

Electronic Thesis and Dissertation Repository

7-18-2014 12:00 AM

Cell Biology of the Entry of *Bdellovibrio* and Like Organisms

Ryan M. Chanyi, *The University of Western Ontario*

Supervisor: Dr. Susan Koval, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree
in Microbiology and Immunology

© Ryan M. Chanyi 2014

Follow this and additional works at: <https://ir.lib.uwo.ca/etd>



Part of the [Bacteria Commons](#)

Recommended Citation

Chanyi, Ryan M., "Cell Biology of the Entry of *Bdellovibrio* and Like Organisms" (2014). *Electronic Thesis and Dissertation Repository*. 2285.

<https://ir.lib.uwo.ca/etd/2285>

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlsadmin@uwo.ca.

**CELL BIOLOGY OF THE ENTRY OF *BDELLOVIBRIO* AND LIKE
ORGANISMS**

(Integrated Article)

by

Ryan Matthew Chanyi

Graduate Program
in
Microbiology and Immunology

A thesis submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, ON, Canada

© Ryan M Chanyi 2014

Abstract

Bdellovibrio and like organisms (BALOs) are obligate predators of Gram negative bacteria. Predation occurs via a periplasmic or epibiotic life cycle. A periplasmic predator invades the periplasmic space of a prey while an epibiotic predator remains on the exterior.

An analysis of the genome sequences of periplasmic predators, *Bdellovibrio bacteriovorus* and *Bacteriovorax marinus*, and epibiotic predators, *Bdellovibrio exovorus* and *Micavibrio aeruginosavorus* determined that the genome size of epibiotic predators was smaller, while the metabolic networks were highly conserved. No core set of invasion-specific genes was identified.

Both life cycles were characterized by cryoelectron microscopy. The periplasmic predator, *Bd. bacteriovorus*, increased the volume of the periplasmic space prior to invasion. This coincided with a reduction in the size of the protoplast. A plug-like structure was found on the outer surface of bdelloplasts. Cryoelectron tomography suggested the plug originated from the flagellar end of the predator. It appears that upon invasion, *Bd. bacteriovorus* sheds the flagellum, which helps to seal the entry pore.

Analysis of PilT1 and PilT2 mutants of *Bd. bacteriovorus* showed that retraction of type IV pili was not required for invasion into the periplasmic space of the prey. However, a *pilT2* mutant was unable to prey on a biofilm, suggesting that retraction of the pili may be required to penetrate the exopolysaccharide matrix layer.

Stenotrophomonas maltophilia is an emerging multidrug resistant opportunistic pathogen. Nineteen clinical and hospital-environment isolates showed variable abilities to form biofilms. A BALO isolated from the environment preyed upon all strains of *S.*

maltophilia. The predator utilized an epibiotic life cycle and was identified as *Bd. exovor*. *Bd. exovor* FFRS-5 was able to reduce the mass of biofilms formed by almost all strains of *S. maltophilia*, even in the presence of ciprofloxacin and/or kanamycin. Thus, *Bd. exovor* has the potential to be used therapeutically as an antimicrobial agent. These studies have shed light on the attachment and invasion strategies of BALOs and presented the first potential use of *Bd. exovor* as a biocontrol agent.

Keywords: BALOs, *Bdellovibrio*, predation, biofilms, type IV pili, developmental life cycles, cryoelectron microscopy, cryoelectron tomography

Co-Authorship

In chapter 2, the whole genome sequencing of *Bd. exovorus* JSS and *M. aeruginosavorus* EPB, the alignment of the contigs and the closing of the genome by PCR were performed by our collaborators in the laboratory of Dr. Edouard Jurkevitch at the Hebrew University of Jerusalem. All data on metabolic pathways presented in chapter 2 were my contributions to the manuscript. In chapter 4, the isolation of the environmental predator, *Bd. exovorus* FFRS-5 was performed by a student volunteer in our lab, Fulburt Fu, under the supervision of an MSc candidate in our lab, Diane Szmielt. The initial predation efficiency assays of colony forming units before and after predation of the 19 *S. maltophilia* isolates were performed by a 4th-year thesis student in Dr. Koval's laboratory, Zach Findlay.

Dedication

I dedicate this thesis to my family, especially my wife Melissa. Without their support and polite motivation I would still be writing this dedication.

Acknowledgements

I thank my wife Melissa, who has stood by my side through all these years. Along with Charly, both have given me inspiration and unconditional love and support. I thank my Mum and Dad, who taught me the value of hard work. I also thank my brothers and sister. They have been by my side and lent a helping hand whenever I needed it, and sometimes when I did not.

I also thank my supervisor, Dr. Susan Koval. Her editing expertise is responsible for crafting this into a coherent thesis. MSc student Diane Szmiett brought some life into the laboratory so I was not the only graduate student there. Through the years there have been many volunteer and 4th-year honors thesis students who have made the time in the laboratory enjoyable: Amy Dresser, Jennah Dharamshi, Emma Farago, Zack Findlay, Ian Lobb, Sarah Walker, Jodie Baer and Fulbert Fu. Cheryl Ewanski's baking has been enjoyed immensely. I thank the honorary Koval lab members Stacey Xu, Ryan Lum-Tai and Matt Woods and the entire Creuzenet lab for sharing their incubator, plate reader and most importantly their multi-channel pipette.

My advisory committee members Dr. Linn and Dr. Trick have provided essential feedback towards the completion of this thesis. Our collaborators, Dr. Joanna Brooke (DePaul University, USA), Dr. Edouard Jurkevitch (Hebrew University of Jerusalem, Israel) and Dr. Cezar Khursigara (University of Guelph, Canada) have given valuable expertise to expand the *Bdellovibrio* field.

And finally, as any graduate student should, I thank Starbucks and Tim Hortons.

Table of Contents

Abstract	ii
Co-Authorship	iv
Dedication	v
Acknowledgments	vi
Table of Contents	vii
List of Tables	xi
List of Figures	xii
List of Abbreviations	xiv
Chapter 1: Introduction	1
1.1. Predatory prokaryotes.....	1
1.2. <i>Bdellovibrio</i> and like organisms (BALOs).....	2
1.3. Life cycles.....	6
1.3.1. Periplasmic life cycle.....	6
1.3.1.1. Finding a prey.....	6
1.3.1.2. Attachment.....	9
1.3.1.3. Invasion.....	11
1.3.1.4. Bdelloplast formation.....	12
1.3.1.5. Growth phase.....	12
1.3.1.6. Septation and release of progeny.....	14
1.3.2. Epibiotic life cycle.....	14
1.3.3. <i>Bdellovibrio</i> encystment.....	17
1.3.4. Prey independent mutants.....	18
1.3.5. Resistance to predation.....	19
1.4. Genome Sequence of <i>Bdellovibrio bacteriovorus</i> HD100.....	20
1.4.1. Motility.....	20
1.4.2. Chemotaxis.....	22
1.4.3. Type IV pili.....	22
1.4.4. Secretion systems.....	23
1.4.5. Hydrolytic enzymes.....	24
1.4.6. Transport proteins.....	24
1.5. Cryoelectron Microscopy.....	26
1.5.1. Benefits.....	26
1.5.2. Disadvantages.....	26
1.5.3. Vitreous ice.....	27
1.5.4. High pressure freeze substitution.....	28
1.5.5. Tomography.....	29
1.5.6. Cryoelectron microscopy and <i>Bdellovibrio bacteriovorus</i>	29
1.6. Type IV pili.....	30
1.6.1. Major pilins.....	33
1.6.2. Minor pilins.....	34
1.6.3. Structural pilus proteins.....	35
1.6.4. Extension.....	35
1.6.5. Retraction and twitching motility.....	36

1.6.6. PilT proteins.....	37
1.6.7. Regulation.....	38
1.7. <i>Stenotrophomonas maltophilia</i>	39
1.7.1. The organism.....	39
1.7.2. Biocontrol agent.....	39
1.7.3. Intrinsic resistance.....	40
1.7.3.1. Bioremediation.....	40
1.7.3.2. Antibiotic resistance.....	40
1.7.3.3. Predation on <i>Stenotrophomonas maltophilia</i>	41
1.7.4. Opportunistic pathogen and involvement in cystic fibrosis	41
1.7.5. Biofilm formation.....	42
1.7.6. Current effective therapeutics.....	43
1.8. Aims and objectives.....	43
1.8.1. Objective 1.....	44
1.8.2. Objective 2.....	44
1.8.3. Objective 3.....	45
1.9. References.....	45
Chapter 2: Two approaches to a prokaryotic predatory life cycle: Cryoelectron microscopy and comparative genomics of periplasmic and epibiotic predators.....	60
2.1. Introduction.....	60
2.2. Materials and Methods.....	63
2.2.1. Bacterial strains, media and growth conditions.....	63
2.2.2. Isolation of <i>E. coli</i> X-1488 mini-cells	64
2.2.3. Semi-synchronous cocultures	64
2.2.4. Plunge freezing	65
2.2.5. High pressure freeze substitution.....	65
2.2.6. Electron microscopy.....	66
2.2.7. Genome sequencing.....	67
2.2.8. Comparative genomic analysis	67
2.3. Results.....	68
2.3.1. Cryoelectron microscopy of epibiotic predation of <i>Bd. exovorus</i> JSS on <i>C. crescentus</i>	68
2.3.2. Novel insights of the periplasmic life cycle revealed by cryoelectron microscopy.....	71
2.3.3. Cryoelectron tomography – Identification of a plug-like structure on bdelloplasts.....	79
2.3.4. Comparative genomics: Metabolic analysis.....	88
2.4. Discussion.....	92
2.4.1. Cryoelectron microscopy.....	92
2.4.2. Comparative genomics	94
2.5. References.....	96

Chapter 3: Characterization of PilT1 and PilT2 in the bacterial predator <i>Bdellovibrio bacteriovorus</i>	100
3.1. Introduction.....	100
3.2. Material and Methods.....	104
3.2.1. Bacterial strains, media and culture conditions.....	104
3.2.2. DNA isolation.....	106
3.2.3. Polymerase chain reaction.....	107
3.2.4. Construction of pRCT1 and pRCT2.....	107
3.2.5. Bacterial transformations.....	109
3.2.6. Conjugal matings.....	111
3.2.7. Counterselection.....	112
3.2.8. Western blot.....	112
3.2.9. Predation assays.....	113
3.2.10. Biofilm predation assay.....	114
3.2.11. Immunofluorescence microscopy.....	115
3.2.12. Electron microscopy.....	115
3.2.13. Construction of pBADGr:: <i>pilT1</i> and pBADGr:: <i>pilT2</i>	117
3.2.14. Subsurface twitching motility assay.....	117
3.3. Results.....	118
3.3.1. Comparative analysis of active domains in <i>pilT</i> genes of <i>Bd. bacteriovorus</i>	118
3.3.2. Verification of Δ <i>pilT</i> mutants	119
3.3.3. Growth and predation assay of Δ <i>pilT</i> mutants	122
3.3.4. Presence of type IV pili on Δ <i>pilT</i> mutants.....	129
3.3.5. Biofilm predation assay.....	137
3.3.6. Scanning electron microscopy	140
3.3.7. Expression of <i>Bd. bacteriovorus</i> PilT1 or PilT2 in <i>P. aeruginosa</i>	147
3.4. Discussion.....	147
3.5. References.....	152
Chapter 4: Dual use of <i>Bdellovibrio exovorus</i> and antibiotics against the opportunistic pathogen <i>Stenotrophomonas maltophilia</i>	155
4.1. Introduction.....	155
4.2. Materials and Methods.....	158
4.2.1. Bacterial strains, media and culture conditions.....	158
4.2.2. Isolation of environmental predators	158
4.2.3. Identification of environmental isolates.....	160
4.2.4. Prey range assessment	161
4.2.4.1. Agar predation assay	161
4.2.4.2. Liquid cocultures.....	161
4.2.5. <i>Bd. exovorus</i> FFRS-5 predation efficiency on <i>S. maltophilia</i> isolates.....	162
4.2.6. Predation assay with antibiotics.....	163
4.2.7. Biofilm disruption assay.....	163
4.2.8. Electron microscopy.....	165

4.3. Results.....	165
4.3.1. Isolation and identification of environmental predators.....	165
4.3.2. Prey range assessment of <i>Bd. exovorus</i> FFRS-5.....	166
4.3.3. Electron microscopy.....	166
4.3.4. Predation efficiency of <i>Bd. exovorus</i> FFRS-5 on <i>S. maltophilia</i> isolates.....	169
4.3.5. Predation kinetics of <i>Bd. exovorus</i> FFRS-5 on <i>S. maltophilia</i> isolates.....	173
4.3.6. Predation of <i>S. maltophilia</i> isolates by <i>Bd. exovorus</i> FFRS-5 in the presence of antibiotics.....	177
4.3.7. Biofilm formation of <i>S. maltophilia</i> isolates	179
4.3.8. Exposure of preformed biofilms of <i>S. maltophilia</i> to ciprofloxacin and/or kanamycin.....	182
4.3.9. Disruption of biofilms by <i>Bd. exovorus</i> FFRS-5.....	182
4.3.10. Enumeration of residual biofilm.....	190
4.4. Discussion.....	191
4.5. References.....	198
Chapter 5: General Discussion	202
5.1. Cryoelectron microscopy.....	202
5.2. Role of TFP in predation by <i>Bd. bacteriovorus</i>	204
5.3. Bacterial predators for therapeutic use	209
5.4. Final statement.....	212
5.5. References.....	213
Curriculum Vitae	217

List of Tables

Table 1:	Comparison of metabolic pathways present in epibiotic and periplasmic predators.....	89
Table 2:	Bacterial strains and plasmids used in Chapter 2... ..	105
Table 3:	PCR oligonucleotide primers used for amplification of <i>pilT1</i> and <i>pilT2</i>	108
Table 4:	Predation of <i>E. coli</i> ML35 by Δ <i>pilT</i> mutants.....	130
Table 5:	Differences in surface piliation depending on quantitative method used.....	136
Table 6:	Bacterial strains used in Chapter 4.....	159
Table 7:	<i>Bd. exovorus</i> FFRS-5 able to prey on all <i>S. maltophilia</i> isolates....	170
Table 8:	Predation by <i>Bd. exovorus</i> FFRS-5 on <i>S. maltophilia</i> X26332 in the presence of antibiotics.....	178
Table 9:	<i>Bd. exovorus</i> FFRS-5 is able to prey on preformed biofilms of all <i>S. maltophilia</i> isolates in the presence of ciprofloxacin and/or kanamycin.....	189

List of Figures

Figure 1:	Electron micrographs of BALOs.....	4
Figure 2:	Periplasmic life cycle of <i>Bdellovibrio</i> and like organisms.....	8
Figure 3:	Epibiotic life cycle of <i>Bdellovibrio exovorus</i>	16
Figure 4:	Diagram of the type IV pilus apparatus in <i>Pseudomonas aeruginosa</i>	32
Figure 5:	Cryoelectron microscopy visualization of the epibiotic life cycle....	70
Figure 6:	CEM and TEM images of the growth phase of the epibiotic life cycle.....	73
Figure 7:	Cryoelectron microscopy visualization of the periplasmic life cycle.....	76
Figure 8:	CEM projection images of initial attachment between <i>Bd.</i> <i>bacteriovorus</i> and the <i>E. coli</i> prey cell.....	78
Figure 9:	CEM projection images of plug-like structure.....	81
Figure 10:	CEM projection image of bdelloplast has plug-like structure with flagellum attached.....	83
Figure 11:	Decrease in cell wall rigidity of bdelloplasts viewed by CEM.....	85
Figure 12:	Cryoelectron tomographic reconstruction of plug-like structure.....	87
Figure 13:	Location of main type IV pili genes in <i>Bd. bacteriovorus</i>	103
Figure 14:	PCR confirmation of $\Delta pilT1$, $\Delta pilT2$ and $\Delta pilT1pilT2$	121
Figure 15:	No cross reactivity of <i>P. aeruginosa</i> anti-PilT antibody with the PilT proteins of <i>Bd. bacteriovorus</i>	124
Figure 16:	Axenic growth curve of <i>Bd. bacteriovorus</i> 109JA and <i>pilT</i> mutants.....	126
Figure 17:	Effect of mutations in <i>pilT</i> genes on predation by <i>Bd.</i> <i>bacteriovorus</i> on <i>E. coli</i> prey cells.....	128
Figure 18:	No difference in PilA levels in <i>pilT</i> mutants.....	132

Figure 19:	Type IV pili on <i>Bd. bacteriovorus</i> much smaller than those of <i>P. aeruginosa</i>	135
Figure 20:	Immunofluorescence microscopy of <i>pilT</i> mutants with anti-PilA antibodies.....	139
Figure 21:	Biofilm formation by <i>E. coli</i> strains.....	142
Figure 22:	Disruption of the preformed <i>E. coli</i> CO1 biofilm by predation by <i>Bd. bacteriovorus</i> and the <i>pilT</i> mutants.....	144
Figure 23:	Scanning electron microscopy of predation on preformed biofilms of <i>E. coli</i> CO1.....	146
Figure 24:	Neither PilT1 nor PilT2 are able to complement twitching motility in a <i>P. aeruginosa</i> PilT mutant.....	149
Figure 25:	Epibiotic life cycle of <i>Bd. exovorus</i> FFRS-5 confirmed by thin section transmission electron microscopy.....	168
Figure 26:	<i>Bd. exovorus</i> FFRS-5 is able to prey on all <i>S. maltophilia</i> isolates.....	172
Figure 27:	Predation kinetic assay of <i>Bd. exovorus</i> preying on <i>S. maltophilia</i> isolates.....	175
Figure 28:	Biofilm formation by <i>S. maltophilia</i> isolates.....	181
Figure 29:	Predation by <i>Bd. exovorus</i> FFRS-5 on preformed biofilms of <i>S. maltophilia</i> isolates with and without antibiotics.....	184
Figure 30:	Enumeration of residual <i>S. maltophilia</i> in biofilms.....	193

List of Abbreviations

BALOs	<i>Bdellovibrio</i> and like organisms
bp	Base pair
BLAST	Basic local assignment search tool
CEM	Cryoelectron microscopy
CET	Cryoelectron tomography
CFU	Colony forming unit
ddH ₂ O	Double distilled water
DNA	Deoxyribonucleic acid
DNB	Dilute nutrient broth
EM	Electron microscopy
h	Hour(s)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HM	HEPES with Calcium and Magnesium
HPFS	High pressure freeze substitution
kb	Kilobase pair
kDa	Kilodalton
LB	Luria-Bertani
LPS	Lipopolysaccharide
mbp	Mega base pair
min	Minute(s)
NB	Nutrient broth
NB-YE	Nutrient broth-yeast extract
OMP	Outer membrane protein
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque forming unit
pH	Potency of hydrogen
PY	Peptone-yeast extract (<i>Bdellovibrio</i>)
PYE	Peptone-yeast extract-Magnesium-Calcium (<i>Caulobacter</i>)

RNA	Ribonucleic acid
RPM	Rotations per minute
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Second(s)
UA	Uranyl acetate
TAE	Tris-acetate-EDTA
TBS	Tris buffered saline
TEM	Transmission electron microscopy
TMP-SMX	Trimethoprim/sulfamethoxazole
TTBS	Tween tris buffered saline
v/v	volume/volume
w/v	weight/volume
<i>g</i>	gravitational force

Chapter 1

1. Introduction

1.1. Predatory prokaryotes

Predation among prokaryotes is a major driving force behind shaping microbial communities, thereby affecting their structure and diversity. However, there are two major differences between eukaryotic and prokaryotic predators: the latter prey on cells that are larger than itself and direct contact is not always required.

The Gram positive predator *Streptoverticillium* is a facultative predator that lyses colonies of *Micrococcus luteus* (Casida, 1980). Lysis of colonies may not involve direct contact between the predator and prey, as a diffusible factor may cause cell lysis. *Agromyces ramosus* is another Gram positive facultative predator that preys on various Gram negative and Gram positive bacteria as well as yeast (Casida, 1983).

The phylum *Proteobacteria* contains the majority of the Gram negative predators, with the δ -proteobacterial predators dominating. There are four basic strategies of predation among Gram negative predators; three require direct contact while one does not. *Myxococcus xanthus* uses a wolf-pack predation strategy in which a group of *M. xanthus* secretes hydrolytic enzymes into the environment, which causes prey cell damage and release of nutrients (Berleman and Kirby, 2009). Direct invasion of the prey cell cytoplasm by one predator, *Daptobacter*, has also been reported although it has not been studied further (Guerrero *et al.*, 1986). Epibiotic predation requires direct contact with the outer membrane of a prey cell and direct secretion of hydrolytic enzymes into the prey cell. The nutrients are then transported back into the attached predator and replication similar to binary fission occurs (e.g., *Bdellovibrio exovorus* and

Vampirococcus) (Guerrero *et al.*, 1986; Koval *et al.*, 2013). The final and most widely studied predation strategy involves direct entry into the periplasmic space of the prey by *Bdellovibrio* and like organisms (BALOs) (Starr and Baigent, 1966).

1.2. *Bdellovibrio* and like organisms (BALOs)

While isolating bacteriophage active against *Pseudomonas syringae* pv. *phaseolicola*, Heinz Stolp noticed plaques that developed on a double layer agar plate three days after initial incubation (Stolp and Petzold, 1962). This cell lysis was studied further because “the belated generation of the plaque spoke against the existence of phage activity”. After isolating the agent involved, Stolp observed a small, rapidly moving microorganism he later identified as *Bdellovibrio bacteriovorus*, named after its cellular morphology and way of life (“bdella” Greek for “leech”). The prey penetration and intraperiplasmic nature of *Bd. bacteriovorus* was later described by Starr and Baigent (1966).

Bdellovibrio bacteriovorus is a small (0.25µm wide and up to 2µm long), aerobic Gram negative obligate predator of other Gram negative bacteria. It is highly motile via a single, polar, sheathed flagellum (Fig. 1) that has a characteristic damped waveform pattern (Thomashow and Rittenberg, 1985). *Bd. bacteriovorus* is ubiquitous, found in bulk soil and the rhizosphere (Jurkevitch *et al.*, 2000), raw sewage and activated sludge (Afinogenova *et al.*, 1981; Fratamico and Cooke, 1996; Fry and Staples, 1974; Fry and Staples, 1976;) and marine (Kelley *et al.*, 1997; Pan *et al.*, 1997; Taylor *et al.*, 1974), brackish (Schoeffield and Williams, 1990; Williams, 1988) and fresh waters (Afinogenova *et al.*, 1981; Richardson, 1990; Varon and Shilo, 1980). It has also been identified in more extreme environments such as hypersaline waters (Piñeiro *et al.*, 2004;

Figure 1. Electron micrographs of BALOs. A) BALOs isolated from sewage in enrichment cultures with *E. coli* prey cells. Note the vibroid shape with a single polar sheathed flagellum; B) *Bd. bacteriovorus* 109J. Note the characteristic dampened waveform flagellum. Samples were negatively stained with uranyl acetate. Micrographs were provided by Dr. S.F. Koval. Scale bar equals approximately 0.5 μm .



Sanchez-Amat and Torrella, 1989), the gills of crabs (Kelley and Williams, 1992), mammalian faeces (Schwudke *et al.*, 2001) and more recently, the gut microbiota of humans (Iebba *et al.*, 2013).

Since the isolation of the first *Bd. bacteriovorus* over half a century ago, two other genera have been proposed, which exhibit properties similar to *Bdellovibrio*. Prior to genotypic methods, classification was mainly based on the obligatory predatory life cycle, small size, vibroid shape and single sheathed flagellum. Originally, all isolates were assigned to a single family, genus and species: *Bd. bacteriovorus* of the family *Bdellovibrionaceae*.

Collectively these predators are now referred to as *Bdellovibrio* and like organisms (BALOs). Since 2000, more comprehensive 16S rRNA gene sequence analysis has allowed the diversification of BALOs to include the family *Bacteriovoraceae* and three genera: *Bdellovibrio* (*Bd.*), *Bacteriovorax* (*Bx.*) and *Peredibacter* (Baer *et al.*, 2000; Davidov and Jurkevitch, 2004; Schwudke *et al.*, 2001; Snyder *et al.*, 2002). To date, there are two species of *Bdellovibrio* (*Bd. bacteriovorus* and *Bd. exovorus*), three species of *Bacteriovorax* (*Bx. stolpii*, *Bx. marinus* and *Bx. litoralis*) and one species of *Peredibacter* (*P. starrii*). Although BALOs are ubiquitous in the environment, only *Bx. marinus* and *Bx. litoralis* have been isolated from marine samples (Williams and Piñeiro, 2007).

As stated earlier, BALOs are obligate predators of other Gram negative bacteria. The prey range is broad and includes free-living bacteria as well as plant, animal and human pathogens (Burger *et al.*, 1968; Dashiff and Kadouri, 2011; Dashiff *et al.*, 2011; Fratamico and Whiting, 1995; Kadouri *et al.*, 2013; Shanks *et al.*, 2013; Shemesh *et al.*,

2003). However, each BALO isolate has a slightly different 'menu'. There are multiple examples where prey ranges overlap between two BALOs belonging to different genera as well as examples where the isolates are very closely related but have completely different prey ranges. Because of this diversity, the prey range cannot be used as a taxonomic marker.

