been exciting, interesting and difficult, and it is by no means over.

7.0 Statements of Support

August 7th 2014

I agree that the author contributions stated in your thesis are correct.

Carey Lambert

August 7th, 2014.

I am writing to confirm that I agree with the author contributions as you have described them for all papers on which I had the pleasure of co-authoring with you.

I agree that the statements you have made about your role in the research and writing of each paper is correct, and hope that you will successfully obtain your PhD based on the large amount you have contributed to the field.

All the best,

Laura Hobley

July 22nd 2014

I am happy to confirm that the thesis you sent to me accurately reflects your contribution to:

Atterbury RJ, Hobley L, Till R, Lambert C, Capeness MJ, Lerner TR, Fenton AK, Barrow P, Sockett RE. "Effects of orally administered Bdellovibrio bacteriovorus on the well-being and Salmonella colonization of young chicks".
Appl Environ Microbiol. 2011 Aug 15;77(16):5794-803.

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Statement on Michael Capeness co-authored manuscripts

To whom it may concern,

I hereby confirm that Michael Capeness has provided an accurate description of author contributions in the papers I have co-authored with him included in his thesis, namely "The Structure of an Unconventional HD-GYP Protein from Bdellovibrio Reveals the Roles of Conserved Residues in this Class of Cyclic-di-GMP Phosphodiesterases" and "Activity of Bdellovibrio hit Locus Proteins, Bd0108 and Bd0109, Links Type IVa Pilus Extrusion/Retraction Status to Prey-Independent Growth Signalling".

Yours sincerely

Andrew L Lovering, PhD

Lecturer in Structural Biology

Dated 22/7/2014

School of Life Sciences
University of Nottingham
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Dr Chien-Yi Chang
Centre for Bacterial Cell Biology
Newcastle University
Newcastle upon Tyne
NE2 4AX
22 July 2014

To whom it may concert,

I, Chien-Yi Chang, is the first author of the paper titled "The *Bdellovibrio bacteriovorus* twin-arginine transport system has roles in predatory and prey-independent growth" which was published in Microbiology (Microbiology 2011 157(11):3079-93). I confirm that Michael James Capeness actively involved in this study and played a crucial role in characterizing gene expressions and constructing a novel plasmid for *Bdellovibrio* studies. I also confirm that the statement related to the above paper in Capeness' PhD thesis is accurate.

Sincerely,

Chien-Yi Chang

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