1.3. Life cycles

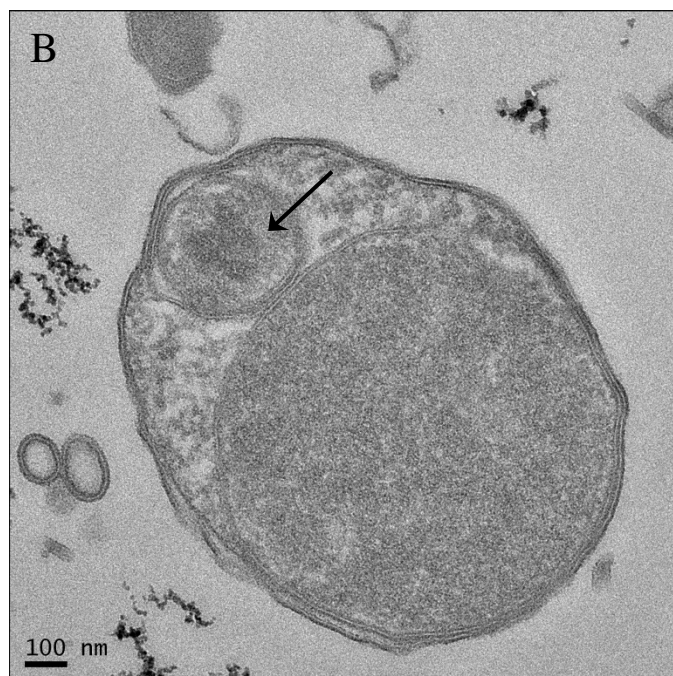
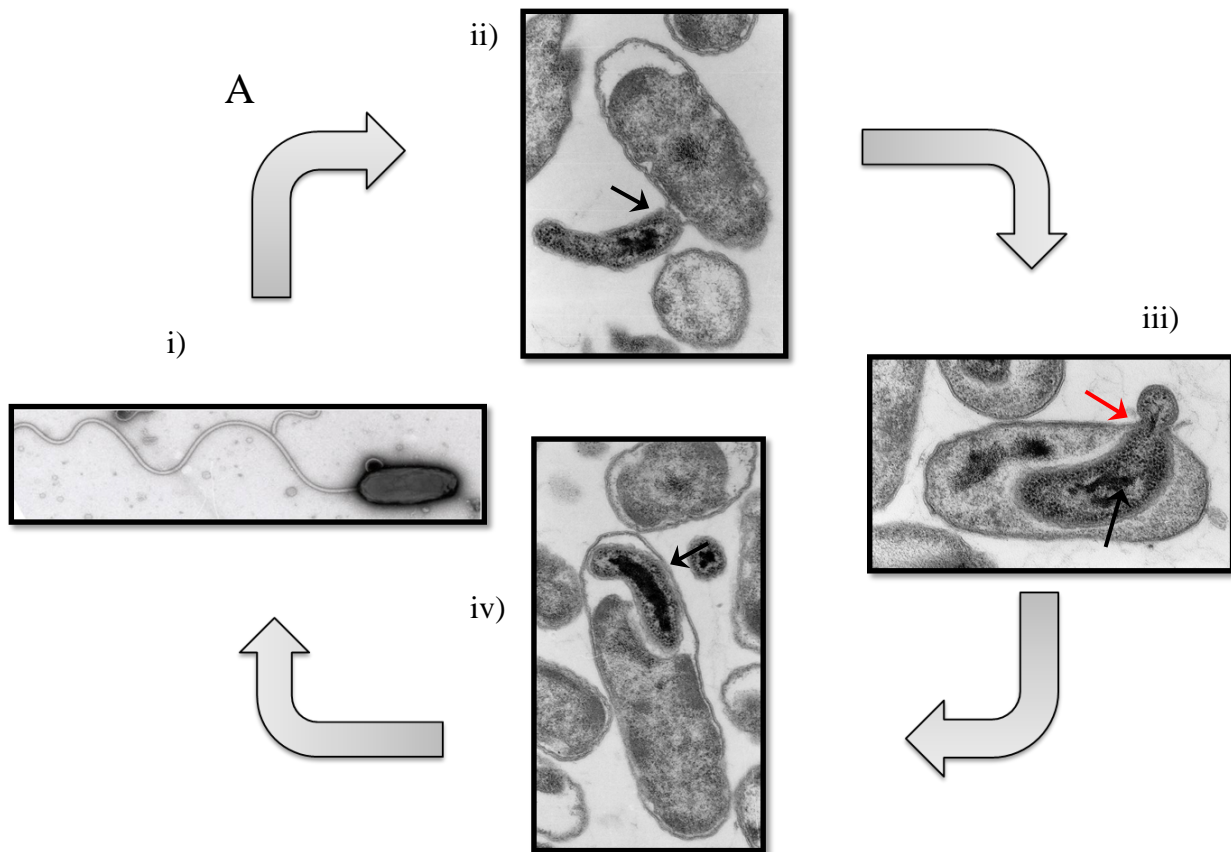
1.3.1. Periplasmic life cycle

The most common life cycle found among BALOs is the periplasmic life cycle (Fig. 2). *Bd. bacteriovorus* is the model genus and species used to study the periplasmic life cycle of BALOs. This life cycle has two main stages: a motile non-growing attack phase and an intraperiplasmic growth phase (Ruby, 1992).

1.3.1.1. Finding a prey

Finding a prey cell relatively quickly is important for survival of BALOs. Recently released progeny are considered to be in a starvation state because there is no DNA replication occurring. It was recognized early in *Bdellovibrio* research that chemotaxis may play an important role in finding potential prey. Four early studies from the Conti lab identified chemotactic responses of *Bd. bacteriovorus* towards yeast extract (Straley and Conti, 1974), amino acids (LaMarre *et al.*, 1977), prey cells (Straley and Conti, 1977) and pure compounds such as: acetone, propionate, thioacetate, malonate and D-glucose-6-phosphate (Straley *et al.*, 1979). However, chemotaxis toward prey occurred only at very high prey cell concentration and would occur whether a cell was susceptible to predation or not. These studies are inconclusive because they were

Figure 2. Periplasmic life cycle of *Bdellovibrio* and like organisms. A) Predation by *Bd. bacteriovorus* (black arrow) on *Delftia acidovorans* i) Motile attack phase; ii) Attachment to potential prey; iii) Invasion into periplasmic space through entry pore (red arrow); iv) Bdelloplast formation. Note that the bdelloplast of *D. acidovorans* does not form a rounded structure B) Characteristic spherical bdelloplast structure, formed by predation of *Bd. bacteriovorus* (arrow) on *E. coli*.



performed using a prey-independent laboratory strain, *Bx. stolpii* UKi2 (formerly *Bd. stolpii*), not representing a true obligate predator. Lambert *et al.* (2003) demonstrated that the loss of Mcp2 decreased predation efficiency by *Bd. bacteriovorus* 109J on *Escherichia coli*. Due to the abundance of *mcp* genes, it is difficult to determine the role each plays in the recognition and detection of prey. As many prey cells would be chemotactic towards these same compounds, increased cell density would allow a higher prevalence of random collisions, allowing predation to occur at an increased rate.

Aerotaxis may play a role in locating a prey cell; however, Kadouri and Tran (2013) have recently shown that *Bd. bacteriovorus* is capable of predation on a biofilm in low oxygen concentrations. Anaerobic bacteria present in the oral cavity are susceptible to predation by BALOs, indicating that a complete aerobic environment may not be a requirement for the recognition of a prey cell (Dashiff and Kadouri, 2011).

1.3.1.2. Attachment

Upon collision with a potential prey cell, there is a brief reversible attachment termed the recognition period. The recognition period is non-specific as a BALO is able to attach to non-prey cells (other Gram negative cells), Gram positive bacteria and abiotic materials in the medium (e.g., salt crystals). This attachment becomes irreversible if the cell is deemed a suitable prey. Temperature, pH and composition of the medium play important roles in attachment; however, these factors mostly inhibit motility of the BALO, which decreases the probability of a random collision occurring (Varon and Shilo, 1968).

Abram and Davis (1970) revealed the presence of small fibers on the pole of the attack phase cell opposite the flagellum. As the non-flagellated pole mediates

attachment, Abram *et al.* (1974) believed these fibers were directly involved in the attachment process although this was only speculation. Evans *et al.* (2007) showed that these fibers were necessary for predation to occur. Mahmoud and Koval (2010) identified these small fibers as type IV pili (TFP). Pre-incubation with antibodies against the main pilin subunit, PilA, inhibited attachment and therefore predation could not occur. As demonstrated in many organisms, including *P. aeruginosa*, TFP have an interesting property that allows them to retract. It has been speculated that after attachment, retraction of the TFP may pull the predator into the periplasmic space of the prey cell. However, until the current studies, no experimental evidence has been provided to support this function.

Cell wall mutants of *E. coli* K-12 and *Salmonella enterica* serovar Typhimurium LT2 were examined to determine if BALOs recognize specific receptor sites on the surface of prey cells. Because rough lipopolysaccharide (LPS) mutants of *Salmonella* were susceptible to predation, it was believed attachment must be mediated by the LPS core, not the O-antigen side chains (Schelling and Conti, 1986). Sequential deletions of sugar residues in the LPS core decreased attachment by *Bd. bacteriovorus*, indicating some level of receptor specificity in the LPS core. However, for *Bx. stolpii* (formerly *Bd. stolpii*), predation occurred even in *S. enterica* SL1102, a deep rough mutant lacking heptose; however, increased incubation time was required. *E. coli* K-12 mutants lacking specific outer membrane porins were susceptible to predation with no decreased efficiency detected. Therefore, these results were inconclusive regarding the contribution of LPS and outer membrane porins as potential receptors as different BALO species gave conflicting results.

1.3.1.3. Invasion

For many years, BALOs were thought to use their flagellar rotation to power the invasion process via a drilling action. Abram *et al.* (1974) noticed that plasmolysed cells of *Aquaspirillum serpens*, *E. coli* and *P. fluorescens* whose protoplasts (cytoplasm) had been separated from the cell walls were unable to be used as prey. This indicated that direct contact with the prey protoplast may be required for penetration to occur, suggesting that factors other than flagellar rotation may be involved. It was not until 2006 that Lambert *et al.* conclusively demonstrated that motility was not required for predation. A non-motile mutant was able to predate but was not able to find the prey cell efficiently.

Transcriptomic studies have shown that many hydrolytic enzymes are upregulated upon initial BALO attachment (Karunker *et al.*, 2013; Lambert *et al.*, 2010). Hydrolytic enzymes are applied in a localized manner to limit excessive damage to the prey cell and unregulated diffusion of periplasmic or cytoplasmic constituents. This results in a small pore through which the BALO can squeeze into the periplasmic space.

Beginning at the irreversible attachment stage and throughout the invasion process, room is created in the periplasmic space of the prey. This can be observed by electron microscopy as a distinct separation between the inner and outer membranes. If rod shaped, the prey cell also begins to round as cross links in the peptidoglycan are being cleaved (Lerner *et al.*, 2012). Although there is some contact between the BALO and the cytoplasmic membrane of the prey, the predator will penetrate only the outer membrane, residing in the periplasmic space for the duration of the life cycle (Burnham *et al.*, 1968).

1.3.1.4. Bdelloplast formation

Once the BALO is inside the periplasmic space, the pore is resealed. The flagellum is lost during the invasion process but it is not known at which stage. Throughout the entire process the prey cell is being modified into a protected enclosure, a bdelloplast, which will allow optimal growth of the predator. The bdelloplast is osmotically stable and resistant to further invasion by another BALO. N-deacetylation of the peptidoglycan during entry inhibits further degradation of peptidoglycan to maintain a rounded cell shape without premature lysis (Thomashow and Rittenberg, 1978b). It is important to note that not all prey cells will round-up during the life cycle. The Koval laboratory has found that *Delftia acidovorans* can be used very efficiently as a prey cell but it maintains a rod-shaped morphology throughout the entire life cycle (Fig. 2). The formation of a bdelloplast signifies the end of the attack phase and the beginning of the growth phase.

Lerner *et al.* (2012) used a more detailed approach and deleted two peptidoglycan hydrolases, *Bd0816* and *Bd3459*. Double mutants did not enter prey or round-up the bdelloplast as efficiently and the prevalence of multi-infection increased. These proteins show homology to PBP4 endopeptidases, which control cell wall maintenance. However, they do not contain the regulatory domain and the active site is more exposed. Lerner *et al.* (2012) believed these modifications allow degradation of diverse peptidoglycan structures, beneficial for the wide prey range exhibited by *Bd. bacteriovorus*.

1.3.1.5. Growth phase

At the initiation of the growth phase, *Bd. bacteriovorus* upregulates hydrolytic enzymes that are used to degrade the macromolecules of the prey cell. Lambert *et al.*

(2010) used microarrays to identify 479 genes that were expressed during the growth phase, termed the “predatosome”. This work was expanded by Karunker *et al.* (2013) using RNA-seq analysis to describe two transcriptomic profiles exhibited by *Bd. bacteriovorus*. One profile is specialized towards the growth phase, which is mutually exclusive from the genes that are upregulated during the attack phase. There were 1557 genes that were active during the growth phase, 353 genes active during the attack phase and 430 genes expressed in both. Generally, genes expressed during the growth phase were associated with replication and division such as ribosomal proteins, RNA polymerase components, peptidoglycan metabolism and transport mechanisms.

The breakdown of the prey macromolecules by an arsenal of hydrolytic enzymes provides *Bd. bacteriovorus* with the building blocks to synthesize DNA, RNA, lipids and proteins (Gray and Ruby, 1991; Hespell *et al.*, 1975; Kuenen and Rittenberg, 1975; Rittenberg and Hespell, 1975; Ruby and McCabe, 1986; Thomashow and Rittenberg, 1978a; Thomashow and Rittenberg, 1978b; Thomashow and Rittenberg, 1978c). To increase efficiency of growth, *Bd. bacteriovorus* is able to transport nucleotides and phospholipid precursors as monophosphates (Rittenberg and Langley, 1975; Ruby *et al.*, 1985).

The mechanism of transport of nutrients into *Bd. bacteriovorus* is unknown. A tight interaction between the predator’s outer membrane and the prey cell’s protoplast may allow direct import through all membranes. However, close proximity between the membranes is not always observed when cells are visualized by electron microscopy. Another method would be diffusion of nutrients into the periplasm and then uptake into *Bd. bacteriovorus* by the many transport proteins known to be upregulated. This would

increase surface area allowing better growth since the protoplast is shrinking while the predator is elongating. Supporting this idea is the observation that *Bd. bacteriovorus* inserts a porin-like protein into the cytoplasmic membrane of the prey (Barel *et al.*, 2005). It has been debated if the predator is able to confiscate outer membrane proteins (OMPs) of the prey and incorporate them for their own use. However, these studies are believed to be misleading, due to the prey cell remnants that remain in suspension in the cocultures after predation is complete and thus contributing to the analyses.

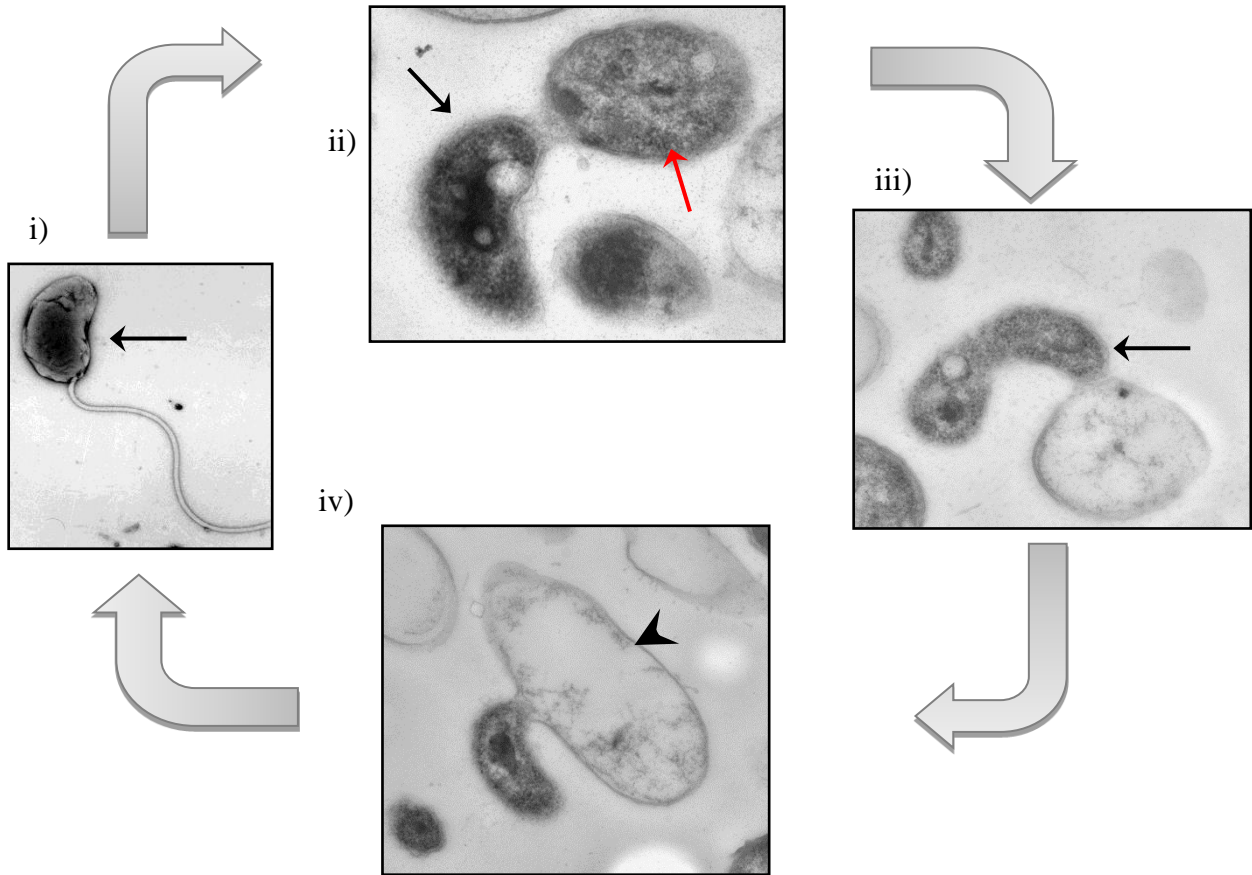
1.3.1.6. Septation and release of progeny

Upon an unknown signal, multiple septa are formed and progeny cells are produced simultaneously (Eksztejn and Varon, 1977). The number of progeny formed depends on the original size of the prey but does vary (Kessel and Shilo, 1976). Fenton *et al.* (2010) showed that anywhere between 2 and 9 progeny *Bd. bacteriovorus* could be released from a single infection of *E. coli*. Their flagella are formed while inside the bdelloplasts. Fenton *et al.* (2010) also showed release of progeny is not through mass degradation of the bdelloplast but through discreet exit pores. Flagellar motility is required for successful release of progeny from the bdelloplast as downregulation of *motA* impaired motility and escape (Flanagan *et al.*, 2004). The progeny are then released as attack phase predators and the life cycle repeats. Overall the entire process takes approximately three hours from initial attachment until release of progeny.

1.3.2. Epibiotic life cycle

Koval and Hynes (1991) described a BALO that did not form a bdelloplast during predation on *Caulobacter crescentus* (Fig. 3). This epibiotic predator shared 93% 16S rRNA gene sequence similarity to *Bd. bacteriovorus*. Based on molecular data and the

Figure 3. Epibiotic life cycle of *Bd. exovorus*. i) Motile attack phase cell (black arrow);
ii) Attachment of *Bd. exovorus* to a prey cell, *Stenotrophomonas maltophilia* (red arrow);
iii) Replication of *Bd. exovorus* by binary fission; iv) Prey cell cytoplasmic content depleted, leaving behind an empty ghost cell (black arrowhead).



different life cycle, Koval *et al.* (2013) proposed that this predator belonged to a novel species, *Bd. exovor*. Unlike with *Vampirococcus*, an electron dense bridge attaching the predator to the prey cell is not observed for *Bd. exovor*. How this predator remains attached to the surface is not known as membrane fusion does not occur between predator and prey. During epibiotic growth, a long aseptate filament is not formed but rather division occurs by binary fission. Chanyi *et al.* (2013) isolated bacteria from raw sewage that could be used as prey for both *Bd. exovor* and *Bd. bacteriovorus*. *Bd. exovor* uses the epibiotic life cycle for survival while *Bd. bacteriovorus* uses the periplasmic life cycle for survival, demonstrating that the life cycle is a characteristic of the predator and not the prey. Koval *et al.* (2013) is the first description of a BALO using a life cycle divergent from the periplasmic life cycle.

1.3.3. *Bdellovibrio* encystment

With the exception of *Bd. exovor*, there does not appear to be large divergence in the stages of the life cycle across the BALO family. However, one strain isolated by Burger *et al.* (1968), *Bdellovibrio* sp. strain W, forms large, electron dense resting bodies termed bdello cysts (Hoeniger *et al.*, 1972; Tudor and Conti, 1977a). Bdello cysts are slightly larger than the vegetative cell and contain 3-fold more carbohydrate but similar levels of protein and RNA. The increase in carbohydrate was attributed to the increase in cell wall structure surrounding the bdello cyst (Tudor, 1980; Tudor and Bende, 1986). Encystment did provide increased resistance to temperature and nutrient deprivation but it did not protect from ultra-violet radiation or sonication (Tudor and Conti, 1977a). Germination of the bdello cysts involved a breakdown of the outer membrane and release of a motile attack phase cell (Tudor and Conti, 1977b). This could be induced in the

presence of L-glutamine, L-asparagine, glycine, L-phenylalanine, L-serine, L-valine, L-threonine, L-methionine and the monovalent ions K^+ and NH_4^+ (Tudor and Conti, 1978). Unfortunately no further work has been performed on the encystment process of this strain.

1.3.4. Prey independent mutants

Since the discovery of BALOs, it has been shown that spontaneous mutants arise in the predator population that allow them to grow in the absence of a prey cell (axenic growth) if residing in rich medium (Seidler and Starr, 1969; Varon *et al.*, 1974). These prey-independent mutants arise at a frequency of 10^{-6} to 10^{-7} . This suggested that this phenotype arose due to a single mutational event. These predators were facultative prey-independent mutants, such that when placed in buffer with a prey cell the predatory life style was retained. Prolonged cultivation in axenic conditions eventually results in complete loss of predatory activity.

Cotter and Thomashow (1992) identified a short open reading frame that was mutated in different prey-independent clones. They termed this genetic region the host-interaction locus (*hit*). Using modern next-generation sequencing technology, Wurtzel *et al.* (2010) found that 89% of prey-independent isolates had mutations in the *hit* locus. Interestingly there were different mutations observed. The most prevalent mutation (46%) was a 42 bp deletion caused by a 10 bp direct repeat flanking the region. Also, 11% of isolates had mutations elsewhere in the genome conferring the prey-independent phenotype. This supports the work by Barel and Jurkevitch (2001) who found prey-independent mutants with a complete *hit* locus. The *hit* locus has been identified only in *Bd. bacteriovorus* although prey-independent phenotypes have been observed in other

BALO species (e.g., *Bx. stolpii* UKi2). There are likely similar genetic elements in the other BALO genomes that have not been identified yet. However, prey-independent variants of the epibiotic predator *Bd. exovorus* JSS have not yet been isolated (Pasternak *et al.*, 2014).

There has been recent debate about the prevalence of prey-independent BALOs in nature. Historically, all BALOs were prey-dependent upon initial isolation from environmental samples and thus it was concluded the prey-independent strains did not exist in nature. Hobbey *et al.* (2012) then described *Bd. bacteriovorus* Tiberius, which was isolated from a source rich in diverse bacteria and organic pollutants (the River Tiber). This predator grew axenically and predatorily while in coculture with *E. coli*. The genome sequence of strain Tiberius revealed the possibility of both ancient and recent lateral gene transfer. Lateral gene transfer was originally thought unlikely due to the predatory life cycle of BALOs but the dual life cycle of this predator makes it possible. It is unknown if other strains exhibit a similar phenomenon.

1.3.5. Resistance to predation

There has never been a report of a bacterium that is susceptible to predation by a BALO becoming resistant and maintaining that resistance through multiple generations of culturing. A phenomenon known as plastic phenotypic resistance occurs in cocultures when there are prey cells remaining after predation is allowed to continue to completion (Shemesh and Jurkevitch, 2004). When isolated out of coculture and grown independently, the progeny of the resistant prey cell are once again susceptible to predation. The presence of a paracrystalline protein surface layer (S-layer) is the only known natural resistance to predation. Koval and Hynes (1991) showed that S-layers are

anti-predation devices and even localized disruption of the S-layer is enough to allow predation to happen. Koval and Bayer (1997) went on to show the thick polysaccharide capsule present on many environmental Gram negative isolates is not a barrier to predation.

1.4. Genome sequence of *Bdellovibrio bacteriovorus*

With next generation sequencing technologies in full swing, four fully annotated genome sequences of BALOs have become available in the past three years. These sequences have revealed many insights into the survival of BALOs in nature, prey-independence, metabolism and their life cycle. *Bd. bacteriovorus* HD100, the type strain, was the first to be sequenced (Rendulic *et al.*, 2004). Typically, intracellular pathogens have smaller genomes, however, strain HD100 encodes for 3,584 proteins found within the 3.8 mbp genome. These values are comparable to much larger cells such as *E. coli*, despite *Bd. bacteriovorus* being a fraction of the physical size. Strain Tiberius is highly conserved despite being able to grow both prey-dependently and independently. At just under 4.0 mbp, the strain Tiberius genome is slightly larger, encoding 3,738 proteins (Hobley *et al.*, 2012). The other two genomes will be discussed in detail later (Chapter 2) as they pertain to work accomplished through this thesis.

1.4.1. Motility

BALOs are highly motile, capable of reaching speeds in excess of 100 times their body length per second. To put this into perspective, this is the equivalent of a 183 cm tall man running 658 km/h.

It was originally believed that this motility was the driving force behind penetration into the prey cell periplasmic space; using brute force to ram the prey cell and

then drill into the cell using a corkscrew motion powered by the flagellum. Often when *Bd. bacteriovorus* attaches to a prey cell in liquid coculture, it can be seen pushing the prey cell around the culture. However, Lambert et. al (2006) showed that this was not the case. Inactivation of one of the six flagellins, *fliC3*, produced progeny that made a flagellar sheath but were not motile. These cells were able to prey on *E. coli* but only when placed directly on lawns of prey cells. This demonstrated that flagellar motility is not the driving force behind penetration; however, motility plays a key role in successfully finding the prey cell. Disruption of *fliC1* resulted in cells that were slightly inhibited in motility, swimming at 92% the speed of the wild-type. This phenotype was exacerbated when *fli5* was disrupted. Cells produced a truncated flagellum (2.3 μm) and were able to swim at 80% the speed of wildtype. Disruption of *fliC2,4* and *6* did not result in an observable phenotype, showcasing the redundancy in flagellins in the *Bd. bacteriovorus* genome.

Flagellar motility is not the only source of movement for BALOs. Twitching motility involves TFP and will be discussed in Section 1.4.3. Lambert *et al.* (2011) describes a third type of motility called gliding motility. Gliding motility is a very slow movement that is used on solid surfaces while searching for prey. Lambert *et al.* (2011) found in *Bd. bacteriovorus* HD100 that the *fliC3* mutant (lacks flagellar motility) and a *pilA* mutant (lacks twitching motility) were able to use gliding motility, averaging 20 $\mu\text{m}/\text{h}$. Addition of A22, an MreB-specific inhibitor, abolished gliding motility. Therefore, MreB cytoskeletal integrity is required for gliding motility despite not requiring either the flagellum or the TFP apparatus. The factors involved in gliding motility or its function are not known.

1.4.2. Chemotaxis

The genome for *Bd. bacteriovorus* HD100 contains 20 methyl-accepting chemotaxis genes (Rendulic *et al.*, 2004). Fourteen of these appear to be conserved in the genome of strain Tiberius, with two genes, *Bd1872* and *Bd1873*, fused into a single chemotaxis gene in strain Tiberius, *Bdt1841*. There are four chemotaxis proteins found in strain HD100 that are not in Tiberius: *bd0262*, *bd0932*, *bd2596* and *bd2622*. Likewise, there are four chemotaxis proteins found within strain Tiberius that are absent from HD100: *bdt1069*, *bdt1102*, *bdt1169*, *bdt1593*. The different environments in which strain HD100 and Tiberius are found, terrestrial and aquatic, respectively, may require chemotactic responses to different stimuli.

Straley *et al.* (1979) also recognized that all tested strains of *Bd. bacteriovorus* had aerotaxis. The genomes of both strain HD100 and Tiberius contain a putative aerotaxis sensor (Aer). Aer is found in other systems, including *E. coli* and *P. aeruginosa*, to signal through chemotactic responses to move towards optimal oxygen concentrations (Rebbapragada *et al.*, 1997; Hong *et al.*, 2004). However, rather than avoiding undesirable conditions, aerotaxis may be a mechanism to accumulate in areas of high prey cell concentration as Kadouri and Tran (2013) showed that predation continued on biofilms at 3% oxygen concentration.

1.4.3. Type IV pili

The attachment between predator and prey is not well understood. Because BALOs have a wide prey range that differs among closely related BALOs, the identification of a receptor on a prey cell is a difficult task (Tudor and McCann, 2007). Analysis of the genome of *Bd. bacteriovorus* identified many TFP gene clusters, which

are candidate structures to allow for bacterial adhesion to other cells (Craig *et al.*, 2004). In *Bd. bacteriovorus*, two main operons encode many of the *pil* genes with others interspersed throughout the genome. This is similar to the organisation of *pil* genes in *P. aeruginosa* (Burrows, 2005). The larger operon contains 8 genes (*pilHIDMNOPQ*), many of which are structural and form the main TFP apparatus. The second operon contains the motor proteins, base protein and a two-component regulatory element (*pilBTCSR*). A few other *pil* genes are found scattered in the genome (*pilA*, *pilG*, *pilM* and *pilT*). These genes and their location are highly conserved in the genome of *Bd. bacteriovorus* Tiberius (Hobley *et al.*, 2012).

1.4.4. Secretion systems

Due to its life cycle, *Bd. bacteriovorus* must secrete many proteins and enzymes that could be harmful to itself. Within the genome, many proteins are found that encode for type I and type II secretion systems, autotransporters (type V secretion) as well as the twin arginine translocation (TAT) system (Barabote *et al.*, 2007). Type I secretion involves direct export of substances from the cytoplasm out of the cell, across both membranes. Type II secretion involves the *sec*-pathway for periplasmic translocation. Both systems allow for the export of potentially destructive proteins (e.g., DNases, RNases, proteases, lipases and toxins) to the extracellular environment (Sandkvist *et al.*, 1997; Sandkvist, 2001). Proteins that use type V secretion are generally large proteins that have three distinct domains: an N-term *sec*-secretion signal peptide, a secreted passenger domain and a C-term translocator domain. Autotransport requires the *sec*-pathway to gain access to the periplasmic space via recognition of the N-terminal signal sequence. The C-terminal translocation domain inserts itself into the outer membrane,

translocating the passenger domain to the exterior of the cell where it can be cleaved. The translocation domain remains in the outer membrane of the cell. The TAT system transports folded proteins. *Bd. bacteriovorus* contains *tatA1*, *tatA2* and *tatBC* along with 21 potential TAT-transported proteins (Chang *et al.*, 2011). The TAT system was shown to be essential for both host-dependent and host-independent growth. No homologues of type III, type IV or type VI secretion systems have been identified in any of the sequenced *Bd. bacteriovorus* strains, *Bd. exovorus* JSS or *Bx. marinus* SJ.

1.4.5. Hydrolytic enzymes

Necessary for survival, all sequenced BALO strains contain a large arsenal of hydrolytic enzymes (~150). *Bd. bacteriovorus* has been the most highly studied in this respect (Lambert and Sockett, 2013; Lambert *et al.*, 2010; Lerner *et al.*, 2012; Rendulic *et al.*, 2004). Of the 150 hydrolytic enzymes present, proteins with homology to proteases and peptidases represent the largest group. Time course transcriptomic studies revealed that only 11 of the 150 enzymes are upregulated upon initial attachment to the prey (Lambert *et al.*, 2010). These enzymes include a Zn-metallo-protease, cysteine-protease, several serine-proteases, 2 endonucleases, a helicase and two other hydrolases. This may represent a core set of enzymes required for prey cell penetration, while others may be involved in degradation of the prey cell cytoplasmic content and release of progeny from the bdelloplast. The analysis by Lambert *et al.* (2010) confirms an earlier report by Gloor *et al.* (1974), which found metallo- and serine-proteases in BALO strains.

1.4.6. Transport proteins

The genome of *Bd. bacteriovorus* encodes a disproportionate number of cytoplasmic membrane channels and outer membrane porins. A detailed analysis

(Barabote *et al.*, 2006) revealed far more TonB/ExbBD-type systems and MotAB-type systems within *Bd. bacteriovorus* when compared to *E. coli*. For survival, *Bd. bacteriovorus* must secrete hundreds of proteins to degrade the cytoplasmic content of the prey, and then it must transport all the material back to the periplasmic space and then into its cytoplasm. The majority of the predicted transporters fall into the ABC-type transporters and the major facilitator superfamily (MFS) permeases. Barabote *et al.* (2007) suggest that *Bd. bacteriovorus* is capable of transporting hydrophobic and amphipathic drugs, amino acids, peptides, lipoproteins, carbohydrates, phosphate, polyamines, nitrate, thiamine, iron and ribonucleotides. Complete flagellar and fimbrial protein export systems are also present.

Although controversial, there is one previous report (Tudor and Karp, 1994) that *Bd. bacteriovorus* can insert an OmpF-like protein into the prey cell cytoplasmic membrane. This presumably allows entry to cytoplasmic contents and transport of degraded material back (Tudor and Karp, 1994). Acquisition of prey-derived OmpF and OmpC proteins into the outer membrane of *Bd. bacteriovorus* (Diedrich *et al.*, 1983; Diedrich *et al.*, 1984; Talley *et al.*, 1986) has been even more controversial; however, with more advanced analytical techniques it is now believed no prey-derived proteins are acquired by *Bd. bacteriovorus* (Barel *et al.*, 2005; Beck *et al.*, 2004; Rayner *et al.*, 1985).

Modern genomic approaches have taught us a great deal about the genetic makeup of these predators. Similarly, with significant advances in imaging technology, one can hope these approaches will allow further understanding of the physiology of these predators.

1.5. Cryoelectron Microscopy

The term cryoelectron microscopy (CEM) is fairly broad and includes many different methods ranging from imaging intact tissue sections to plunge-frozen samples of bacteria, viruses or individual proteins. Specific techniques such as cryoelectron tomography (CET), single particle electron microscopy and electron crystallography are all part of CEM. For the purposes of this study, CEM will be used to refer to plunge-frozen bacterial samples while CET will refer to tomographic reconstructions of CEM samples.

1.5.1. Benefits

The use of CEM has become widespread within the past few years. This is due to the many benefits when compared to conventional transmission electron microscopy (TEM; Milne *et al.*, 2013). When organic matter is visualized by any electron microscopy method there is constant damage being done to the sample by the electrons used to image it. Generally a negative stain is applied, which reduces the electron damage; however, this has inherent problems. The use of cryogenic temperatures alleviates some of these issues. Samples are visualized in their natural state without the use of fixatives, heavy metal stains or cryoprotectants and samples are not adhered to a solid surface. Instead, cells are frozen in vitreous ice within the small holes of specially designed electron microscopy grids and contrast is obtained from the natural elements within the cell. All this allows for the visualization of cell and cellular structures at a near native state.

1.5.2. Disadvantages

Despite its many advantages, CEM does have a few drawbacks. Although somewhat alleviated, CEM samples are still exposed to radiation damage by electrons

(Milne *et al.*, 2013). Advancements in CCD arrays used to capture the images allows for lower electron intensities and shorter exposure times, both decreasing the damage applied to the sample without the need for a heavy metal stain. Because samples are embedded in a thin layer of ice, thicker samples become increasingly more difficult to image. New technologies such as focussed ion-beam milling with gallium can be used to remove thin layers of sample to reduce the thickness. Sample preparation involves cryofixation; therefore, the temperature of the sample must never rise above -155°C , even when transferring the samples from liquid nitrogen storage into the electron microscope.

Specially designed cryoholders have been developed to maintain these temperatures; however, one must work very quickly to avoid ice damage. Imaging samples without the use of heavy metal stains is difficult due to poor signal to noise ratio. Microscopes equipped with an energy filter increase this ratio allowing better image contrast and resolution; however, these are expensive and not essential for obtaining CEM images. Surprisingly, a problem with CEM is that the samples are maintained at cryogenic temperatures. Any moisture in the air will condense onto the sample and form ice contamination, which can obstruct the sample. It is important to work in a humidity-controlled environment and avoid excess moisture.

1.5.3. Vitreous ice

Vitreous ice occurs when water is frozen so quickly that the water molecules cannot rearrange. It acts more similar to a liquid with very high viscosity rather than solid ice. Typically when water is frozen, the ice has a crystal lattice structure. When this is visualized using an electron microscope it scatters all the electrons and an image cannot be obtained. For vitreous ice to be produced, the water in the sample must be

cooled at an extremely fast rate. This is accomplished by plunging a grid with a very thin layer of sample into liquid ethane surrounded by liquid nitrogen. Although liquid nitrogen is colder, its heat transfer properties are poor and therefore liquid ethane is used. Samples are stored in liquid nitrogen and cannot become warmer than -155°C or the vitreous ice can revert to crystalline ice. Of interest, ice frozen slowly has a hexagonal structure while vitreous ice warmed up slightly will take on a cubic structure.

1.5.4. High pressure freeze substitution

As described above, sample thickness plays a crucial role in the ability to use CEM techniques. High pressure freeze substitution (HPFS) is a compromise that allows one to visualize thicker specimens with decreased sample artifacts. For this method, a concentrated sample is placed in a small disk that holds less than $2\ \mu\text{l}$ of liquid. The sample is then exposed to over 2000 bar of pressure (30,000 psi) under cryogenic temperatures (-180°C). This “slam-freezes” the sample. It is then placed into a freeze substitution medium, generally containing 95% acetone with uranyl acetate and osmium tetroxide. Over the course of three days the temperature of the sample is slowly brought up to room temperature. Samples are then infiltrated and embedded similarly to conventional thin-section TEM sample preparation. These sections are heavy metal stained and viewed at room temperature. The artefacts associated with HPFS are far less than the artefacts typically observed with thin-sections. HPFS maintains better structural details and using heavy metal staining, similar to conventional thin sectioning, allows for greater contrast and image resolution. This is currently the gold-standard in visualization techniques of prokaryotic and eukaryotic specimens.

1.5.5. Tomography

Cryoelectron tomography can be used to obtain three dimensional image reconstructions of macromolecular complexes but also can be used for viruses and bacterial samples. Projection images are collected at different tilt-angles relative to the incident electron beam. Fiducial gold is used to align each image at different angles. Computer programs have been developed to aid in this process and create three dimensional maps based on the images. In the case of macromolecular complexes, image averaging can be used to obtain a detailed three dimensional structure. At increasing angles, the thickness of the sample plays a large role in the quality of the images that can be obtained. For thicker samples, high pressure freeze substitution (HPFS) can be used and tomograms can be obtained from sections. Although there are artefacts associated with this technique, it is currently the best option for larger cells.

In the near future, cryoelectron microscopy of vitreous sections will become the gold-standard of CEM. This requires samples to be frozen in vitreous ice before being cryogenically sectioned, not an easy task. There are many challenges with this technique that have to be addressed. Until these problems are fixed, CEM, CET and HPFS are the best methods for imaging bacterial cells at a near-native state.

1.5.6. Cryoelectron microscopy and *Bdellovibrio bacteriovorus*

Conventional electron microscopy has been instrumental to our understanding the various stages of the BALO life cycles. CEM and CET provide the next steps in the evolution of microscopic imaging. Due to the small size of the attack phase cells, *Bd. bacteriovorus* is a model organism to visualize using CEM and CET (Borgnia *et al.*, 2008). It is known that BALOs squeeze into the periplasmic space of the prey and,

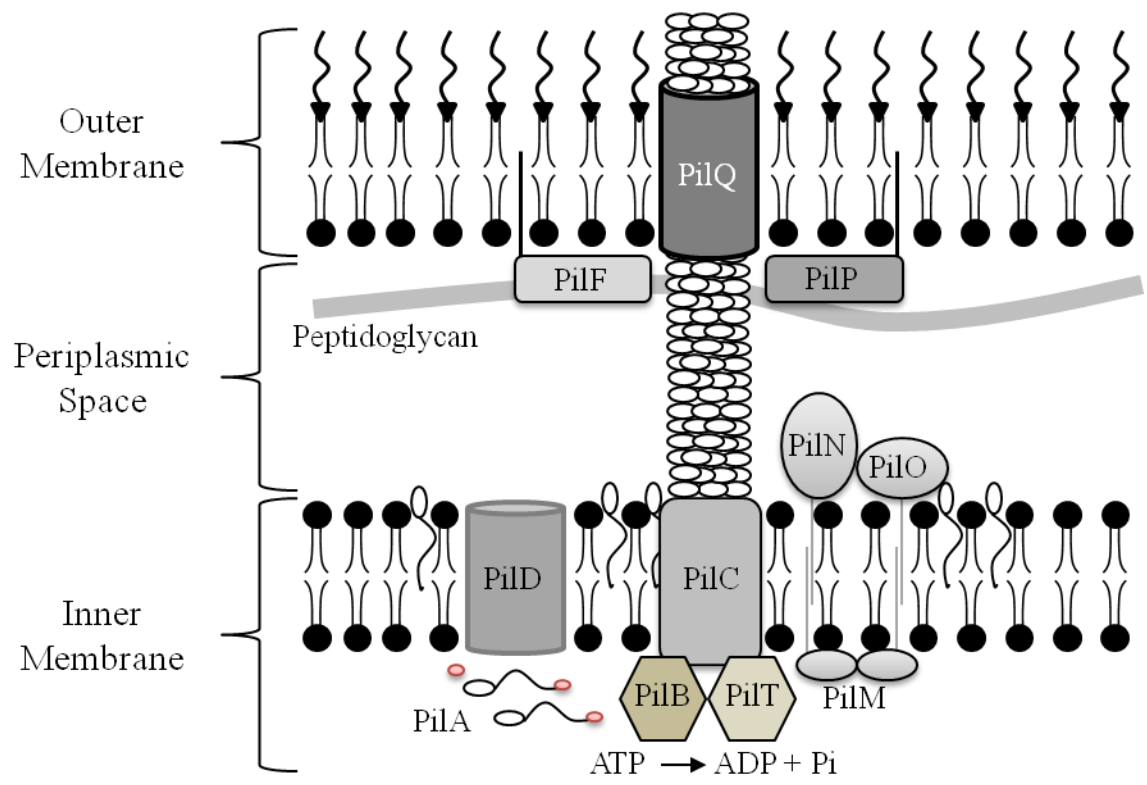
therefore, the cell wall architecture of the predator must remain flexible. Borgnia *et al.* (2008) demonstrated this flexibility showing that *Bd. bacteriovorus* is able to wrap around the edge of a carbon film, an observation one would not expect to see when visualizing cells with typical envelope rigidity. Butan *et al.* (2011) used CEM and CET to show that *Bd. bacteriovorus* has a helical nucleoid organisation that is mediated by MreB. These studies of attack phase cells demonstrate the quality of images one is able to obtain with CEM and CET. It may be possible when imaging predator-prey interactions to observe structures on the BALO surface that may prove essential for attachment and invasion.

1.6. Type IV pili

Type IV pili (TFP) are small (5-8 nm diameter) fibers that can be found on Gram-negative and Gram-positive bacteria, most notably *P. aeruginosa*, *N. gonorrhoeae* and *V. cholerae* (Burrows, 2005). These narrow fibers have a remarkable tensile strength, able to withstand forces in excess of 100 pN per single fiber (Maier *et al.*, 2002). Although the length of the pilus varies, *P. aeruginosa* TFP are on average 5 μm in length (Sherker and Berg, 2001). TFP are important in colonization, establishment of biofilms, uptake of extracellular DNA, phage adsorption and twitching motility. Due to these reasons, TFP have been associated with pathogenicity (Craig *et al.*, 2004). Twitching motility involves successive cycles of extension, adherence and retraction. Therefore, the maintenance of proper function requires many proteins working as a complex. In *P. aeruginosa* there are close to 20 proteins involved in formation, regulation and proper function of the TFP apparatus (Fig. 4). There is a high degree of sequence conservation of the pilins and the

Figure 4. Diagram of the type IV pilus apparatus in *Pseudomonas aeruginosa*.

Prepilin leader sequence of PilA is cleaved (and subsequently methylated) by PilD. Processed PilA is assembled at the PilC base protein by the PilNMO complex and the PilB ATPase. The pilus is extruded through the PilQ outer membrane porin, which is stabilized by PilP (Collins *et al.*, 2001). The fiber is retracted through the action of the PilT ATPase, where the mature pilin is recycled back into the inner membrane.



pilus assembly complex with the components of the type II secretion system, phage assembly proteins and archaeal flagella. Because the nomenclature varies from organism to organism and has not yet been determined for BALOs, the well characterized TFP system in *P. aeruginosa* has been adopted for *Bd. bacteriovorus* and by extension to the other BALOs.

1.6.1. Major pilins

There are two major types of TFP: type IVa and type IVb. This distinction is based on the structure of the major pilin protein, PilA in *P. aeruginosa*. Type IVa pilins have a short 5-6 amino acid leader peptide that gets cleaved, leaving an N-terminal phenylalanine residue. Also, at 150 amino acids, type IVa pilins are shorter in length. Type IVa pili are found on a wide variety of Gram-negative bacteria including *P. aeruginosa*, *Neisseria* spp., *Moraxella bovis* and *Bd. bacteriovorus* (Henrichsen, 1975; Henrichsen, 1983; Meyer *et al.*, 1984). Type IVb pilins have a longer 15-30 amino acid leader peptide and are on average about 40 amino acid residues longer. The N-terminal residue is more variable and can be methionine, leucine or valine. Interestingly, type IVb pilins are generally associated with pathogenic bacteria that colonize the human intestine, such as *V. cholerae*, *Salmonella enterica* serovar Typhi and both enteropathogenic and enterotoxigenic *E. coli* (Shi and Sun, 2002; Zhang *et al.*, 2000).

The overall pilin structure is fairly conserved consisting of an N-terminal alpha-helix and a globular head. The D-region is on one side of the head domain, which is hidden due to pilin-pilin interactions, exposed only on the tip where adherence occurs. Cysteine residues present in the disulfide bonded loop region are responsible for the pilin

interactions (Harvey *et al.*, 2009). These features are of great interest for preventing adherence to implanted medical devices.

As described above, both type IVa and IVb pilins contain an N-terminal leader peptide which is recognized by the PilD pre-pilin peptidase found in the cytoplasmic membrane. PilD binds to the leader peptide and translocates the PilA protein across the cytoplasmic membrane. The leader peptide is cleaved from the protein and the protein is incorporated into the outer leaflet of the cytoplasmic membrane. During this process, the new N-terminal residue is methylated (Burrows, 2005). This process is dependent on a glutamic acid residue at the +5 position of the new peptide. A pool of prepared pilin can be found in the cytoplasmic membrane where it can be readily used for the formation of the pilus fiber.

1.6.2. Minor pilins

Minor pilins are proteins that are less abundant than the major PilA structural subunit but are essential for the formation of a functional pilus. Giltner *et al.* (2010) used immunogold labelling to show the *P. aeruginosa* minor pilins FimU, PilV, PilW, PilX and PilE were incorporated into the growing filament. The stoichiometric ratios of the pilins are essential for function. A stable complex is believed to form when the correct ratios are present. This then allows for binding of the major pilin subunit. Overexpression of PilV, PilW or PilE resulted in cells that had pili but were non-motile. With altered stoichiometric ratios the pili were extended but were unable to retract, possibly due to interfering with efficient depolymerisation of the fiber subunits. Cells in which FimU or PilX are overexpressed exhibited wildtype motility but had a shorter pilus.

1.6.3. Structural pilus proteins

The initial scaffold in the cytoplasmic membrane is formed by two copies of PilC. This baseplate allows for the pilins to be added or removed depending on the motor protein functioning. Mutations within PilC protein can disrupt both elongation and retraction of the fiber indicating a direct interaction between PilC and the motor proteins (Takhar *et al.*, 2013). As the pilins are added to the base, they extend through the PilQ secretin. This multimeric protein forms a gated channel that allows for the pilus to extend through the outer membrane. PilP is a periplasmic lipoprotein that inserts itself in the inner leaflet of the outer membrane, which helps the multimerization of the PilQ secretin and provides structural support.

1.6.4. Extension

There are two major motor proteins located in the cytoplasm that drive extension and retraction of the pilus, PilB and PilT, respectively. PilB is a nucleotide binding protein with ATPase activity that powers the extension of the pilus by polymerizing the mature pilins at the PilC base. The initial steps in this process have only recently been discovered in *Thermus thermophilus* by Karuppiyah *et al.* (2013) and require PilM, PilN and PilO. PilM is a cytoplasmic lipoprotein that anchors itself to the cytoplasmic membrane by binding the PilN protein. Both PilN and PilO proteins contain a cytoplasmic tail, a transmembrane domain and a periplasmic domain. Two PilM proteins bind to the cytoplasmic tails of two PilN proteins. This then allows for two PilO proteins to bind this complex. However, this displaces the PilN periplasmic domains, leaving two separate PilMNO complexes. Each of these complexes is able to bind a single PilA protein, which is located in the outer leaflet of the inner membrane. Now the pilin can be

added to the elongating fiber using the PilB ATPase; however, it is not known how the bound PilAMNO complex would release the PilA subunit to the elongating fiber. Although a similar mechanism may be functioning in *P. aeruginosa* (Ayers *et al.*, 2009), deletion of PilT in a PilNMOP mutant restores 10-15% surface piliation (Takhar *et al.*, 2013). It is not known if the fibers are able to self-assemble in the absence of PilNMOP.

1.6.5. Retraction and twitching motility

Less is known about the mechanistic detail of how the pilins are depolymerized from the elongated pilus. However, what is known is that this energy-dependent process requires the hexameric cytoplasmic PilT ATPase to remove the pilins at the base of the fiber. Once removed, pilins are recycled back into the cytoplasmic stores of mature pilin proteins in the cytoplasmic membrane. This process may be similar to the extension process described above in reverse; however, further work is required. Deletion of the PilT gene in *P. aeruginosa* abolishes retraction; however, TFP are produced. Cells become hyperpiliated and cannot use twitching motility (Burrows, 2005; Kaiser, 2000; Merz *et al.*, 2000). Similar results have been observed in *Synechocystis* (Okamoto and Ohmori, 2002), *Microcystis aeruginosa* (Nakasugi *et al.*, 2007), *Acinetobacter baumannii* (Clemmer *et al.*, 2011), *Myxococcus xanthus* (Wu and Kaiser, 1995; Wu *et al.*, 1997) and *N. gonorrhoeae* (Wolfgang *et al.*, 1998) to name a few examples. In *P. aeruginosa* the increase in TFP of the hyperpiliated mutants increases adherence of the cells and results in an increased ability to form a biofilm (Chiang and Burrows, 2003; O'Toole and Kolter, 1998).

1.6.6 PilT Proteins

The PilT proteins belong to a large group of proteins known as the P-loop NTPase family. Proteins within this family are used to energize type II secretion, type III secretion, type IV secretion and TFP (Mattick, 2002; Peabody *et al.*, 2003). There are three members of the P-loop NTPase family within the TFP apparatus: PilB, PilU and PilT. As stated above, PilT forms a homo-hexamers in the cytoplasm at the base of the TFP apparatus. Functionally, P-loop NTPase proteins are defined by four main domains: Walker A motif, Walker B motif, Asp box and His box. The Walker A motif (GXXXXGK(T/S)) is a highly conserved nucleotide-binding domain that interacts with the phosphate residues of the ATP substrate (Robien *et al.*, 2003). The lysine residue is essential for this interaction (Hanson and Whiteheart, 2005).

The Walker B motif is not as highly conserved but can be condensed to a group of at least four hydrophobic residues ending with aspartic acid and glutamate (hhhhDE). The aspartic acid residue is responsible for coordinating the Mg^{+} required for ATP binding while the glutamate residue is necessary for ATP hydrolysis (Yeo *et al.*, 2000). The Asp box contains three invariant acidic residues while the His box has two invariant His-residues. Site-directed mutagenesis of the third acidic residues of the Asp box or the second His-residues in the His box abolished ATPase activity of PilT *in vitro* (Chiang *et al.*, 2008). Using the crystal structure of the PilT protein in *Aquifex aeolicus* to model these mutations, it was revealed that the Asp and His boxes form a catalytic pocket that surrounds the ligand (Herdendorf *et al.*, 2002). Hydrolysis of ATP is thought to create a structural change in the PilT hexamer, that allows for the release of a single pilin protein

from the fiber, releasing it into the outer leaflet of the inner membrane (Misic *et al.*, 2010).

1.6.7. Regulation

Due to the ability of TFP to adhere so well to both biotic and abiotic surfaces, it does not benefit a cell to always express them. There are many different proteins that regulate different aspects of the TFP system. The simplest regulatory pathway involves a negative feedback loop in which excess PilA negatively regulates the transcription of *pilA*. Bertrand *et al.* (2010) found that the transcription of *pilA* is upregulated in PilA and PilT deletion mutants, suggesting that excess PilA negatively regulates itself.

The Chp chemosensory system has also been shown to regulate TFP. The core signaling components include ChpA, a putative histidine kinase and two CheY-like response regulators encoded by *pilG* and *pilH*. PilG is involved in promoting the extension of TFP, possibly by activating PilB (Darzins, 1993; Bertrand *et al.*, 2010). On the other hand, PilH activates PilT, which increases retraction of the pilus. Of interest, this activation of retraction does not alter *pilA* transcription despite having increased levels of free PilA. Bertrand *et al.* (2010) attribute this to some unknown positive regulation of *pilA* by the PilH protein.

Expression of PilA is also regulated by the alternative sigma factor 54 (σ^{54}) and another two-component system transcribed by *pilS* and *pilR*. PilS is a sensor kinase that autophosphorylates upon an unknown signal. The phosphate is transferred to the response regulator, PilR, which will then bind upstream of *pilA* to activate transcription (Hobbs *et al.*, 1993).

1.7. *Stenotrophomonas maltophilia*

1.7.1. The organism

Stenotrophomonas maltophilia is a γ -proteobacterium first characterized in 1961 as *Pseudomonas maltophilia* (Hugh and Ryschenkow, 1961). The species was later assigned to *Xanthomonas* and finally moved to the genus *Stenotrophomonas* (Palleroni and Bradbury, 1993). Generally regarded as non-pathogenic, *S. maltophilia* can be readily isolated from the rhizosphere of many plant species where it plays a fundamental role in nitrogen and sulphur cycles. *S. maltophilia* has a remarkable ability to adapt to harsh and nutrient-limited environments and can survive in chlorhexidine-cetrimide topical antiseptics (Wishart and Riley, 1976), hand-washing soap (Klausner *et al.*, 1999) and contact lens solution (Furuhata *et al.*, 2010).

1.7.2. Biocontrol agent

Stenotrophomonas spp. have been proposed as biocontrol agents as their addition to the roots of some plants (wheat, tomato, lettuce, sweet pepper, melon, celery and carrot) has been shown to increase growth and influence the development of certain plants (Ryan *et al.*, 2009). Messiha *et al.* (2007) found that *S. maltophilia* was an effective antagonist of *Ralstonia solanacearum*, the causative agent of potato brown rot. The authors attributed this interference to possible antibiotics produced by strains of *S. maltophilia*, although not conclusively demonstrated. Many strains of *S. maltophilia* scavenge iron from siderophores produced by other microorganisms, such as ferrichrome produced by the phytopathogenic fungus *Ustilago maydis* (Ardon *et al.*, 1997). This promotes the growth of *S. maltophilia* while limiting the growth of the pathogen by reducing iron availability. Another study found that *S. maltophilia* C5 produced a

chitinase that was able to suppress summer patch disease in Kentucky bluegrass by activating disease resistance genes within the plant (Kobayashi *et al.*, 2002). These studies show that *S. maltophilia* can influence the growth of plant pathogens by directly killing the competitor with secreted molecules or indirectly by stealing iron or enhancing the plant's response by activating resistance genes.

1.7.3. Intrinsic resistance

1.7.3.1. Bioremediation

Isolates of *S. maltophilia* have been studied as potential agents to help with bioremediation. Many can degrade polycyclic aromatic hydrocarbons, *p*-nitrophenol, 4-chlorophenol, selenium compounds, benzene, toluene and ethylbenzene. Although there is some degree of intraspecies heterogeneity, many strains of *S. maltophilia* have developed tolerance to multiple heavy metals such as cadmium, lead, zinc, cobalt, mercury and silver (Clausen, 2000; Pages *et al.*, 2008). Arsenic and tellurium resistance has also been documented (Bachate *et al.*, 2009; Botes *et al.*, 2007; Ryan *et al.*, 2009). However, the attributes that are attractive to exploit, namely its ability to colonize and survive, have placed *S. maltophilia* under watch as an opportunistic pathogen (Brooke, 2012).

1.7.3.2. Antibiotic resistance

Not surprisingly, *S. maltophilia* is also highly resistant to many clinically relevant antibiotics including trimethoprim-sulfamethoxazole (TMP-SMX), β -lactam antibiotics, macrolides, cephalosporins, fluoroquinolones, aminoglycosides, carbapenems, chloramphenicol, tetracycline and polymyxins. This resistance is largely due to low membrane permeability, efflux pumps and the production of β -lactamases and antibiotic

modifying enzymes. There is a high degree of heterogeneity among individual *S. maltophilia* strains; however, resistance genes are not found within mobile genetic elements, which indicate that these genes were not acquired in the clinical setting but selected for over time. There is intense microbiological activity in the rhizosphere and these genes may give *S. maltophilia* an advantage in this niche.

1.7.3.3. Predation on *Stenotrophomonas maltophilia*

Protozoa in the soil can have a dramatic effect on the bacteria present. *S. maltophilia* R551-3 has a six gene cluster that encodes *reb* genes involved in the production of refractile inclusion bodies (R-bodies). R-bodies are toxic to species of *Paramecium*, a unicellular ciliate protozoan (Heruth *et al.*, 1994). Conceivably, these proteins may protect *S. maltophilia* from grazing by soil protozoans.

S. maltophilia has been used once as a prey cell to isolate BALOs (Lambina *et al.*, 1982) but the plaque-forming predator isolated was identified as *Micavibrio admirandus*, an α -proteobacterium and thus not a δ -proteobacterial BALO. Dashiff *et al.* (2011) tested the ability of *Bd. bacteriovorus* 109J to prey on 6 clinical *S. maltophilia* isolates. Predation was not observed on either planktonic cells or surface-attached lawn cells.

1.7.4. Opportunistic pathogen and involvement in cystic fibrosis

Infection by *S. maltophilia* is almost entirely hospital-associated and confined within immunocompromised patients. Infections have been associated with bacteremia and pneumonia with a high mortality rate (Paez and Costa, 2008). A recent study by Behnia *et al.* (2014) found that the causative agent for 85% of their patients diagnosed with nosocomial pneumonia was due to a Gram-negative culprit. Analysis of their bronchoalveolar lavage and sputum cultures found 40% of patients contained *P.*

aeruginosa, 34% had *S. maltophilia* and 32% had *Acinetobacter baumannii*. These findings are similar to those of many previous studies on respiratory infections (Chawla *et al.*, 2013; Kim *et al.*, 2014; Lakatos *et al.*, 2014; Soubirou *et al.*, 2014; Trandafir *et al.*, 2013).

Although the majority of infections are found within patients who have an indwelling device and are primarily due to biofilms forming on the device, upwards of 25% of cystic fibrosis patients are colonized with *S. maltophilia* (Amin and Waters, 2014; Davies and Rubin, 2007; Samonis *et al.*, 2012; Steinkamp *et al.*, 2005). Surprisingly, however, the presence of *S. maltophilia* in CF patients is controversial as its presence does not always correlate with severity of disease and lung damage (Goss *et al.*, 2002; Goss *et al.*, 2004; Karpati *et al.*, 1994).

1.7.5. Biofilm formation

The classification of *S. maltophilia* as an opportunistic pathogen is almost entirely reliant upon its ability to form biofilms. It possesses many antibiotic resistance genes but does not contain any characterized virulence factors (Brooke, 2012). Its ability to colonize implanted medical devices and persist are partly due to the presence of various pili or fimbriae that allow surface adhesion, auto-aggregation and biofilm formation to occur. The SMF1 fimbriae have been identified in almost all clinical strains of *Stenotrophomonas* (Ryan *et al.*, 2009), showcasing the necessity for surface adherence in a successful infection. Due to the structure of the biofilm and dormancy of many cells within it, many antibiotics are either ineffective or cannot penetrate deep enough to be effective.

1.7.6. Current effective therapeutics

Unfortunately, if a therapeutic agent is effective against *S. maltophilia*, it is not long before resistance is acquired. Although resistance has been shown, currently the best therapy involves the use of trimethoprim-sulphamethoxazole (TMP-SMX) (Brooke, 2012). Trimethoprim is a dihydrofolate reductase inhibitor that prevents the reduction of dihydrofolic acid to tetrahydrofolic acid, which is an essential precursor to thymidine synthesis. Sulfamethoxazole inhibits dihydropteroate synthetase, an enzyme upstream in the same pathway. Administered together, TMP-SMX act synergistically. Alternatively ticarcillin-clavulanate (β -lactam and β -lactamase inhibitor), gatifloxacin (4th generation fluoroquinolone), moxifloxacin (4th generation fluoroquinolone), minocycline (tetracycline) and tigecycline (glycylcycline) can be administered but the duration for which these will remain effective before resistance is acquired is unknown.

1.8 Aims and objectives

This thesis has two main aims:

- 1) To further our understanding of the attachment and invasion of *Bdellovibrio* and like organisms.
- 2) To assess the potential use of *Bd. exovorius* as an antimicrobial agent.

To address these aims, there are three major objectives:

- (i) To compare and contrast the periplasmic and epibiotic life cycles.
- (ii) To identify the role of type IV pili during predation by *Bd. bacteriovorus*
- (iii) To explore the use of *Bd. exovorius* to disrupt biofilms of *Stenotrophomonas maltophilia* in the presence of antibiotics.

Objective 1.

Since the isolation of the first BALO in 1962 and until 1989, all predators isolated from environmental samples displayed the typical periplasmic life cycle. With the isolation and characterization of the epibiotic predator, *Bd. exovorus* JSS^T, many interesting questions were raised. In collaboration with Dr. E. Jurkevitch at the Hebrew University of Jerusalem, the genome sequence of *Bd. exovorus* was obtained. A comparative genomics approach was undertaken to examine similarities and differences between two epibiotic predators, *Bd. exovorus* and *M. aeruginosavorus*, and two periplasmic predators, *Bd. bacteriovorus* and *Bx. marinus*. From this, genes required for the invasion process could be distinguished from those required for predation to occur.

In the Koval laboratory, it is important to look at cultures and not completely rely on bioinformatic analyses to understand structure and function. To do this, a collaboration with Dr. C. Khursigara at the University of Guelph was established to use cryoelectron microscopy and tomography to look at the epibiotic and periplasmic life cycles. These methods have previously been used to look at attack phase cells of *Bd. bacteriovorus*; however, other stages of the life cycle were never examined. I have used cryoelectron microscopy of vitreous frozen samples to look at both life cycles, the gold standard in imaging techniques. I have taken this one step further and used high pressure freeze substitution and tomography to obtain three-dimensional images of different stages of the periplasmic and epibiotic life cycles. These studies are presented in Chapter 2.

Objective 2

The attack phase pole of *Bd. bacteriovorus* has always been an area of interest to understand how these predators recognize and invade the prey cell. Evans *et al.* (2007)

and Mahmoud and Koval (2010) established that polar fibers seen on the attack phase cells were TFP and essential for predation to occur. However, the role that TFP play has not been established. To determine the function of TFP single markerless in-frame deletion mutants of the *pilT1* and *pilT2* genes and a double deletion mutant were created. The ability of these mutants to prey on *E. coli* in liquid cocultures, on agar plates and within a biofilm was analysed. Transmission electron microscopy and immunofluorescent microscopy was used to analyse variations in surface piliation among the *pilT* mutants and Western blot analysis to identify differences in total cellular TFP levels. These studies are presented in Chapter 3.

Objective 3

Stenotrophomonas maltophilia is an emerging multi-drug resistant opportunistic pathogen. Current therapies are quickly becoming obsolete and new methods of controlling these infections must be studied. In collaboration with Dr. J. Brooke from DePaul University, a BALO was isolated from an enrichment culture which could use *S. maltophilia* as a prey cell. The prey range was tested against a bank of 19 clinical *S. maltophilia* isolates. The ability for each *S. maltophilia* isolate to form a biofilm was analyzed along with the ability of the predator to prey on these biofilms. This was repeated in the presence of kanamycin and ciprofloxacin. This study is presented in Chapter 4.

1.9. References

Abram, D. and Davis, B.K. (1970) Structural properties and features of parasitic *Bdellovibrio bacteriovorus*. *J Bacteriol* **104**: 948-965.

Abram, D., Melo, J.C. and Chou, D. (1974) Penetration of *Bdellovibrio bacteriovorus* into host cells. *J Bacteriol* **118**: 663-680.

Afinogenova, A.V., Romai Penabad, S., Konovalona, S.M., Churkrina, L.G. and Lambina, V.A. (1981) Comparative characteristics of strains of *Bdellovibrio* isolated from river water and sewage. *Mikrobiologia* **50**: 378-385.

Amin, R. and Waters, V. (2014) Antibiotic treatment for *Stenotrophomonas maltophilia* in people with cystic fibrosis. *Cochrane Database Syst Rev* **4**: CD009249.

Ardon, O., Weizman, H., Libman, J., Shanzer, A., Chen, Y. and Hadar, Y. (1997) Iron uptake in *Ustilago maydis*: studies with fluorescent ferrichrome analogues. *Microbiology* **143**: 3625-3631.

Ayers, M., Sampaleanu, L.M., Tammam, S., Koo, J., Harvey, H., Howell, P.L. and Burrows, L.L. (2009) PilM/N/O/P proteins form an inner membrane complex that affects the stability of the *Pseudomonas aeruginosa* type IV pilus secretin. *J Mol Biol* **394**: 128-142.

Bachate, S.P., Cavalca, L. and Andreoni, V. (2009) Arsenic-resistant bacteria isolated from agricultural soils of Bangladesh and characterization of arsenate-reducing strains. *J Appl Microbiol* **107**: 145-156.

Baer, M.L., Ravel, J., Chun, J., Hill, R.T. and Williams, H.N. (2000) A proposal for the reclassification of *Bdellovibrio stolpii* and *Bdellovibrio starrii* into a new genus, *Bacteriovorax* gen. nov. as *Bacteriovorax stolpii* comb. nov. and *Bacteriovorax starrii* comb. nov., respectively. *Int J Syst Evol Microbiol* **1**: 219-224.

Barabote, R.D., Rendulic, S., Schuster, S.C. and Saier Jr., M.H. (2007) Comprehensive analysis of transport proteins encoded within the genome of *Bdellovibrio bacteriovorus*. *Genomics* **90**: 424-446.

Barel, G. and Jurkevitch, E. (2001) Analysis of phenotypic diversity among host-independent mutants of *Bdellovibrio bacteriovorus* 109J. *Arch Microbiol* **176**: 211-216.

Barel, G., Sirota, A., Volpin, H. and Jurkevitch, E. (2005) Fate of predator and prey proteins during growth of *Bdellovibrio bacteriovorus* on *Escherichia coli* and *Pseudomonas syringae* prey. *J Bacteriol* **187**: 329-335.

Beck, S., Schwudke, D., Strauch, E., Appel, B. and Linscheid, M. (2004) *Bdellovibrio bacteriovorus* strains produce a novel major outer membrane protein during predacious growth in the periplasm of prey bacteria. *J Bacteriol* **186**: 2766-2773.

Behnia, M., Logan, S.C., Fallen, L. and Catalano, P. (2014) Nosocomial and ventilator-associated pneumonia in a community hospital intensive care unit: a retrospective review and analysis. *BMC Res Notes* **7**: 232.

- Berleman, J.E. and Kirby, J.R. (2009) Deciphering the hunting strategy of a bacterial wolfpack. *FEMS Microbiol Rev* **33**: 942-957.
- Bertrand, J.J., West, J.T. and Engel, J.N. (2010) Genetic analysis of the regulation of type IV pilus function by the Chp chemosensory system of *Pseudomonas aeruginosa*. *J Bacteriol* **192**: 994-1010.
- Borgnia, M.J., Subramanian, S. and Milne, J.L. (2008) Three-dimensional imaging of the highly bent architecture of *Bdellovibrio bacteriovorus* by using cryo-electron tomography. *J Bacteriol* **190**: 2588-2596.
- Botes, E., Van Heerden, E. and Litthauer, D. (2007) Hyper-resistance to arsenic in bacteria isolated from an antimony mine in South Africa. *South African Journal of Science*. **103**: 279-281.
- Bradley, D.E. (1980) A function of *Pseudomonas aeruginosa* PAO polar pili: twitching motility. *Can J Microbiol* **26**: 146-154.
- Brooke, J.S. (2012) *Stenotrophomonas maltophilia*: an emerging global opportunistic pathogen. *Clin Microbiol Rev* **25**: 2-41.
- Burger, A., Drews, G. and Ladwig, R. (1968) Host range and infection cycle of a newly isolated strain of *Bdellovibrio bacteriovorus*. *Arch Microbiol* **61**: 261-279.
- Burnham, J.C., Hashimoto, T. and Conti, S.F. (1968) Electron microscope observations on the penetration of *Bdellovibrio bacteriovorus* into gram-negative bacterial hosts. *J Bacteriol* **96**: 1366-1381.
- Burrows, L.L. (2005) Weapons of mass retraction. *Mol Microbiol* **57**: 878-888.
- Butan, C., Hartnell, L.M., Fenton, A.K., Bliss, D. and Sockett, R.E. (2011) Spiral architecture of the nucleoid in *Bdellovibrio bacteriovorus*. *J Bacteriol* **193**: 1341-1350.
- Casida, L.E. (1980) Bacterial predators of *Micrococcus luteus* in soil. *Appl Environ Microbiol* **39**: 1035-1041.
- Casida, L.E. (1983) Interaction of *Agromyces ramosus* with other bacteria in soil. *Appl Environ Microbiol* **46**: 881-888.
- Chang, C.Y., Hopley, L., Till, R., Capeness, M., Kanna, M., Burt, W., Jagtap, P., Aizawa, S.I. and Sockett, R.E. (2011) The *Bdellovibrio bacteriovorus* twin-arginine transport system has roles in predatory and prey-independent growth. *Microbiology* **157**: 3079-3093.
- Chanyi, R.M., Ward, C., Pechey, A. and Koval, S.F. (2013) To invade or not to invade: two approaches to a prokaryotic predatory life cycle. *Can J Microbiol* **59**: 273-279.

Chawla, K., Vishwanath, S. and Munim, F.C. (2013) Nonfermenting Gram-negative bacilli other than *Pseudomonas aeruginosa* and *Acinetobacter* spp. causing respiratory tract infections in a tertiary care center. *J Glob Infect Dis* **5**: 144-148.

Chiang, P. and Burrows, L.L. (2003) Biofilm formation by hyperpiliated mutants of *Pseudomonas aeruginosa*. *J Bacteriol* **185**: 2374-2378.

Chiang, P., Sampaleanu, L.M., Ayers, M., Pahuta, M., Howell, P.L. and Burrows, L.L. (2008) Functional role of conserved residues in the characteristic secretion NTPase motifs of the *Pseudomonas aeruginosa* type IV pilus motor proteins PilB, PilT and PilU. *Microbiology* **154**: 114-126.

Clausen, C.A. (2000) Isolating metal-tolerant bacteria capable of removing copper, chromium and arsenic from treated wood. *Waste Manage Res* **18**: 264-268.

Clemmer, K.M., Bonomo, R.A. and Rather, P.N. (2011) Genetic analysis of surface motility in *Acinetobacter baumannii*. *Microbiology* **157**: 2534-2544.

Collins, R.F., Davidsen, L., Derrick, J.P., Ford, R.C. and Tonjum, T. (2001) Analysis of the PilQ secretin from *Neisseria meningitidis* by transmission electron microscopy reveals a dodecameric quaternary structure. *J Bacteriol* **183**: 3825-3832.

Cotter, T.W. and Thomashow, M.F. (1992) Identification of a *Bdellovibrio bacteriovorus* genetic locus, *hit*, associated with the host-independent phenotype. *J Bacteriol* **174**: 6018-6024.

Craig, L., Pique, M.E. and Tainer, J.A. (2004) Type IV pilus structure and bacterial pathogenicity. *Nat Rev Microbiol* **2**: 363-378.

Darzins, A. (1993) The *pilG* gene product, required for *Pseudomonas aeruginosa* pilus production and twitching motility, is homologous to the enteric, single-domain response regulator CheY. *J Bacteriol* **175**: 5934-5944.

Dashiff, A. and Kadouri, D.E. (2011) Predation of oral pathogens by *Bdellovibrio bacteriovorus* 109J. *Mol Oral Microbiol* **26**: 19-34.

Dashiff, A., Junka, R.A., Libera, M. and Kadouri, D.E. (2011) Predation of human pathogens by the predatory bacteria *Micavibrio aeruginosavorus* and *Bdellovibrio bacteriovorus*. *J Appl Microbiol* **110**: 431-444.

Davidov, Y. and Jurkevitch, E. (2004) Diversity and evolution of *Bdellovibrio*-and-like-organisms (BALOs), reclassification of *Bacteriovorax starrii* as *Peredibacter starrii* gen. nov., comb. nov., and description of the *Bacteriovorax-Peredibacter* clade as *Bacteriovoracaceae* fam. nov. *Int J Syst Evol Microbiol* **54**: 1439-1452.

Davies, J.C. and Rubin, B.K. (2007) Emerging and unusual Gram-negative infections in cystic fibrosis. *Semin Respir Crit Care Med* **28**: 312-321.

- Diedrich, D.L., Portnoy, C.A. and Conti, S.F. (1983) *Bdellovibrio* possesses a prey-derived OmpF protein in its outer membrane. *Curr Microbiol* **8**: 51-56.
- Diedrich, D.L., Duran, C.P. and Conti, S.F. (1984) Acquisition of *Escherichia coli* outer membrane proteins by *Bdellovibrio* sp. strain 109D. *J Bacteriol* **159**: 329-334.
- Eksztejn, M. and Varon, M. (1977) Elongation and cell division in *Bdellovibrio bacteriovorus*. *Arch Microbiol* **114**: 175-181.
- Evans, K.J., Lambert, C. and Sockett, R.E. (2007) Predation by *Bdellovibrio bacteriovorus* HD100 requires type IV pili. *J Bacteriol* **189**: 4850-4859.
- Fenton, A.K., Kanna, M., Woods, R.D., Aizawa, S.I. and Sockett, R.E. (2010) Shadowing the actions of a predator: backlit fluorescent microscopy reveals synchronous nonbinary septation of predatory *Bdellovibrio* inside prey and exit through discrete bdelloplast pores. *J Bacteriol* **192**: 6329-6335.
- Flannagan, R.S., Valvano, M.A. and Koval, S.F. (2004) Downregulation of the *motA* gene delays the escape of the obligate predator *Bdellovibrio bacteriovorus* 109J from bdelloplasts of bacterial prey cells. *Microbiology* **150**: 649-656.
- Fratamico, P.M. and Whiting, R.C. (1995) Ability of *Bdellovibrio bacteriovorus* 109J to lyse gram-negative food-borne pathogenic and spoilage bacteria. *J Food Protection* **58**: 160-164.
- Fratamico, P.M. and Cooke, P.H. (1996) Isolation of bdellovibrios that prey on *Escherichia coli* O157:H7 and *Salmonella* species and application for removal of prey from stainless steel surfaces. *J Food Safety* **16**: 161-173.
- Fry, J. and Staples, D.G. (1974) The occurrence and role of *Bdellovibrio bacteriovorus* in a polluted river. *Water Res* **8**: 1029-1035.
- Fry, J. and Staples, D.G. (1976) Distribution of *Bdellovibrio bacteriovorus* in sewage works, river, water and sediments. *Appl Environ Microbiol* **31**: 469-474.
- Furuhata, K., Ishizaki, N., Kawakami, Y. and Fukuyama, M. (2010) Bacterial contamination of stock solutions in storage cases for contact lens, and the disinfectant-resistance of isolates. *Biocontrol Sci* **15**: 81-85.
- Giltner, C.L., Habash, M. and Burrows, L.L. (2010) *Pseudomonas aeruginosa* pilins are incorporated into type IV pili. *J Mol Biol* **398**: 444-461.
- Gloor, L., Klubek, B. and Seidler, R.J. (1974) Molecular heterogeneity of the bdellovibrios: metallo and serine proteases unique to each species. *Arch Microbiol* **95**: 45-56.

Goss, C.H., Otto, K., Aitken, M.L. and Rubinfeld, G.D. (2002) Detecting *Stenotrophomonas maltophilia* does not reduce survival of patients with cystic fibrosis. *Am J Respir Crit Care Med* **166**: 356-361.

Goss, C.H., Mayer-Hamblett, N., Aitken, M.L., Rubinfeld, G.D. and Ramsey, B.W. (2004) Association between *Stenotrophomonas maltophilia* and lung function in cystic fibrosis. *Thorax* **59**: 955-959.

Gray, K.M. and Ruby, E.G. (1991) Intercellular signalling in the *Bdellovibrio* developmental growth cycle. In: Microbial cell-cell interactions. Dworkin, M., ed. American Society for Microbiology pp. 333-366.

Guerrero, R., Pedros-Alio, C., Esteve, I., Mas, J., Chase, D. and Margulis, L. (1986) Predatory prokaryotes, predation and primary consumption evolved in bacteria. *Proc Natl Acad Sci USA* **83**: 2138-2142.

Hanson, P.I. and Whiteheart, S.W. (2005) AAA+ proteins: have engine, will work. *Nat Rev Mol Cell Biol* **6**: 519-529.

Harvey, H., Habash, M., Aidoo, F. and Burrows, L.L. (2009) Single-residue changes in the C-terminal disulfide-bonded loop of the *Pseudomonas aeruginosa* type IV pilin influence pilus assembly and twitching motility. *J Bacteriol* **191**: 6513-6524.

Henrichsen, J. (1975) The occurrence of twitching motility among gram-negative bacteria. *Acta Pathol Microbiol Scand Sect B* **83**: 171-178.

Henrichsen, J. (1983) Twitching motility. *Annu Rev Microbiol* **37**: 81-93.

Herdendorf, T.J., McCaslin, D.R. and Forest, K.T. (2002) *Aquifex aeolicus* PilT, homologue of a surface motility protein, is a thermostable oligomeric NTPase. *J Bacteriol* **184**: 6465-6471.

Heruth, D.P., Pond, F.R., Dilts, J.A. and Quackenbush, R.L. (1994) Characterization of genetic determinants for R body synthesis and assembly in *Caedibacter taeniospiralis* 47 and 116. *J Bacteriol* **176**: 3559-3567.

Hespell, R.B., Miozzari, G.F. and Rittenberg, S.C. (1975) Ribonucleic acid destruction and synthesis during intraperiplasmic growth of *Bdellovibrio bacteriovorus*. *J Bacteriol* **123**: 481-491.

Hobbs, M., Collie, E.S., Free, P.D., Livingston, S.P. and Mattick, J.S. (1993) PilS and PilR, a two-component transcriptional regulatory system controlling expression of type IV fimbriae in *Pseudomonas aeruginosa*. *Mol Microbiol* **7**: 669-682.

- Hobley, L., Lerner, T.R., Williams, L.E., Lambert, C., Till, R., Milner, D.S., Basford, S.M., Capeness, M.J., Fenton, A.K., Atterbury, R.J., Harris, M.A. and Sockett, R.E. (2012) 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. *BMC Genomics* **13**: 670.
- Hoeniger, J., Ladwig, R. and Moor, H. (1972) The fine structure of resting bodies of *Bdellovibrio* sp. strain W developed in *Rhodospirillum rubrum*. *Can J Microbiol* **18**: 87-92.
- Hong, C.S., Shitashiro, M., Kuroda, A., Ikeda, T., Takiguchi, N., Ohtake, H. and Kato, J. (2004) Chemotaxis proteins and transducers for aerotaxis in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **231**: 247-252.
- Hugh, R. and Ryschenkow, E. (1961) *Pseudomonas maltophilia*, an alcaligenes-like species. *J Gen Microbiol* **26**: 123-132.
- Iebba, V., Santangelo, F., Totino, V., Nicoletti, M., Gagliardi, A., De Biase, R.V., Cucchiara, S., Nencioni, L., Conte, M.P. and Schippa, S. (2013) Higher prevalence and abundance of *Bdellovibrio bacteriovorus* in the human gut of healthy subjects. *PLoS One* **8**: e61608.
- Jurkevitch, E. (2006) The Genus *Bdellovibrio*, In *The Prokaryotes: a Handbook on the Biology of Bacteria*, 3rd edn, vol. 7, pp. 12–30. (Ed.) M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrandt. New York: Springer.
- Jurkevitch, E., Minz, D., Ramati, B. and Barel, G. (2000) Prey range characterization, ribotyping and diversity of soil and rhizosphere *Bdellovibrio* spp. isolated on phytopathogenic bacteria. *Appl Environ Microbiol* **66**: 2365-2371.
- Kadouri, D.E. and Tran, A. (2013) Measurement of predation and biofilm formation under different ambient oxygen conditions using a simple gasbag-based system. *Appl Environ Microbiol* **79**: 5264-5271.
- Kadouri, D.E., To, K., Shanks, R.M. and Doi, Y. (2013) Predatory bacteria: a potential ally against multidrug-resistant Gram-negative pathogens. *PLoS One* **8**: e63397.
- Kaiser, D. (2000) Bacterial motility: How do pili pull? *Curr Biol* **10**: 777-780.
- Karpati, F., Malmborg, A.S., Alfredsson, H., Hjelte, L. and Strandvik, B. (1994) Bacterial colonisation with *Xanthomonas maltophilia*—a retrospective study in a cystic fibrosis patient population. *Infection* **22**: 258-263.
- Karunker, I., Rotem, O., Dori-Bachash, M., Jurkevitch, E. and Sorek, R. (2013) A global transcriptional switch between the attack and growth forms of *Bdellovibrio bacteriovorus*. *PLoS One* **8**: e61850.

Karuppiah, V., Collins, R.F., Thistlethwaite, A., Gao, Y. and Derrick, J.P. (2013) Structure and assembly of an inner membrane platform for initiation of type IV pilus biogenesis. *Proc Natl Acad Sci USA* **110**: e4638-4647.

Kelley, J.I. and Williams, H.N. (1992) Bdellovibrios in *Callinectes sapidus*, the Blue Crab. *Appl Environ Microbiol* **58**: 1408-1410.

Kelley, J., Turng, B., Williams, H. and Baer, M. (1997) Effects of temperature, salinity and substrate on the colonization of surfaces *in situ* by aquatic bdellovibrios. *Appl Environ Microbiol* **63**: 84-90.

Kessel, M. and Shilo, M. (1976) Relationship of *Bdellovibrio* elongation and fission to host cell size. *J Bacteriol* **128**: 477-480.

Kim, T., Chong, Y.P., Park, S.Y., Jeon, M.H., Choo, E.J., Chung, J.W., Lee, H.K., Moon, C., Kim, D.M., Peck, K.R. and Kim, Y.S. (2014) Risk factors for hospital-acquired pneumonia caused by carbapenem-resistant Gram-negative bacteria in critically ill patients: a multicenter study in Korea. *Diagn Microbiol Infect Dis* **78**: 457-461.

Klausner, J.D., Zukerman, C., Limaye, A.P. and Corey, L. (1999) Outbreak of *Stenotrophomonas maltophilia* bacteremia among patients undergoing bone marrow transplantation: association with faulty replacement of hand washing soap. *Infect Control Hosp Epidemiol* **20**: 756-758.

Kobayashi, D.Y., Reedy, R.M., Bick, J. and Oudemans, P.V. (2002) Characterization of a chitinase gene from *Stenotrophomonas maltophilia* strain 34S1 and its involvement in biological control. *Appl Environ Microbiol* **68**: 1047-1054.

Koval, S.F. (2007) The search for hunters: culture-dependent and -independent methods for analysis of *Bdellovibrio* and like organisms. In: *Predatory prokaryotes – Biology, ecology and evolution*. Jurkevitch, E. (ed.) Springer Microbiology Monographs. pp. 191-211.

Koval, S.F. and Hynes, S.H. (1991) Effect of paracrystalline protein surface layers on predation by *Bdellovibrio bacteriovorus*. *J Bacteriol* **173**: 2244-2249.

Koval, S.F. and Bayer, M.E. (1997) Bacterial capsules: no barrier against *Bdellovibrio*. *Microbiology* **143**: 749-753.

Koval, S.F., Hynes, S.H., Flannagan, R.S., Pasternak, Z., Davidov, Y., and Jurkevitch, E. (2013) *Bdellovibrio exovorus* sp. nov., a novel predator of *Caulobacter crescentus*. *IJSEM* **63**: 146-151.

- Kuenen, J.G. and Rittenberg, S.C. (1975) Incorporation of long-chain fatty acids of the substrate organism by *Bdellovibrio bacteriovorus* during intraperiplasmic growth. *J Bacteriol* **121**: 1145-1157.
- Lakatos, B., Jakopp, B., Widmer, A., Frei, R., Pargger, H., Elzi, L. and Battegay, M. (2014) Evaluation of treatment outcomes for *Stenotrophomonas maltophilia* bacteraemia. *Infection* [E-pub ahead of print].
- LaMarre, A.G., Straley, S.C. and Conti, S.F. (1977) Chemotaxis toward amino acids by *Bdellovibrio bacteriovorus*. *J Bacteriol* **131**: 201-207.
- Lambert, C. and Sockett, R.E. (2013) Nucleases in *Bdellovibrio bacteriovorus* contribute towards efficient self-biofilm formation and eradication of preformed prey biofilms. *FEMS Microbiol Lett* **340**: 109-116.
- Lambert, C., Smith, M.C.M. and Sockett, R.E. (2003) A novel assay to monitor predator-prey interactions for *Bdellovibrio bacteriovorus* 109J reveals a role for methyl-accepting chemotaxis proteins in predation. *Environ Microbiol* **5**: 127-132.
- Lambert, C., Evans, K.J., Till, R., Hobley, L., Capeness, M., Rendulic, S., Schuster, S.C., Aizawa, S. and Sockett, R.E. (2006) Characterizing the flagellar filament and the role of motility in bacterial prey-penetration by *Bdellovibrio bacteriovorus*. *Mol Microbiol* **60**: 274-286.
- Lambert, C., Chang, C.Y., Capeness, M.J. and Sockett, R.E. (2010) The first bite – Profiling the predatosome in the bacterial pathogen *Bdellovibrio*. *PLoS One* **5**: e8599.
- Lambert, C., Fenton, A.K., Hobley, L. and Sockett, R.E. (2011) Predatory *Bdellovibrio* bacteria use gliding motility to scout for prey on surfaces. *J Bacteriol* **193**: 139-141.
- Lambina, V.A., Afinogenova, A.V., Romai Penabad, S., Konovalova, S.M. and Pushkareva, A.P. (1982) *Micavibrio admirandus* gen. et sp. nov. *Mikrobiologiya* **51**: 114-117.
- Lerner, T.R., Lovering, A.L., Bui, N.K., Uchida, K., Aizawa, S.I., Vollmer, W. and Sockett, R.E. (2012) Specialized peptidoglycan hydrolases sculpt the intra-bacterial niche of predatory *Bdellovibrio* and increase population fitness. *PLoS Pathog* **8**: e1002524.
- Mahmoud, K.K. and Koval, S.F. (2010) Characterization of type IV pili in the life cycle of the predator bacterium *Bdellovibrio*. *Microbiology* **156**: 1040-1051.
- Maier, B., Potter, L., So, M., Seifert, H.S. and Sheetz, M.P. (2002) Single pilus motor forces exceed 100 pN. *Proc Natl Acad Sci USA* **99**: 16012-16017.
- Mattick, J.S. (2002) Type IV pili and twitching motility. *Ann Rev Microbiol* **56**: 289-314.

- Messiha, N.A.S., van Diepeningen, A.D., Farag, N.S., Abdallah, S.A., Janse, J.D. and van Bruggen, A.H.C. (2007) *Stenotrophomonas maltophilia*: a new potential biocontrol agent of *Ralstonia solanacearum*, causal agent of potato brown rot. *Eur J Plant Pathol* **118**: 211-225.
- Merz, A.J., So, M. and Sheetz, M.P. (2000) Pilus retraction powers bacterial twitching motility. *Nature* **407**: 98-102.
- Meyer, T.F., Billyard, E., Haas, R., Storzbach, S. and So, M. (1984) Pilus genes of *Neisseria gonorrhoeae*: chromosomal organization and DNA sequence. *Proc Natl Acad Sci USA* **81**: 6110-6114.
- Milne, J.L., Borgnia, M.J., Bartesaghi, A., Tran, E.E., Earl, L.A., Schauder, D.M., Lenqyel, J., Pierson, J., Patwardhan, A. and Subramanian, S. (2013) Cryo-electron microscopy—a primer for the non-microscopist. *FEBS J* **280**: 28-45.
- Misic, A.M., Satyshur, K.A. and Forest, K.T. (2010) *P. aeruginosa* PilT structures with and without nucleotide reveal a dynamic type IV pilus retraction motor. *J Mol Biol* **400**: 1011-1021.
- Nakasugi, K., Alexova, R., Svenson, C.J. and Neilan, B.A. (2007) Functional analysis of PilT from the toxic cyanobacterium *Microcystis aeruginosa* PCC 7806. *J Bacteriol* **189**: 1689-1697.
- Okamoto, S. and Ohmori, M. (2002) The cyanobacterial PilT protein responsible for cell motility and transformation hydrolyzes ATP. *Plant Cell Physiol* **43**: 1127-1136.
- O'Toole, G.A. and Kolter, R. (1998) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol* **30**: 295-304.
- Paez, J.I. and Costa, S.F. (2008) Risk factors associated with mortality of infections caused by *Stenotrophomonas maltophilia*: a systematic review. *J Hosp Infect* **70**: 101-108.
- Pages, D., Rose, J., Conrod, S., Cuine, S., Carrier, P., Heulin, T. and Achouak, W. (2008) Heavy metal tolerance in *Stenotrophomonas maltophilia*. *PLoS One* **3**: e1539.
- Palleroni, N.J. and Bradbury, J.F. (1993) *Stenotrophomonas*, a new bacterial genus for *Xanthomonas maltophilia* (Hugh 1980) Swings *et al.*, 1983. *Int J Syst Bacteriol* **43**: 606-609.
- Pasternak, Z., Njagi, M., Shani, Y., Chanyi, R., Rotem, O., Lurie-Weinberger, M.N., Koval, S., Pietrokovski, S., Gophna, U. and Jurkevitch, E. (2014) In and out: an analysis of epibiotic vs periplasmic bacterial predators. *ISME J* **8**: 625-635.

- Pan, C.L., Hsu, Y.L., Tsai, G.J., Kuo, H.J., Chang, C.M., Wang, F.J. and Wu, C.S. (1997) Isolation and identification of *Bdellovibrio* from coastal areas of Taiwan. *Fish Sci* **63**: 52-59.
- Peabody, C.R., Chung, Y.J., Yen, M.R., Vidal-Ingigliardi, D., Pugsley, A.P. and Saier, M.H. (2003) Type II protein secretion and its relationship to bacterial type IV pili and archaeal flagella. *Microbiology* **149**: 3051-3072.
- Piñeiro, S.A., Sahaniuk, G.E., Romberg, E. and Williams, H. (2004) Predation pattern and phylogenetic analysis of *Bdellovibrionaceae* from the Great Salt Lake, Utah. *Curr Microbiol* **48**: 113-117.
- Rayner, J.R., Cover, W.H., Martinez, R.J. and Rittenberg, S.C. (1985) *Bdellovibrio bacteriovorus* synthesizes an OmpF-like outer membrane protein during both axenic and intraperiplasmic growth. *J Bacteriol* **163**: 595-599.
- Rebbapragada, A., Johnson, M.S., Harding, G.P., Zuccarelli, A.J., Fletcher, H.M., Zhulin, I.B. and Taylor, B.L. (1997) The Aer protein and the serine chemoreceptor Tsr independently sense intracellular energy levels and transduce oxygen, redox, and energy signals for *Escherichia coli* behavior. *Proc Natl Acad Sci USA* **94**: 10541-10546.
- Rendulic, S., Jagtap, P., Rosinus, A., Eppinger, M., Baar, C., Lanz, C., Keller, H., Lambert, C., Evans, K.J., Goesmann, A., Meyer, F., Sockett, R.E. and Schuster, S.C. (2004) A predator unmasked: Life cycle of *Bdellovibrio bacteriovorus* from a genomic perspective. *Science* **303**: 689-692.
- Richardson, I.R. (1990) The incidence of *Bdellovibrio* spp. in man-made water systems: coexistence with legionellas. *J Appl Bacteriol* **53**: 134-140.
- Rittenberg, S.C. and Langley, D. (1975) Utilization of nucleoside monophosphates per se for intraperiplasmic growth of *Bdellovibrio bacteriovorus*. *J Bacteriol* **121**: 1137-1144.
- Rittenberg, S.C. and Hespell, R.B. (1975) Energy efficiency of intraperiplasmic growth of *Bdellovibrio bacteriovorus*. *J Bacteriol* **121**: 1158-1165.
- Robien, M.A., Krumm, B.E., Sandkvist, M. and Hol, W.G. (2003) Crystal structure of the extracellular protein secretion NTPase EpsE of *Vibrio cholerae*. *J Mol Biol* **333**: 657-674.
- Ruby, E. G. (1992) The Genus *Bdellovibrio*. In *The Prokaryotes*, 2nd edn, pp. 3400–3415. (Ed.) A. Balows, H. G. Truper, M. Dworkin, W. Harder & K.-H. Scheifer. New York: Springer Verlag.
- Ruby, E.G. and McCabe, J.B. (1986) An ATP transport system in the intracellular bacterium, *Bdellovibrio bacteriovorus* 109J. *J Bacteriol* **167**: 1066-1070.

Ruby, E.G., McCabe, J.B. and Barke, J.I. (1985) Uptake of intact nucleoside monophosphates by *Bdellovibrio bacteriovorus* 109J. *J Bacteriol* **163**: 1087-1094.

Ryan, R.P., Monchy, S., Cardinale, M., Taghavi, S., Crossman, L., Avison, M.B., Berg, G., van der Lelie, D. and Dow, J.M. (2009) The versatility and adaptation of bacteria from the genus *Stenotrophomonas*. *Nat Rev Microbiol* **7**: 514-525.

Samonis, G., Karageorgopoulos, D.E., Maraki, S., Levis, P., Dimopoulou, D., Spervasilis, N.A., Kofteridis, D.P. and Falagas, M.E. (2012) *Stenotrophomonas maltophilia* infections in a general hospital: patient characteristics, antimicrobial susceptibility and treatment outcome. *PLoS One* **7**: e37375.

Sanchez-Amat, A. and Torrella, F. (1989) Isolation and characterization of marine and salt pond halophilic bdellovibrios. *Can J Microbiol* **35**: 771-778.

Sankvist, M. (2001) Biology of type II secretion. *Mol Microbiol* **40**: 271-283.

Sandkvist, M., Michel, L.O., Hough, L.P., Morales, V.M., Bagdasarian, M., Koomey, M. and DiRita, V.J. (1997) General secretion pathway (*eps*) genes required for toxin secretion and outer membrane biogenesis in *Vibrio cholerae*. *J Bacteriol* **179**: 6994-7003.

Schelling, M. and Conti, S.F. (1986) Host receptor sites involved in the attachment of *Bdellovibrio bacteriovorus* and *Bdellovibrio stolpii*. *FEMS Microbiol Lett* **36**: 319-323.

Schoeffield, A.J. and Williams, H.N. (1990) Efficiencies of recovery of bdellovibrios from brackish-water environments by using various bacterial species as prey. *Appl Environ Microbiol* **56**: 230-236.

Schwudke, D., Strauch, E., Krueger, M. and Appel, B. (2001) Taxonomic studies of predatory bdellovibrios based on a 16S rRNA analysis, ribotyping and the *hit* locus and characterization of isolates from the gut of animals. *Syst Appl Microbiol* **24**: 385-394.

Seidler, R.J. and Starr, M.P. (1969) Isolation and characterization of host-independent bdellovibrios. *J Bacteriol* **95**: 1952-1955.

Shanks, R.M., Davra, V.R., Romanowski, E.G., Brothers, K.M., Stella, N.A., Godbole, D. and Kadouri, D.E. (2013) An eye to a kill: Using predatory bacteria to control Gram-negative pathogens associated with ocular infections. *PLoS One* **8**: e66723.

Shemesh, Y.S. and Jurkevitch, E. (2004) Plastic phenotypic resistance to predation by *Bdellovibrio* and like organisms in bacterial prey. *Environ Microbiol* **6**: 12-18.

Shemesh, Y.S., Davidov, Y., Koval, S.F. and Jurkevitch, E. (2003) Small eats big: ecology and diversity of *Bdellovibrio* and like organisms, and their dynamics in predator-prey interactions. *Agronomie* **23**: 433-449.

- Sherker, J.M. and Berg, H.C. (2001) Direct observation of extension and retraction of type IV pili. *Proc Natl Acad Sci USA* **98**: 6901-6904.
- Shi, W. and Sun, H. (2002) Type IV pilus-dependent motility and its possible role in bacterial pathogenesis. *Infect Immun* **70**:1-4.
- Snyder, A.R., Williams, H.N., Baer, M.L. and Walker S.O.C. (2002) 16S rDNA sequence analysis of environmental *Bdellovibrio*-and-like organisms (BALOs) reveals extensive diversity. *Int J Syst Evol Microbiol* **52**: 2089-2094.
- Soubirou, J.F., Gault, N., Alfaiate, T., Lolom, I., Tubach, F., Andremont, A., Lucet, J.C., Wolff, M. and Barbier, F. (2014) Ventilator-associated pneumonia due to carbapenem-resistant Gram-negative bacilli in an intensive care unit without carbapenemase-producing *Enterobacteriaceae* or epidemic *Acinetobacter baumannii*. *Scand J Infect Dis* **46**: 215-220.
- Starr, M.P. and Baigent, N.L. (1966) Parasitic interaction of *Bdellovibrio bacteriovorus* with other bacteria. *J Bacteriol* **91**: 2006-2017.
- Steinkamp, G., Wiedemann, B., Rietschel, E., Krahl, A., Gielen, J., Barmeier, H. and Ratjen, F. (2005) Prospective evaluation of emerging bacteria in cystic fibrosis. *J Cyst Fibros* **4**: 41-48.
- Stolp, H. and Petzold, H. (1962) Untersuchungen über einen obligat parasitischen Mikroorganismus mit lytischer Aktivität für *Pseudomonas*-Bakterien. *Phytopathol Z* **45**: 364-390.
- Stolp, H. and Starr, M.P. (1963) *Bdellovibrio bacteriovorus* gen. et sp., a predatory, ectoparasitic, and bacteriolytic microorganism. *Antonie van Leeuwenhoek* **29**: 217-248.
- Straley, S.C. and Conti, S.F. (1974) Chemotaxis in *Bdellovibrio bacteriovorus*. *J Bacteriol* **120**: 549-551.
- Straley, S.C. and Conti, S.F. (1977) Chemotaxis by *Bdellovibrio bacteriovorus* toward prey. *J Bacteriol* **132**: 628-640.
- Straley, S.C., LaMarre, A.G., Lawrence, L.J. and Conti, S.F. (1979) Chemotaxis of *Bdellovibrio bacteriovorus* toward pure compounds. *J Bacteriol* **140**: 634-642.
- Takhar, H.K., Kemp, K., Kim, M., Howell, P.L. and Burrows, L.L. (2013) The platform protein is essential for type IV pilus biogenesis. *J Biol Chem* **288**: 9721-9728.
- Talley, B.G., McDade Jr., R.L. and Diedrich, D.L. (1987) Verification of the protein in the outer membrane of *Bdellovibrio bacteriovorus* as an OmpF protein of its *Escherichia coli* prey. *J Bacteriol* **169**: 694-698.

- Taylor, V.I., Baumann, P., Reichlet, J.L. and Allen, R.D. (1974) Isolation, enumeration and host range of marine bdellovibrios. *Arch Microbiol* **98**: 101-114.
- Thomashow, L.S. and Rittenberg, S.C. (1985) Isolation and composition of sheathed flagella from *Bdellovibrio bacteriovorus* 109J. *J Bacteriol* **163**: 1047-1054.
- Thomashow, M.F. and Rittenberg, S.C. (1978a) Intraperiplasmic growth of *Bdellovibrio bacteriovorus* 109J: solubilisation of *Escherichia coli* peptidoglycan. *J Bacteriol* **135**: 998-1007.
- Thomashow, M.F. and Rittenberg, S.C. (1978b) Intraperiplasmic growth of *Bdellovibrio bacteriovorus* 109J: N-deacetylation of *Escherichia coli* peptidoglycan amino sugars. *J Bacteriol* **135**: 1008-1014.
- Thomashow, M.F. and Rittenberg, S.C. (1978c) Intraperiplasmic growth of *Bdellovibrio bacteriovorus* 109J: attachment of long-chain fatty acids of *Escherichia coli* peptidoglycan. *J Bacteriol* **135**: 1015-1023.
- Trandafir, L.M., Moscalu, M., Diaconu, G., Cirdeiu, E., Tudose, A.A., Coman, G. and Paduraru, D.T. (2013) The impact of respiratory tract infections on the nutritional state of children with cystic fibrosis. *Rev Med Chir Soc Med Nat Iasi* **117**: 863-869.
- Tudor, J.J. (1980) Chemical analysis of the outer cyst wall and inclusion material of *Bdellovibrio* bdello cysts. *Curr Microbiol* **4**: 251-256.
- Tudor, J.J. and Bende, S.M. (1986) The outer cyst wall of *Bdellovibrio* bdello cysts is made *de novo* and not from formed units from the prey cell wall. *Curr Microbiol* **13**: 185-189.
- Tudor, J.J. and Conti, S.F. (1977a) Characterization of bdello cysts of *Bdellovibrio* sp. *J Bacteriol* **131**: 314-322.
- Tudor, J.J. and Conti, S.F. (1977b) Ultrastructural changing during encystment and germination of *Bdellovibrio* sp. *J Bacteriol* **131**: 323-330.
- Tudor, J.J. and Conti, S.F. (1978) Characterization of germination and activation of *Bdellovibrio* bdello cysts. *J Bacteriol* **133**: 130-138.
- Tudor, J.J. and Karp, M.A. (1994) Translocation of an outer membrane protein into prey cytoplasmic membranes by bdellovibrios. *J Bacteriol* **176**: 948-952.
- Tudor, J.J. and McCann, M.P. (2007) Genomic analysis and molecular biology of predatory prokaryotes In: *Predatory prokaryotes – Biology, ecology, and evolution*. Jurkevitch, E. (ed.) Springer Microbiology Monographs. pp. 153-189.

Varon, M. and Shilo, M. (1968) Interaction of *Bdellovibrio bacteriovorus* and host bacteria. I Kinetic studies of attachment and invasion of *Escherichia coli* B by *Bdellovibrio bacteriovorus*. *J Bacteriol* **95**: 744-753.

Varon, M. and Shilo, M. (1980) Ecology of aquatic bdellovibrios. *Adv Aquatic Microbiol* **2**: 1-48.

Varon, M., Dickbusch, S. and Shilo, M. (1974) Isolation of host-dependent and nonparasitic mutants of the facultative parasitic *Bdellovibrio* UKi2. *J Bacteriol* **119**: 635-637.

Williams, H.N. (1988) A study of the occurrence and distribution of bdellovibrios in estuarine sediment over an annual cycle. *Microbial Ecol* **15**: 9-20.

Williams, H.N. and Piñeiro, S. (2007) Ecology of the predatory *Bdellovibrio* and like organisms. In: *Predatory prokaryotes – Biology, ecology and evolution*. Jurkevitch, E. (ed.) Springer Microbiology Monographs. pp. 213-248.

Wishart, M.M. and Riley, T.V. (1976) Infection with *Pseudomonas maltophilia*: hospital outbreak due to contaminated disinfectant. *Med J Aust* **2**: 710-712.

Wolfgang, M., Park, H.S., Hayes, S.F., van Putten, J.P. and Koomey, M. (1998) Suppression of an absolute defect in type IV pilus biogenesis by loss-of-function mutations in PilT, a twitching motility gene in *Neisseria gonorrhoeae*. *Proc Natl Acad Sci USA* **95**: 14973-14978.

Wu, S.S. and Kaiser, D. (1995) Genetic and functional evidence that type IV pili are required for social gliding motility in *Myxococcus xanthus*. *Mol Microbiol* **18**: 547-558.

Wu, S., Wu, J. and Kaiser, D. (1997) The *Myxococcus xanthus pilT* locus is required for social gliding motility although pili are still produced. *Mol Microbiol* **23**: 109-121.

Wurtzel, O., Dori-Bachash, M., Pietrokovski, S., Jurkevitch, E. and Sorek, R. (2010) Mutation detection with next-generation resequencing through a mediator genome. *PLoS One* **5**: e15628.

Yeo, H.J., Savvides, S.N., Herr, A.B., Lanka, E. and Waksman, G. (2000) Crystal structure of the hexameric traffic ATPase of the *Helicobacter pylori* type IV secretion system. *Mol Cell* **6**: 1461-1472.

Zhang, X.L., Tsui, I.S.M., Yip, C.M.C., Fung, A.W.Y., Wong, D.K.H., Dai, X., Yang, Y., Hackett, J. and Morris, C. (2000) *Salmonella enterica* serovar Typhi uses type IVb pili to enter human intestinal epithelial cells. *Infect Immun* **68**: 3067-3073.

Chapter 2

2. Two approaches to a prokaryotic predatory life cycle: Cryoelectron microscopy and comparative genomics of periplasmic and epibiotic predators*

*A part of this chapter has been published in the journal *International Society of Microbial Ecology*

Pasternak, Z., Njagi, M., Shani, Y., **Chanyi, R.**, Rotem, O., Lurie-Weinberger, M.N., Koval, S., Pietrokovski, S., Gophna, U. and Jurkevitch, E. (2014) In and out: an analysis of epibiotic vs periplasmic bacterial predators. *ISME J* **8**: 625-35.

2.1. Introduction

Bacterial predators in the soil are important mediators controlling the structure of the bacterial communities present. *Bdellovibrio* and like organisms (BALOs) are a group of solitary hunters, unlike *Myxococcus xanthus*. Overall there are two predation strategies for BALOs; periplasmic and epibiotic. An individual BALO species is capable of only using either the epibiotic or the periplasmic life cycle. This property is a function of the predator, as the prey cell being attacked has no bearing on the predation strategy (Chanyi *et al.*, 2013). Both life cycles can be broken down into two main phases: attack phase and growth phase. The attack phase of BALOs is highly conserved regardless of the predation strategy that the predator uses. It is defined by a highly motile predator propelled using a single polar sheathed flagellum. This phase is completely dedicated toward finding a prey cell, possibly guided by multiple chemotaxis systems. There is no

DNA replication or cell division occurring during the attack phase. When a prey cell is found, the predator will irreversibly bind to the outer membrane of the prey cell and begin to form an entry pore. This signals the end of the attack phase and the beginning of the growth phase.

It is during the growth phase where the two predation strategies diverge. Epibiotic predators, such as *Bdellovibrio exovorus* JSS, remain attached to the surface of the prey cell throughout the entire growth phase (Koval *et al.*, 2013). Hydrolytic enzymes are secreted into the prey cell to degrade the cytoplasmic contents, the products of which are transported back to *Bd. exovorus* at the cell surface. The predator divides into two daughter cells via binary fission. The progeny released begin as a new attack phase predator while the predator attached to the prey is able to undergo another round of cell division. This process will be repeated until the prey cell is devoid of enough cytoplasmic content to facilitate further *Bd. exovorus* growth.

While *Bd. exovorus* is the only epibiotic predator isolated so far belonging to the BALOs, the majority of BALOs isolated use the periplasmic predation strategy. Periplasmic predators have been isolated that belong to multiple genera and species including: *Bdellovibrio bacteriovorus*, *Bacteriovorax marinus*, *Bacteriovorax litoralis*, *Bacteriovorax stolpii* and *Peredibacter starrii*. As the name implies, periplasmic predators invade the periplasmic space of the prey cell. After attachment, hydrolytic enzymes are secreted by the predator, which creates an entry pore through the peptidoglycan layer of the prey (Lerner *et al.*, 2012). The predator squeezes through this pore, forming an osmotically stable body termed a bdelloplast. During this process the flagellum is lost. However, it is not known precisely when the flagellum is shed and for

what purpose. The cytoplasmic contents of the prey cell are degraded by an arsenal of hydrolytic enzymes (Lambert *et al.*, 2010) and used for predator growth into a long, polynucleoid, aseptate filament (Abram *et al.*, 1974). Upon an unknown, signal multiple septa form simultaneously along the length of the filament forming multiple progeny. The length of the filament and, by association, the number of progeny released from an infection are reliant upon the original size of the prey cell used (Kessel and Shilo, 1976; Shilo, 1969). The progeny are then released through distinct exit pores in the bdelloplast wall (Fenton *et al.*, 2010) and the progeny repeat the cycle.

This study takes advantage of the modern era of whole genome sequencing and bioinformatic analyses. The genome of the epibiotic predator *Bd. exovorus* JSS was sequenced. Using the previously sequenced periplasmic predators *Bd. bacteriovorus* HD100 and *Bx. marinus* SJ, a multi-genomic comparative approach was used to reveal differences between the two predatory life cycles and their molecular basis. Overall the metabolic pathways present in predators with a periplasmic life cycle were fairly conserved within predators with an epibiotic life cycle.

To complement the *in silico* analysis, cryoelectron microscopy (CEM) techniques were used to observe the two life cycles in a near-native state. CEM has previously been used to visualize the attack phase cells of the periplasmic predator *Bd. bacteriovorus* (Borgnia *et al.*, 2008; Butan *et al.*, 2011). Those studies demonstrated the level of structural detail that is obtained in the absence of the artefacts typically associated with electron microscopy techniques. However, only the attack phase cells were observed. To understand the predator-prey interactions associated with both predatory life cycles, CEM of vitreous samples and tomography of high pressure freeze substitution (HPFS) sections

were performed in this study. The analysis revealed that the periplasmic predator begins to modify the interior of the prey cell prior to invasion occurring and these modifications decreased the cell wall rigidity of the prey cell. Most importantly, CEM revealed the presence of a plug-like structure inserted into the bdelloplast wall. This suggests that *Bd. bacteriovorus* invades a prey cell then sheds the flagellum along with some surrounding membrane to seal the entry pore. Tomographic reconstructions of the plug-like structure confirmed that the plug originated from *Bd. bacteriovorus*.

2.2. Materials and Methods

2.2.1. Bacterial strains, media and growth conditions

E. coli ML35 and X-1488 were routinely grown overnight at 30°C in Luria-Bertani (LB) medium. *C. crescentus* CB2A and KSAC were grown in peptone-yeast-extract-magnesium-calcium (PYE) medium (0.2% peptone, 0.1% yeast extract, 0.8 mM MgSO₄·7H₂O, 0.5 mM CaCl₂·2H₂O, pH 7.0; 10% inoculum) overnight at 30°C. When required, *C. crescentus* KSAC was grown overnight in PYE medium supplemented with 25 µg/ml kanamycin. *Bd. bacteriovorus* 109J was maintained in coculture with *E. coli* ML35. *Bd. exovorus* JSS was maintained in coculture with *C. crescentus* CB2A. Cocultures were prepared by mixing a 1:3 (v/v) ratio of predator to prey in HEPES with calcium and magnesium (HM) buffer (3 mM HEPES, 1 mM CaCl₂·2H₂O, 0.1 mM MgSO₄·7H₂O, pH 7.6) and incubated at 30°C overnight (Chanyi *et al.*, 2013). All cultures were incubated with continuous shaking at 180 rpm. *E. coli* X-1488 is a mini-cell producing strain that was used to assess if *Bd. bacteriovorus* could use mini-cells as prey. *C. crescentus* KSAC is an S-layer and hold-fast deficient mutant that was used to prevent formation of rosettes in cocultures.

2.2.2. Isolation of *E. coli* X-1488 mini-cells

E. coli X-1488 ($F^- str^r hst^- hsm^+ minA^- minB^- purE^- pdxC^- his^- ile^- met^- ade^- ura^- r^- m^+_k$) is a mini-cell producing mutant (Meagher *et al.*, 1977). It was grown in LB broth at 30°C with vigorous aeration to an optical density reading (OD₆₀₀) of 0.7–0.9. Subsequent steps were performed on ice or at 4°C. Mini-cells were separated from the rod-shaped cells by differential centrifugation. Cells were harvested by centrifugation at 1 000 g for 10 min. The resulting pellet contained the larger filamentous and rod-shaped cells and was therefore discarded, whereas the supernatant was enriched in mini-cells. The supernatant was transferred into a sterile Eppendorf tube and centrifuged at 14 000 g for 15 min to concentrate the mini-cells. The cell pellet was resuspended in 0.15 M NaCl and centrifuged at 1000 g for 10 min. To increase the purity of mini-cells, this step was repeated twice or until no pellet was detected. The mini-cells were pelleted by centrifugation at 14 000 g for 15 min, washed in 50 mM Tris-Cl, pH 7.5 and pelleted again. The purity of the isolated mini-cells was examined by phase contrast microscopy, and the pellets were stored in glycerol at –80°C.

2.2.3. Semi-synchronous cocultures

In order to increase the prevalence of observing any one specific stage in the life cycle, semi-synchronous cocultures were prepared and sampled at the time point of interest. Twenty milliliter cocultures were prepared as above to produce an active predator culture. To this active culture, concentrated prey cells were added. For *Bd. bacteriovorus*, 6 ml of an overnight *E. coli* ML35 culture was centrifuged at 7 000 g for 10 min. For *Bd. exovorus*, 10 ml of an overnight *C. crescentus* KSAC culture was prepared in the same way. The pellets were resuspended in 1 ml HM buffer and added

directly to the active predator culture. These cultures were incubated overnight at 30°C with vigorous aeration. This increased the predator concentration. For the semi-synchronous culture, 3 ml of the respective prey cell was concentrated as described above and added directly to the predator culture (Time = 0), this produced a coculture with a predator to prey multiplicity of infection of approximately 3 to 1. Again these were incubated at 30°C with aeration and monitored by phase contrast microscopy to observe the stages of the life cycle. When the appropriate stage was viewed, samples were diluted 1/20 and quickly taken for plunge freezing.

2.2.4. Plunge freezing

QUANTIFOIL® R2/2 copper Holey carbon grids (Electron Microscopy Sciences) were used for all CEM and CET studies. Prior to plunge freezing, the EM grids were plasma cleaned to decrease hydrophobicity (Solarus). A manual plunge-freezing apparatus was specially made at the University of Guelph. When samples were ready, the exterior holder was filled with liquid nitrogen. When this equilibrated, the interior holder was filled with gaseous ethane, which, due to the cold, condensed to form liquid ethane. Samples were frozen by placing 5 µl onto an EM grid suspended over the liquid ethane by a pair of tweezers. The liquid was blotted from behind for 3 sec to adhere more cells to the grid. The sample was released and plunged into the liquid ethane. The grids were then stored in liquid nitrogen for a minimum of 1 h to dissipate the residual ethane on the sample.

2.2.5. High pressure freeze substitution

High pressure freeze substitution (HPFS) was performed at the Molecular and Cellular Imaging Facility at the University of Guelph. Samples for HPFS were prepared

as described above for semi-synchronous cocultures. Each sample was concentrated by centrifugation 10-fold prior to HPFS. Samples were frozen with a Leica HPM100 high pressure freezing apparatus. Samples were then transferred to high pressure freeze substitution medium (1% uranyl acetate, 1% osmium tetroxide, 2.5% glutaraldehyde in acetone) and placed into a Leica AFS2 freeze substitution low temperature embedding system. The samples were stored at -90°C for 60 h then slowly warmed to -60°C over the course of 5 h and maintained at this temperature for 8 h. Samples were then slowly warmed to -30°C over 5 h and maintained there for 8 h. The samples were then warmed to 0°C over the course of 5 h and then finally to 20°C after 2 h. Samples were then processed by a method similar to that of room temperature embedding.

The samples were washed three times in 0.1 M cacodylate buffer (pH 7.4) and enrobed in 5% Noble agar. The agar blocks were washed five times in ddH₂O and fixed in 2 % UA for 2 h. The samples were washed in 50% ethanol for 15 min and stored in 70% ethanol overnight at 4°C . Samples were dehydrated further in 85%, 95% and 2 \times 100% ethanol washes, each for 15 min. Samples were placed in propylene oxide for 30 min. Samples were infiltrated with 1:1 epon resin:propylene oxide for 3 h, 3:1 epon resin:propylene oxide overnight and finally with pure epon resin for 6 h. Infiltrated samples were polymerized at 60°C for approximately two days.

2.2.6. Electron microscopy

All samples were visualized at the Molecular and Cellular Imaging Facility at the University of Guelph using an FEI Tecnai G2 F20 field emission electron microscope operating at either 120 kV or 200 kV. Images were obtained using a bottom mount Gatan 4k CCD camera using Tecnai Imaging and Analysis software. Tomograms were

collected at 1° tilt increments from -65 to +65. Image alignment and tomogram reconstructions were performed using eTomo (Mastronarde, 1997) via the IMOD image analysis and modelling package (Kremer *et al.*, 1996).

2.2.7. Genome sequencing

Genome sequences of *Bd. bacteriovorus* HD100 (NC_005363.1) and *Bx. marinus* SJ (NC_016620.1) are available online from the National Centre for Biotechnology Information (NCBI). The genomes of *Bd. exovorus* JSS and *M. aeruginosavorus* EPB were sequenced for the purposes of this study. Genome sequencing was performed at the Genome High-Throughput Sequencing Laboratory at Tel Aviv University with the Genome Analyzer IIx machine (Illumina, San-Diego, CA, USA). Both genomes were assembled by sequentially applying the Abyss (Simpson *et al.*, 2009) and Minimus (Sommer *et al.*, 2007) DNA sequence assemblers. The sequences were joined into a single chromosome by identifying overlapping sequences on the ends of each contig. Directed PCR was used to confirm uncertain regions and to verify the repeats in the *Bx. exovorus* JSS CRISPR region. Whole genome sequence data was sent to us for further analyses.

2.2.8. Comparative genomic analysis

For both genomes, ORF prediction was performed with Prodigal (Hyatt *et al.*, 2010), sequence similarity searches with BLAST (Altschul *et al.*, 1997), and protein domain searches using HMMPFAM (Eddy, 1998) and CDD (Marchler-Bauer *et al.*, 2011). Metabolic reconstructions were performed using the Pathway Tools software package (Karp and Paley, 1996), which uses the BioCyc database collection and extensive manual curation using BLAST. An organism-specific Pathway/Genome

database was constructed using the PathoLogic program (Caspi *et al.*, 2012; Dale *et al.*, 2010; Karp *et al.*, 2002) for each predator. Each pathway was manually searched to identify gaps in the metabolic pathway. If a gap was present, the protein sequence of a highly studied organism (usually *E. coli*) whose function fills this gap was used to perform a standalone reciprocal BLAST search against the predator proteome. Candidate proteins must have an e-value below 10^{-4} , score above 200 and have identity greater than 50%. This allowed for manual identification of genes that would function to complete the pathway, which the automated tools could not. This was performed for each predator, for every metabolic pathway identified. Transcription factors were identified using InterproScan (Jones *et al.*, 2014; Hunter *et al.*, 2011).

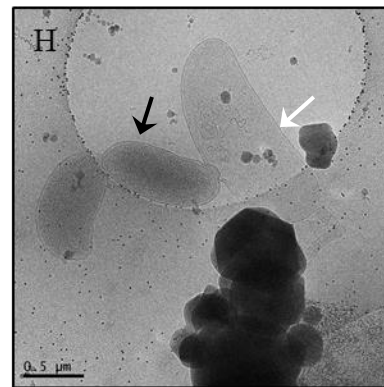
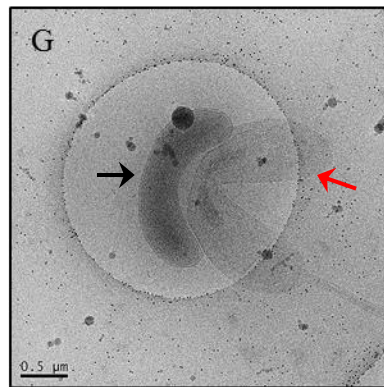
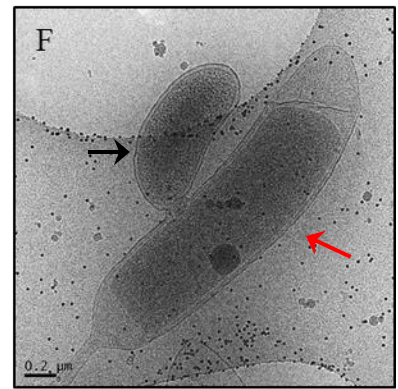
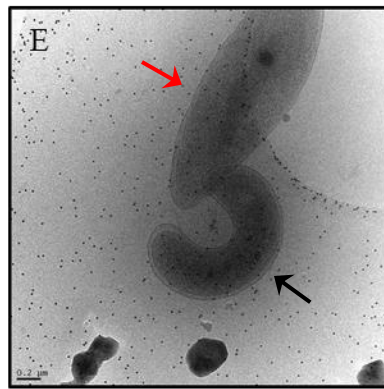
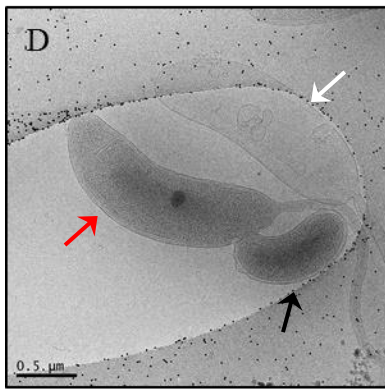
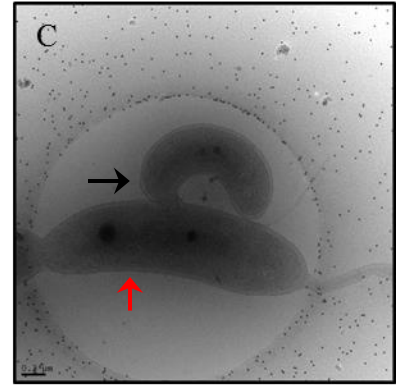
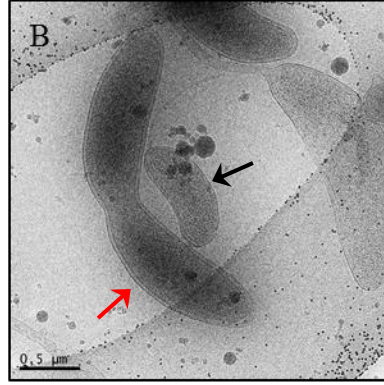
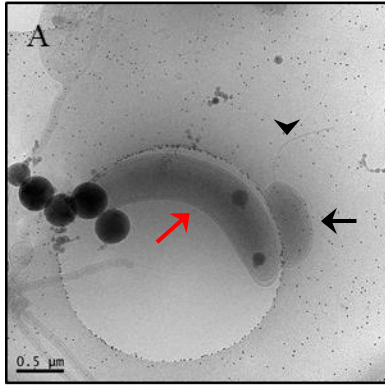
2.3. Results

2.3.1. Cryoelectron microscopy of epibiotic predation of *Bd. exovor* JSS on *C. crescentus*

C. crescentus has a developmental life cycle that consists of a flagellated, motile swarmer cell and a non-motile dividing stalk cell. The stalk cells produce a hold-fast material that forms rosettes. Preparation of samples containing rosettes formed ice that was too thick to effectively image by CEM. Therefore, a hold-fast and S-layer deficient mutant that does not form rosettes was obtained (Courtesy of Dr. John Smit, University of British Columbia), *C. crescentus* KSAC.

The life cycle of *Bd. exovor* JSS on *C. crescentus* KSAC as observed by CEM is illustrated in Figure 5. Attack phase *Bd. exovor* with a flagellum and *C. crescentus* are shown in Figure 5A. Attachment of *Bd. exovor* JSS occurred on both stalk and swarmer cells, and occasionally was observed on pre-divisional cells (Fig. 5B). This is

Figure 5. Cryoelectron microscopy visualization of the epibiotic life cycle. Vitreous samples containing *Bd. exovorus* JSS (black arrow) preying on *C. crescentus* KSAC (red arrow). A) Initial interaction between *Bd. exovorus* and *C. crescentus*; the flagellum of *Bd. exovorus* is still attached (black arrowhead); B) *Bd. exovorus* attached to a pre-divisional cell; C) Irreversible attachment prior to prey cell cytoplasmic degradation; D) Growth stage at which a decrease in cytoplasmic content of the prey cell is visible. Note the empty prey ghost stalked cell beside (white arrow); E) Elongation of *Bd. exovorus*; F) After a-round of binary fission as shown by the significant loss in cytoplasmic content of *C. crescentus*, *Bd. exovorus* remains attached to the prey cell to begin another growth cycle; G) Elongation of *Bd. exovorus* with prey cell cytoplasmic contents nearly depleted; H) Binary fission of *Bd. exovorus*. Note the empty prey ghost cell (white arrow).



interesting as these BALOs generally prey on stationary phase cells, avoiding actively growing prey cells. The attack phase pole of *Bd. exovorus* protruded into the periplasmic space of the prey, and this is where the predator remained for the duration of the life cycle (Fig. 5C). Elongation of *Bd. exovorus* was observed, which coincided with a loss of cytoplasmic content (Fig. 5D, E and G). The protoplast of the prey shrunk toward the predator (Fig. 5F; Fig. 6A). This observation has not been observed using other EM techniques, which usually show a general loss of staining in the cytoplasm of the prey cell during predation (Fig. 6B).

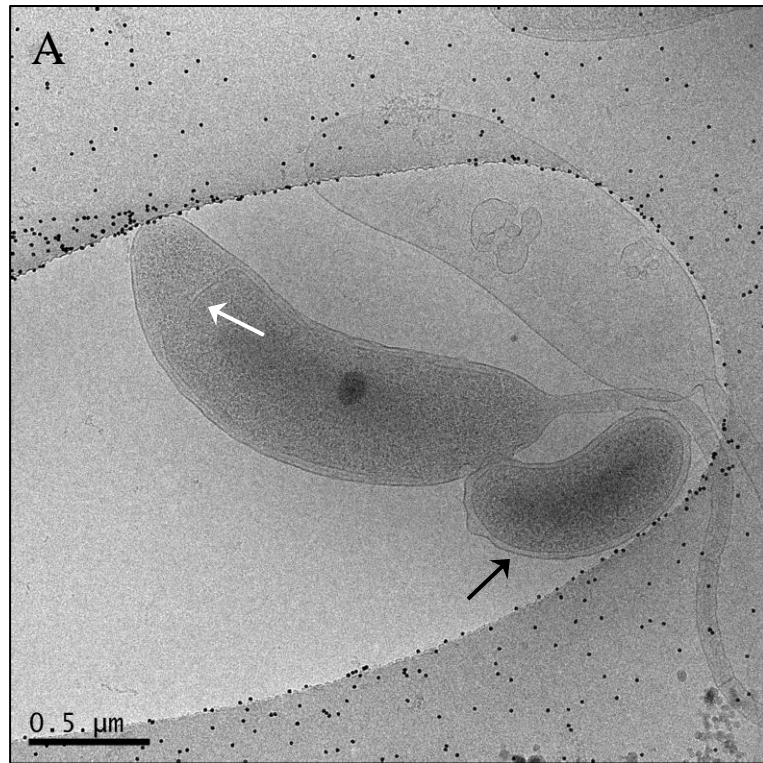
Division of *Bd. exovorus* occurred by binary fission while the predator remained attached to the prey cell. Figure 5F shows a single predator attached to a prey cell with significant loss of cytoplasmic content. This would be a predator that has already divided at least once. Predation continued until there was insufficient prey cell cytoplasmic content to allow further division (Fig. 5G and H). *Bd. exovorus* will form multiple progeny from a single predation event, dividing multiple times during the life cycle. Cryoelectron microscopy of the epibiotic predator *Bd. exovorus* thus confirmed details of this life cycle that had been observed via other microscopy techniques (Koval *et al.*, 2013).

2.3.2. Novel insights of the periplasmic life cycle revealed by cryoelectron microscopy

When using CEM, smaller biological samples give better sample resolution as ice thickness is decreased. Therefore, the small size of the *E. coli* mini-cells was hypothesized to be perfect to visualize the attachment site of *Bd. bacteriovorus* 109J during the initial stages of predation, rather than using the entire *E. coli* cell.

Figure 6. CEM and TEM images of the growth phase of the epibiotic life cycle.

CEM image of vitreous samples (A) show the protoplast (white arrow) of the prey cell shrinks towards the site of attachment with the predator (black arrow). Conventional fixation (B) shows a general decrease in cytoplasmic staining of the prey cell. Courtesy of Dr. Susan Koval. Bar equals 200 nm.



Concentrated mini-cell preparations were purified and cocultures were prepared to observe attachment, invasion and possibly mini-cell lysis. Although some attachment was observed by phase contrast microscopy, *Bd. bacteriovorus* 109J irreversibly attached to a mini-cell was not observed by CEM. Therefore, *E. coli* ML35 was used as the prey cell for CEM and CET was performed on HPFS sections.

A step-wise example of the stages involved in the periplasmic life cycle is shown in Figure 7. These images confirm the stages visualized in thin sections. Preservation of the attack phase cells of *Bd. bacteriovorus* was similar to the quality obtained by Borgnia *et al.* (2008) and Butan *et al.* (2011). Fiducial gold particles were placed onto the CEM grids prior to sample preparation to aid in focussing on images and realignment of tomograms if performed. Interesting details of the stages are discussed in the following figures.

Bd. bacteriovorus begins to modify the interior niche of the prey cell prior to invasion (Fig. 8). Upon attachment, the cytoplasmic membrane of the prey cell begins to lose the close association with the cell wall, primarily at the polar regions (Fig. 8B and C). Prey cell shape is initially maintained (Fig. 8 D).

The protoplast began to form a spherical structure (Fig. 8E and F), which was soon followed by the entire prey cell taking on a spherical shape (Fig. 8F). Throughout this process, *Bd. bacteriovorus* remained on the surface of the prey cell. Finally, when there was sufficient volume in the prey cell periplasmic space, *Bd. bacteriovorus* began to squeeze through the pore (Fig. 8G). During invasion the pore in the prey cell outer membrane was sealed and an osmotically stable bdelloplast was formed.

Figure 7. Cryoelectron microscopy visualization of the periplasmic life cycle. Stages of the periplasmic life cycle visualized through project images taken by cryoelectron microscopy. A) Attack phase cells of *Bd. bacteriovorus* and an uninfected *E. coli* prey cell; B) Initial attachment of *Bd. bacteriovorus* to *E. coli*; C) and D) Invasion into the periplasmic space of *E. coli*; E) Bdelloplast formation; F) Elongation of *Bd. bacteriovorus* using the cytoplasmic contents of *E. coli* for growth; G) Long aseptate filament formed during growth by *Bd. bacteriovorus*; H) Synchronous septation forming multiple progeny simultaneously; I) Attack phase *Bd. bacteriovorus* ready to be released from the bdelloplast.

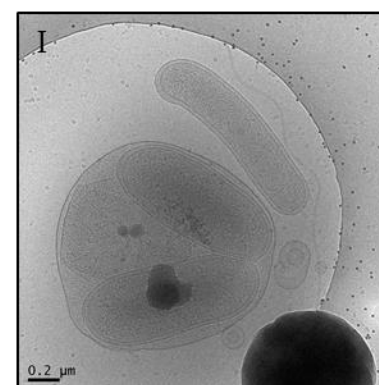
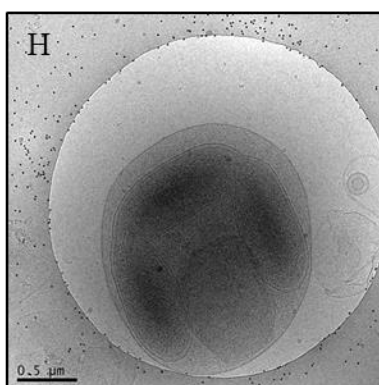
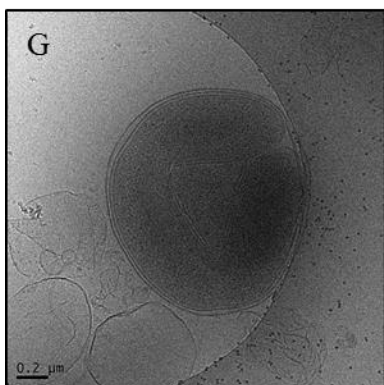
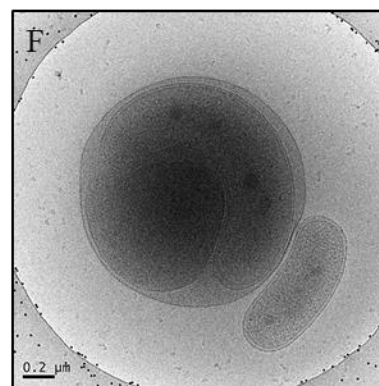
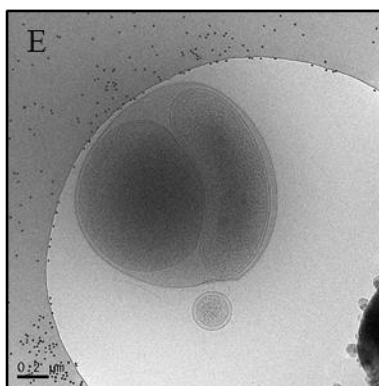
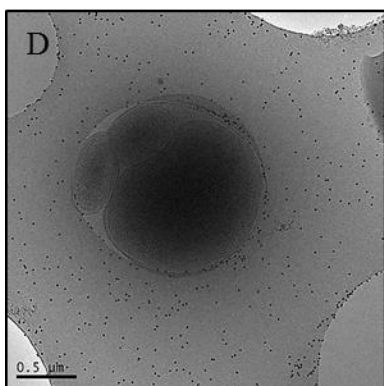
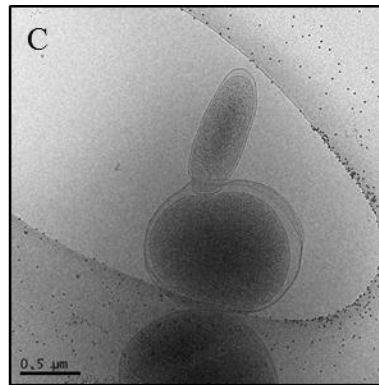
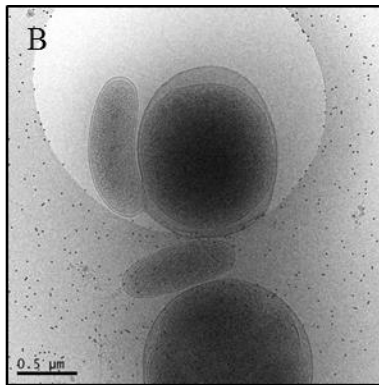
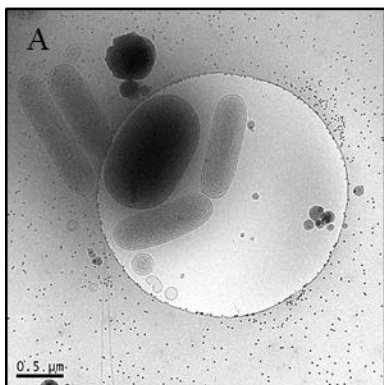
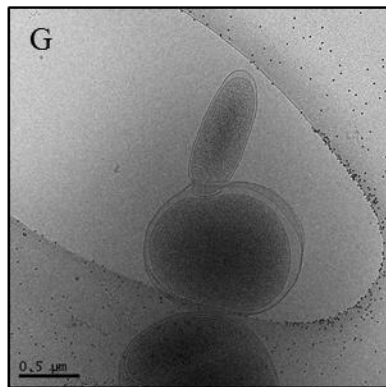
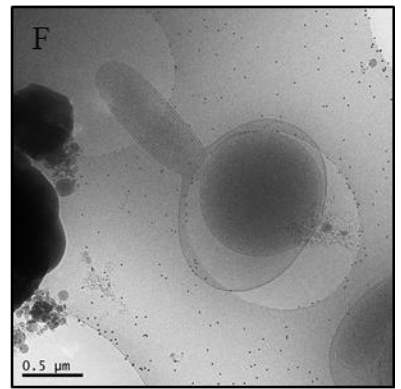
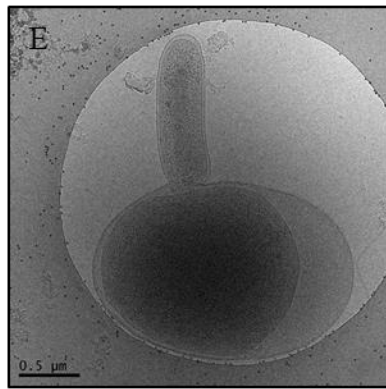
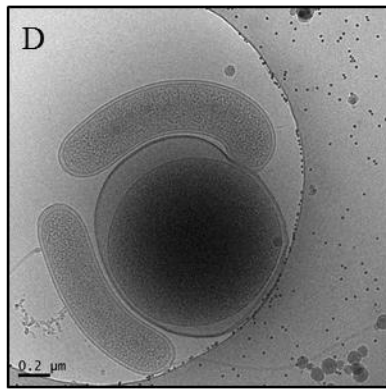
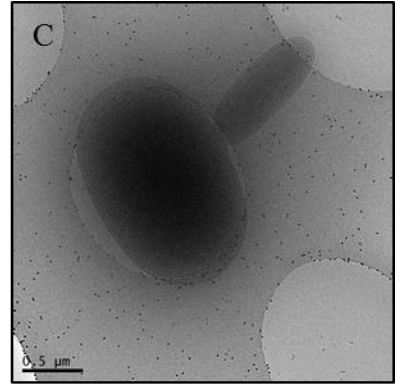
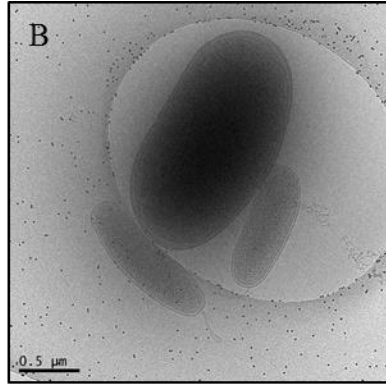
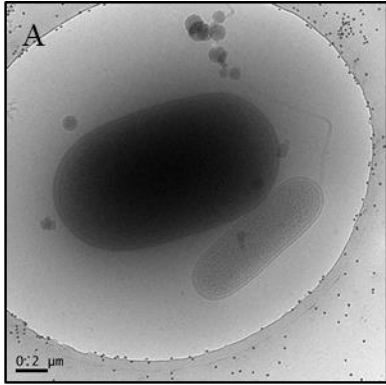


Figure 8. CEM projection images of initial attachment between *Bd. bacteriovorus* and the *E. coli* prey cell. A) Attack phase *Bd. bacteriovorus* near an *E. coli* prey cell; note the flagellum on the predator; B) and C) Initial attachment, the cytoplasmic membrane of the prey cell begins to lose the close association with the cell wall at the polar regions; D) Prey cell shape is initially maintained; E) and F) The protoplast begins to form a spherical structure, followed by the entire cell forming a spherical bdelloplast.



After the bdelloplast was formed and the predator was in the periplasm, a plug-like structure was observed at the outer membrane of the bdelloplast. This plug was observed only on bdelloplasts and always aligned with the predator where it would have invaded the prey cell (Fig. 9). One micrograph showed the same plug-like structure with a sheathed flagellum attached to it (Fig. 10). It is known the flagellum is lost during predation, however, when and why it is lost have never been understood. Finally, the cell wall rigidity of bdelloplasts appeared to be altered as a group of bdelloplasts together changed their shape when clustered together (Fig. 11). The change in cell shape was never observed on uninfected *E. coli* prey cells.

2.3.3. Cryoelectron tomography – Identification of a plug-like structure on bdelloplasts

To investigate the plug-like structure further, cryoelectron tomography of HPFS sections was performed. The outer membrane of the prey cell and the predator were clearly defined with the exception of the region where the plug was located. In Fig. 12A, one side of the plug-like structure aligned with the outer membrane of the prey cell while the other end aligned with the outer membrane of the predator. Although the attachment site was not within the section, a small portion of the flagellum was observed, which may have originated from the plug. In Fig. 12B the same observations can be made; however, in this section the peptidoglycan of the predator was visible. It was located at the base of the plug, still maintaining its curve although not associated with the plug membrane. Again, one side of the plug membrane appeared to be integrated into the outer membrane of the prey cell. The opposite end of the membrane overlapped with the outer membrane of the prey cell but had not yet been fused. Interestingly, the outer membrane of the prey

Figure 9. CEM projection images of plug-like structure. The plug-like structure (black arrow) on the bdelloplast aligns with the invaded *Bd. bacteriovorus*.

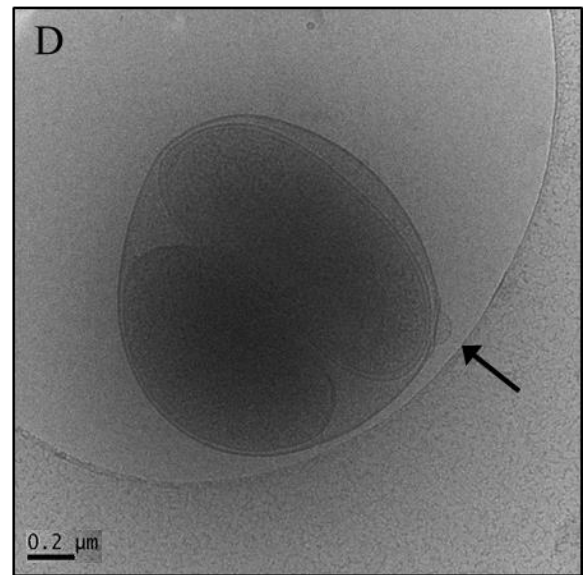
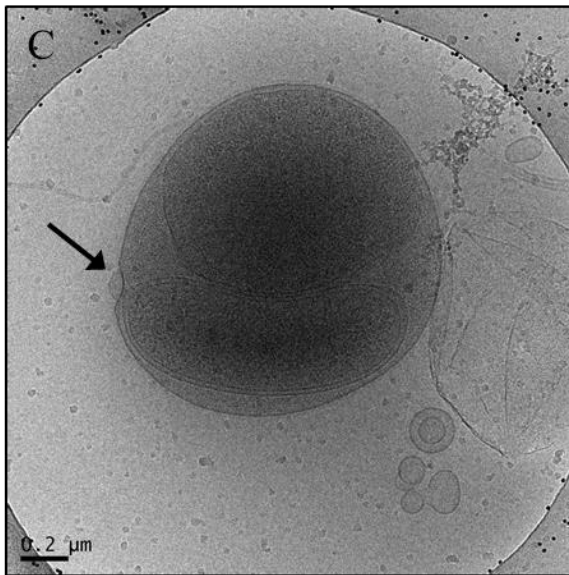
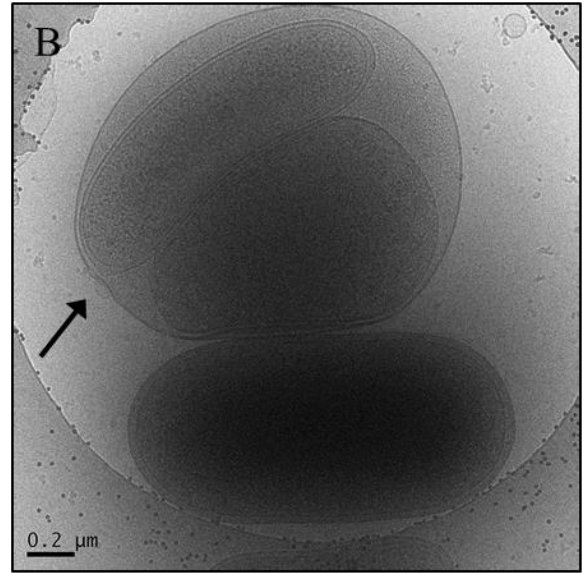
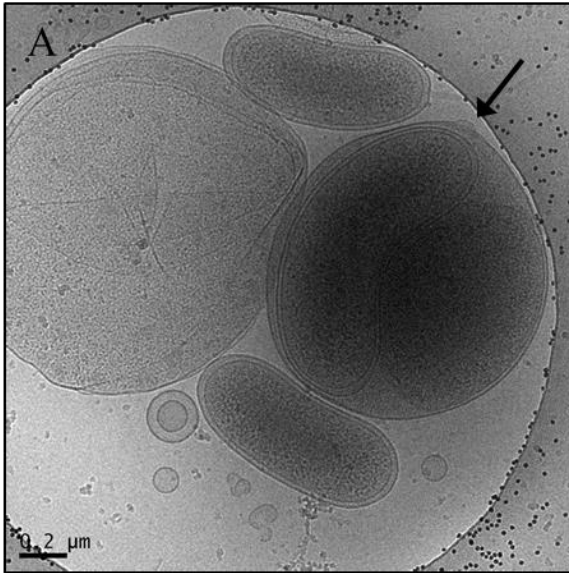


Figure 10. CEM projection image of bdelloplast has plug-like structure with flagellum attached. A sheathed flagellum (white arrow) is attached to the plug-like structure of the bdelloplast (black arrow). The *E. coli* prey cell used is non-flagellated.

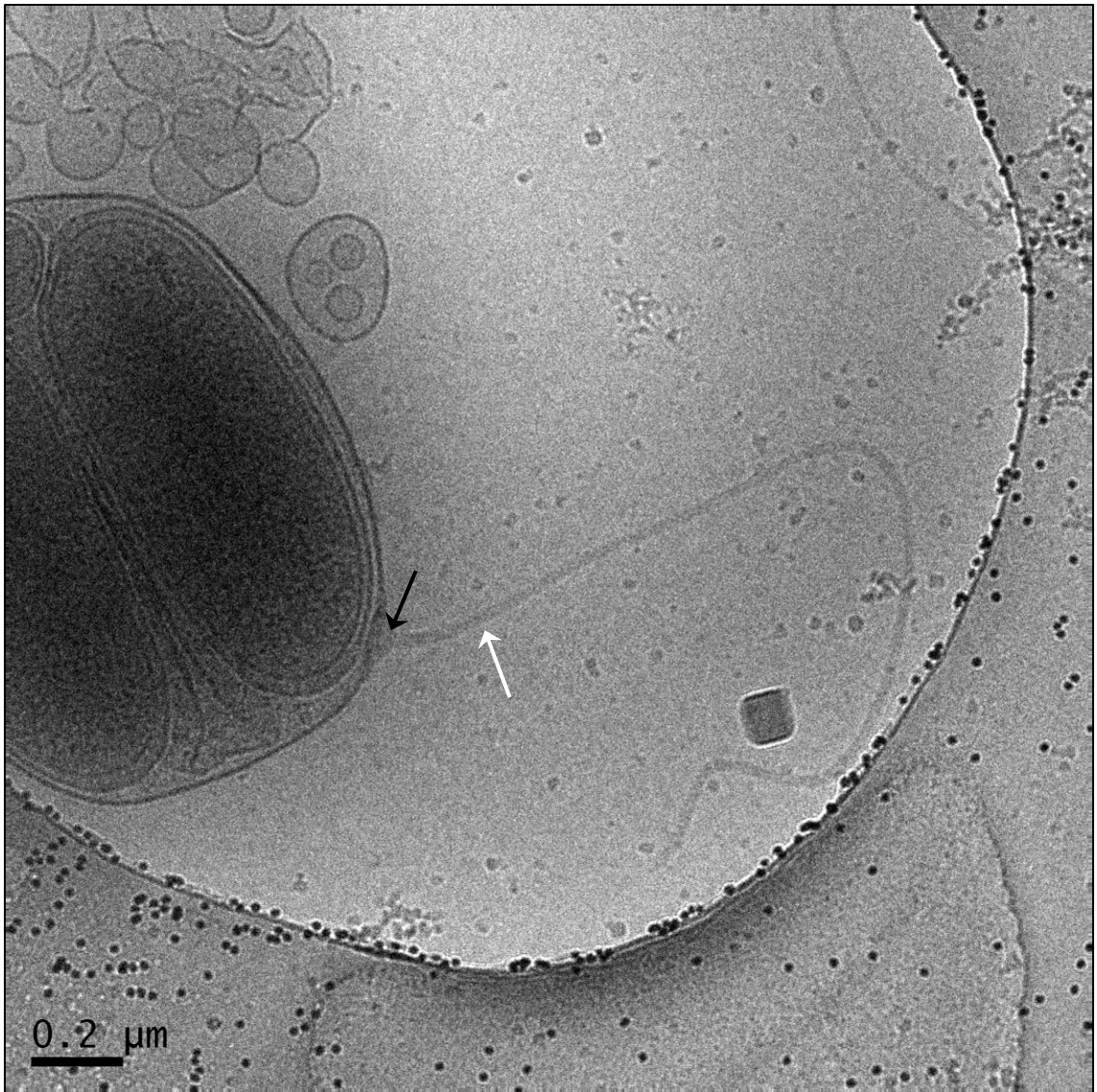


Figure 11. Decrease in cell wall rigidity of bdelloplasts viewed by CEM.
Modifications to the cell wall of bdelloplast increase plasticity, allowing them to conform to external pressure, such as being compressed within a hole in the grid.

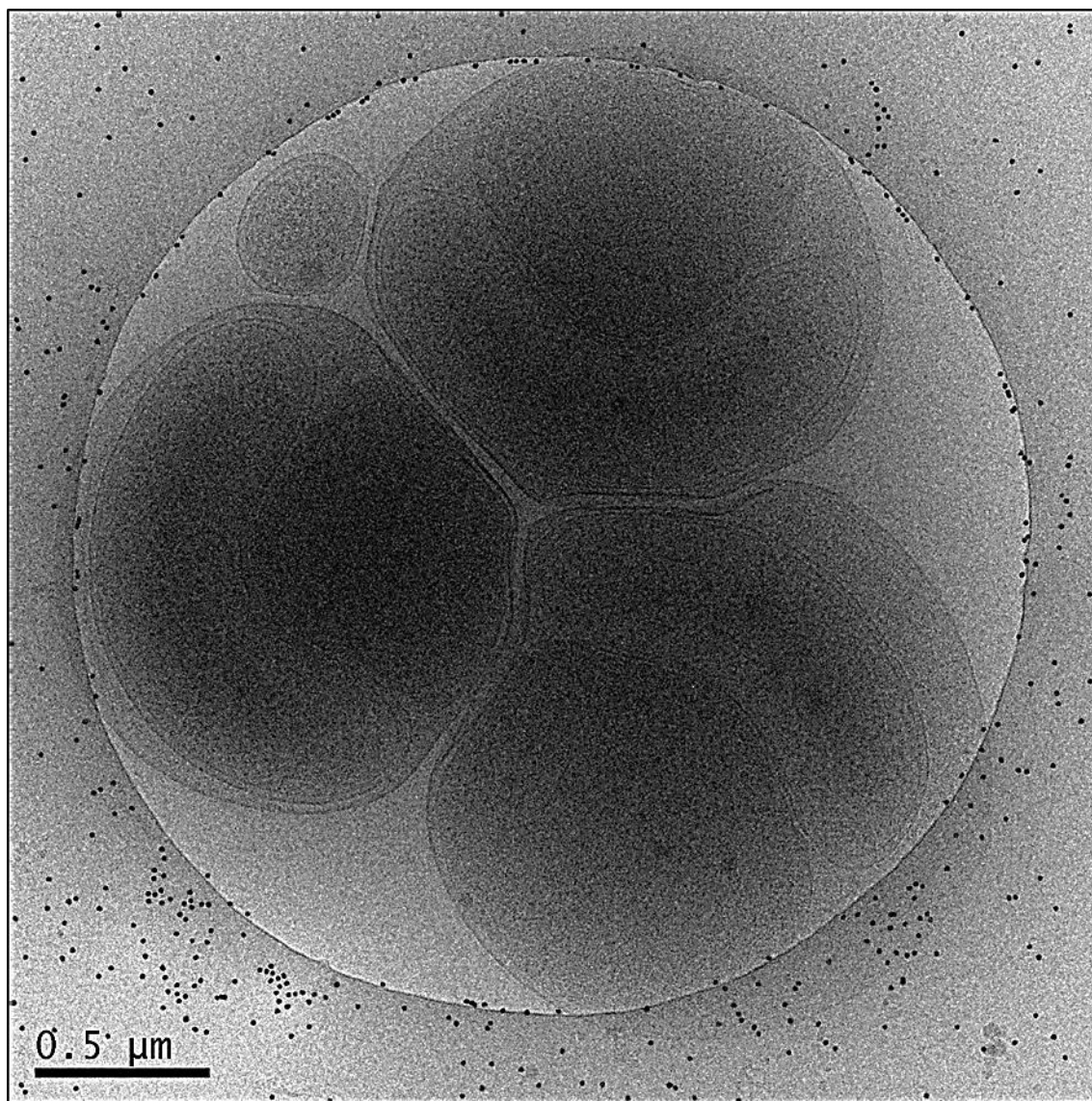
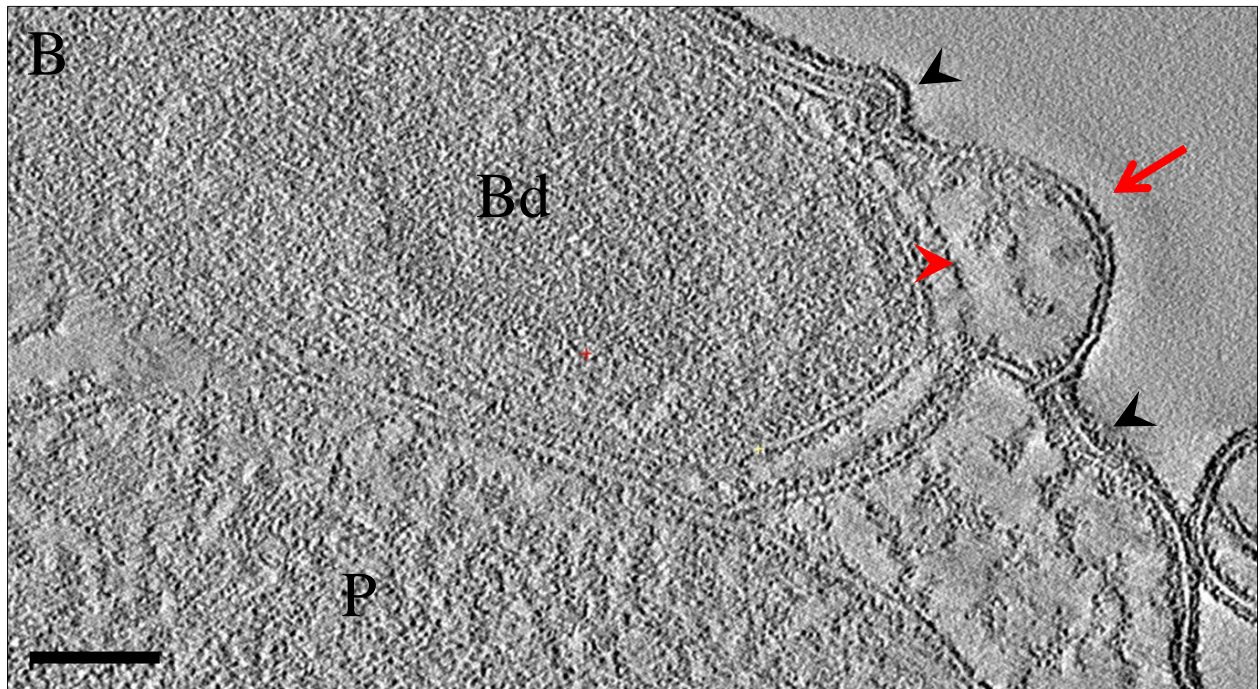
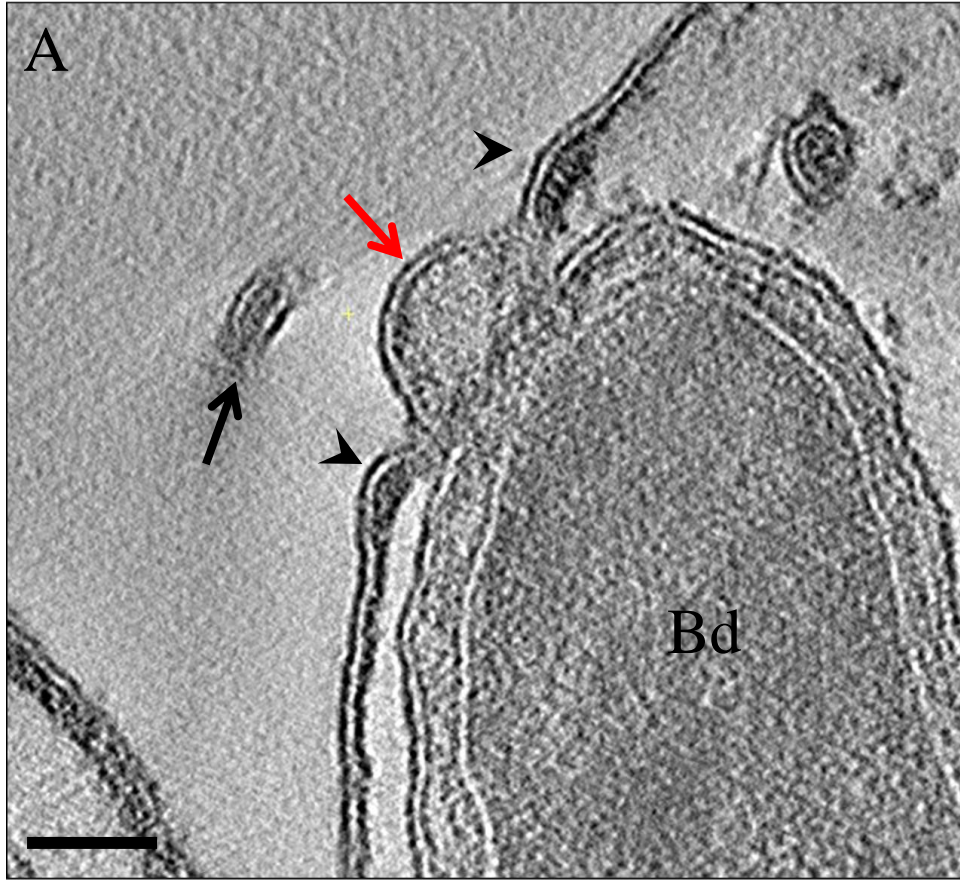


Figure 12. Cryoelectron tomographic reconstruction of plug-like structure. Reconstruction shows *Bd. bacteriovorus* (Bd) inside the periplasmic space of the *E. coli* prey cell with the plug-like structure (red arrow) associated with the *E. coli* outer membrane (black arrowhead). A portion of a sheathed flagellum (black arrow) is present. B) The peptidoglycan (red arrowhead) of *Bd. bacteriovorus* is visible in the area where the plug originated. The protoplast of the prey cell (P) is visible. The scale bar represents approximately 50 nm.



cell may fuse with the outer membrane of the predator. These processes may facilitate the resealing of the pore and provide the reason why the flagellum is shed early in the periplasmic life cycle.

2.3.4. Comparative genomics: Metabolic analysis

My contribution to this study was to perform a detailed metabolic annotation of *Bd. bacteriovorus* HD100^T, *Bd. exovorus* JSS^T, *Bx. marinus* SJ^T and *Micavibrio aeruginosavorus* EPB genomes. The results obtained from *M. aeruginosavorus* will not be discussed in this thesis because it is not a BALO. A detailed list of the metabolic pathways identified is presented in Table 1.

Similar to results discussed in the literature (Crossman *et al.*, 2013; Rendulic *et al.*, 2004), the three BALOs had the full gene pathways for glycolysis and the tricarboxylic acid cycle. Fatty acid elongation and the phosphogluconate pathway were not complete in *Bd. exovorus* JSS. In the periplasmic predators, purine and pyrimidine synthesis was complete; however, *Bd. exovorus* was not able to produce inosine, a necessary precursor for purine biosynthesis. In all three predators, the pathways required to produce phenylalanine, arginine, histidine, tryptophan, valine, isoleucine and tyrosine were not present. However, ABC transporters for branched-chain amino acids were encoded in the genomes of the three predators. Unlike the periplasmic predators, *Bd. exovorus* did not encode the complete pathways for riboflavin and vitamin B6 biosynthesis. All predators had the nicotinate pathway. None of the predators were able to synthesize glycogen or polyhydroxyalkanoate; however, *Bd. bacteriovorus* and *Bx. marinus* do encode for a polyhydroxyalkanoate depolymerase. Both periplasmic predators encode for aerobactin, a siderophore that is upregulated during the growth

Table 1. Comparison of metabolic pathways present in epibiotic and periplasmic predators.

	Periplasmic Predators		Epibiotic Predator
	<i>Bd. bacteriovorus</i> HD100	<i>Bx. marinus</i> SJ	<i>Bd. exovorus</i> JSS
Genome size (mbp)	3.78	3.44	2.66
Proteins	3586	3231	2669
Hydrolytic enzymes	197	176	130
Metabolic Pathway			
Glycolysis	Complete	Complete	Complete
Tricarboxylic Acid (TCA) Cycle	Complete	Complete	Complete
Pentose Phosphate Pathway			
-Oxidative	Not Present	Not Present	Not Present
-Non-oxidative	Complete from D-ribose-5-phosphate	Complete from D-ribose-5-phosphate	Complete from D-ribose-5-phosphate
Fatty Acid Biosynthesis			
Initiation	Complete	Complete	Complete
Saturated Elongation	Partial	Partial	Not Present
Unsaturated Elongation	Very partial	Partial: missing <i>fabA</i> (E.C.5.3.3.14)	Not Present
Fatty Acid Metabolism	Complete pathway for β -oxidation I	Complete pathway for β -oxidation I and oleate β -oxidation I	Complete fatty acid degradation
Vitamins and Cofactor Biosynthesis			
Biotin	Partial pathway from 7-keto-8-aminopelargonate (missing E.C.6.3.3.3) but cannot produce 7-keto-8-aminopelargonate	Not Produced	Not Produced
Riboflavin	Complete	Complete	Not Produced
Thiamine	Not Produced	Not Produced	Not Produced
Nicotinate	Complete (from L-aspartate)	Complete (from L-aspartate)	Complete (from L-aspartate)
Pantothenate and CoA Biosynthesis	Partial pathway for phosphopantothenate (missing <i>panE</i>), Coenzyme A production missing	Missing <i>panE</i> but rest is complete	Complete

Vitamin B6	Complete	Complete	Partial
Lipoic Acid Metabolism	Not Present	Partial (Contains lipA and lipB)	Lipoid Acid Produced
Folate	Very partial	Complete assuming BMS_1883 functions as folC	Complete
Retinol	Not Produced	Not Produced	Not Produced
Amino Acid Biosynthesis			
Alanine	Not Produced	Complete	Complete
Cysteine	Complete	Complete	Complete
Aspartic Acid	Complete	Complete	Complete
Glutamic Acid	Complete	Complete	Complete
Phenylalanine	Not Produced	Not Produced	Not Produced
Glycine	Complete	Complete	Complete
Histidine	Not Produced	Not Produced	Not Produced
Isoleucine	Not Produced	Not Produced	Not Produced
Lysine	Complete	Complete	Possibly
Leucine	Possibly	Not Produced	Not Produced
Methionine	Not Produced	Possibly	Not Produced
Asparagine	Complete	Complete	Not Produced
Proline	Not Produced	Not Produced	Complete (using ProA)
Glutamine	Complete	Complete	Complete
Arginine	Not Produced	Not Produced	Not Produced
Serine	Not Produced	Complete	Complete (from pyruvate)
Threonine	Not Produced	Not Produced	Complete
Valine	Not Produced	Not Produced	Not Produced
Tryptophan	Not Produced	Not Produced	Not Produced
Tyrosine	Complete (from phenylalanine)	Not Produced	Complete (from phenylalanine)
De novo Nucleotide Biosynthesis			
dATP	Complete	Complete	Possible (Complete from inosine)
dGTP	Complete	Complete	Possible (Complete from inosine)
dCTP	Complete	Complete	Complete
dTTP	Complete	Complete	Complete
Respiratory Chains			
Cytochrome B	Present	Present	Not Present
Cytochrome C	Present	Present	Present
Cytochrome D	Not Present	Not Present	Not Present

Cytochrome O	Not Present	Present	Not Present
Succinate Dehydrogenase	Present	Present	Present
Aerobic Metabolism			
Catalase	Present	Present	Not Present
Superoxide Dismutase	Present	Present	Not Present
Peroxides	Present	Present	Not Present
Transporters			
Amino acids	Present	Present	Present
Nucleosides	Not Present	Present	Not Present
Sugars	Present	Not Present	Not Present
Metal Transporters	Copper, Iron, Manganese, Potassium, Zinc	Copper, Iron, Manganese, Molybdenum, Potassium	Iron, Manganese
Lectins	Not Produced	Not Produced	Not Produced
Hemolysin	9 Produced	2 Produced	10 Produced
Colicin Receptor	Not Produced	Not Produced	Not Produced
Siderophores	Produced	Produced	Not Produced
Aerobactin	Produced	Not Produced	Not Produced
Reserve Material			
Polyhydroxyalkanoate	Not Produced	Not Produced	Not Produced
Glycogen	Not Produced	Not Produced	Not Produced
Polyphosphate	Produced	Produced	Not Produced

phase (Karunker *et al.*, 2013).

2.4. Discussion

2.4.1. Cryoelectron microscopy:

In the age of bioinformatic analyses, *in silico* prediction software and result-orientated hypotheses, this study highlights the importance of curiosity-driven experiments and looking at cultures. Understanding complex predatory life cycles requires a multi-faceted approach. Conventional imaging techniques for electron microscopy have taught us a great deal about the periplasmic life cycle of BALOs. In this study, state-of-the-art microscopy techniques were used to provide an unprecedented level of resolution of cells. The results obtained in this study explain important aspects of BALO cell biology.

Films by Stolp (1967a and 1967b) as cited by Shilo (1969), provided evidence that spheroplast formation did not occur until at least 20 to 30 min into the predatory life cycle. However, my projection images demonstrated that the predator began to modify the intracellular niche prior to invasion. It was only after space was created in the periplasm of the prey that *Bd. bacteriovorus* was able to invade, confirming the description by Jurkevitch (2006).

Fenton *et al.* (2010) stated that there is no direct attachment between the predator and the cytoplasmic membrane of the prey cell in the bdelloplast. The images are difficult to interpret in this regard as the predator was always shown along-side the cytoplasmic membrane of the protoplast. However, an electron dense region, which would indicate an anchoring mechanism between the two membranes, similar to that of *Vampirococcus* (Guerrero *et al.*, 1986), was not observed.

It is known that periplasmic predators shed the flagellum early in growth phase. Swarmer cells of *C. crescentus* also shed their flagellum to signal the beginning of the growth phase; a stalk is formed at the site left by the flagellum (Jensen and Shapiro, 1999). It has been believed that *Bd. bacteriovorus* loses the flagellum upon irreversible attachment to the prey cell, when the decision to invade has been determined, again signalling a phase transition. Lambert *et al.* (2006) obtained a negative stain of a bdelloplast with the flagellum of the invaded *Bd. bacteriovorus* protruding out through the bdelloplast wall. Until now, this was believed to be a one-off result, similar to finding a predator with two flagella on the same pole. Cryoelectron microscopy of vitreous samples showed the presence of a structure on the surface of bdelloplasts that was distinctly different from that of the surrounding membrane. This structure was observed only on bdelloplasts and always aligned with the end of the invaded *Bd. bacteriovorus*. This structure is similar to the “scar” described by Shilo (1969). The scar was observed at the site of penetration, possibly indicating some repair mechanism early in the life cycle. Taken together, it is evident that this must be a plug that is being used to seal the pore created during invasion into the periplasmic space. However, whether the plug was a result of the prey cell maintaining its membrane or the predator fixing the pore just created cannot be determined from CEM alone.

E. coli ML35 is non-flagellated (Thomashow and Rittenberg, 1985) and *Bd. bacteriovorus* is one of very few bacterial species with a sheathed flagellum. Therefore, finding the plug-like structure with a sheathed flagellum attached affirms that the plug must originate from the predator. Cryoelectron tomography of HPFS samples was used to look at the membrane architecture of the plug. If it was to form as a result of the prey

cell maintenance, to fix the pore, the membrane of the plug would be continuous with the adjacent membrane of the prey cell at all times. This is not the case as there was overlap between the two membranes where the plug was formed. This study reveals the answer to a fundamental question in BALO cell biology. The plug was first recognized in 1969 (Shilo) and since has never been observed. After 45 years, modern techniques have now allowed us to determine answers we were never able to in the past.

2.4.2. Comparative genomics:

The genomes of periplasmic and epibiotic predators differ in many aspects. The major finding of this study was that epibiotic predators have a smaller genome, encoding fewer proteins than that of periplasmic predators. The original aim of this study was to identify genes present in the periplasmic predators that were missing in the epibiotic predators. Although *Bd. exovorus* has a smaller proteome, a specific cluster of genes missing in epibiotic predators was not found that would explain the inability to invade the periplasmic space.

Maybe not surprisingly, there are very few differences between the metabolic pathways present in epibiotic and periplasmic predators. It appears that the ability to utilize the cellular components of the prey cell is fairly conserved. There are some differences in the results obtained through the *in silico* analysis compared to what is known about the predators. For example, nucleoside transporters were not detected in any of the predatory genomes; however, it is known that *Bd. bacteriovorus* is capable of using nucleoside monophosphates during growth (Rittenberg and Langley, 1975; Ruby *et al.*, 1985). This highlights that, although bioinformatic analyses may be powerful tools to

shed light on a topic, they must be used in conjunction with culture-based biochemical analyses.

Hydrolytic enzymes are very important tools in a predatory life cycle. *Bd. bacteriovorus* encodes 150% more hydrolytic enzymes than does *Bd. exovorus*, which may account for the inability of epibiotic predators to invade prey cells. Many hydrolytic enzymes are required to form the entry pore (Lambert *et al.*, 2010); however, if some essential enzymes are absent, *Bd. exovorus* may not be able to squeeze into the periplasmic space.

Bd. exovorus JSS is unable to produce Vitamin B6 and riboflavin (vitamin B2), essential cofactors involved in central metabolism. The predatory life cycle may supply these nutrients, allowing growth. A prey-independent mutant of *Bd. exovorus* has never been isolated. The addition of iron may also help as *Bd. exovorus* does not produce any siderophores and may not be able to scavenge for iron. Research on prey-dependent processes requires the use of prey-independent strains. It is possible that by supplying these nutrients within the medium, this may allow a prey-independent strain to be isolated.

Evans *et al.* (2007) and Mahmoud and Koval (2010) showed that type IV pili were essential for predation to occur. It was believed the retraction of these fibers may provide the driving force to allow penetration into the periplasmic space. However, type IV pili genes were present in *Bd. exovorus* in a very similar genomic arrangement to that found in *Bd. bacteriovorus*. Thus further study is required to determine how the periplasmic predators invade.

2.5. References

- Abram, D., Melo, J.C. and Chou, D. (1974) Penetration of *Bdellovibrio bacteriovorus* into host cells. *J Bacteriol* **118**: 663-680.
- Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389-3402.
- Borgnia, M.J., Subramanian, S. and Milne, J.L. (2008) Three-dimensional imaging of the highly bent architecture of *Bdellovibrio bacteriovorus* by using cryo-electron tomography. *J Bacteriol* **190**: 2588-2596.
- Butan, C., Hartnell, L.M., Fenton, A.K., Bliss, D. and Sockett, R.E. (2011) Spiral architecture of the nucleoid in *Bdellovibrio bacteriovorus*. *J Bacteriol* **193**: 1341-1350.
- Caspi, R., Altman, T., Dreher, K., Fulcher, C.A., Subhraveti, P., Keseler, I.M., Kothari, A., Krummenacker, M., Latendresse, M., Mueller, L.A., Ong, Q., Paley, S., Pujar, A., Shearer, A.G., Travers, M., Weerasinghe, D., Zhang, P. and Karp, P.D. (2012) The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Res* **40**: D742-753.
- Chanyi, R.M., Ward, C., Pechey, A. and Koval, S.F. (2013) To invade or not to invade: two approaches to a prokaryotic predatory life cycle. *Can J Microbiol* **59**: 273-279.
- Crossman, L.C., Chen, H., Cerdeño-Tárraga, A.M., Brooks, K., Quail, M.A., Piñeiro, S.A., Hobley, L., Sockett, R.E., Bentley, S.D., Parkhill, J., Williams, H.N. and Stine, O.C. (2013) A small predatory core genome in the divergent marine *Bacteriovorax marinus* SJ and the terrestrial *Bdellovibrio bacteriovorus*. *ISME J* **7**: 148-160.
- Dale, J.M., Popescu, L. and Karp, P.D. (2010) Machine learning methods for metabolic pathway prediction. *BMC Bioinformatics* **11**: 15
- Eddy, S.R. (1998) Profile hidden Markov models. *Bioinformatics* **14**: 755-763.
- Evans, K.J., Lambert, C. and Sockett, R.E. (2007) Predation by *Bdellovibrio bacteriovorus* HD100 requires type IV pili. *J Bacteriol* **189**: 4850-4859.
- Fenton, A.K., Kanna, M., Woods, R.D., Aizawa, S.I. and Sockett, R.E. (2010) Shadowing the actions of a predator: backlit fluorescent microscopy reveals synchronous nonbinary septation of predatory *Bdellovibrio* inside prey and exit through discrete bdelloplast pores. *J Bacteriol* **192**: 6329-6335.
- Guerrero, R., Pedros-Alio, C., Esteve, I., Mas, J., Chase, D. and Margulis, L. (1986) Predatory prokaryotes, predation and primary consumption evolved in bacteria. *Proc Natl Acad Sci USA* **83**: 2138-2142.

Hobley, L., Lerner, T.R., Williams, L.E., Lambert, C., Till, R., Milner, D.S., Basford, S.M., Capeness, M.J., Fenton, A.K., Atterbury, R.J., Harris, M.A. and Sockett, R.E. (2012) 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. *BMC Genomics* **13**: 670.

Hunter, S., Jones, P., Mitchell, A., Apweiler, R., Attwood, T.K., Bateman, A., Bernard, T., Binns, D., Bork, P., Burge, S., de Castro, E., Coggill, P., Corbett, M., Das, U., Daugherty, L., Duquenne, L., Finn, R.D., Fraser, M., Gough, J., Haft, D., Hulo, N., Kahn, D., Kelly, E., Letunic, I., Lonsdale, D., Lopez, R., Madera, M., Maslen, J., McAnulla, C., McDowall, J., McMenamin, C., Mi, H., Mutowo-Muellenet, P., Mulder, N., Natale, D., Orengo, C., Pesseat, S., Punta, M., Quinn, A.F., Rivoire, C., Sangrador-Vegas, A., Selengut, J.D., Sigrist, C.J.A., Scheremetjew, M., Tate, J., Thimmajananathan, M., Thomas, P.D., Wu, C.H., Yeats, C. and Yong, S.Y. (2011). InterPro in 2011: new developments in the family and domain prediction database. *Nucleic Acids Research* **40**: D306-D312.

Hyatt, D., Chen, G.L., Locascio, P.F., Land, M.L., Larimer, F.W. and Hauser, L.J. (2010) Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* **11**: 119

Jensen, R.B. and Shapiro, L. (1999) The *Caulobacter crescentus smc* gene is required for cell cycle progression and chromosome segregation. *Proc Natl Acad Sci USA* **96**: 10661-10666.

Jones, P., Binns, D., Chang, H.Y., Fraser, M., Li, W., McAnulla, C., McWilliam, H., Maslen, J., Mitchell, A., Nuka, G., Pesseat, S., Quinn, A.F., Sangrador-Vegas, A., Scheremetjew, M., Yong, S.Y., Lopez, R., and Hunter, S. (2014) InterProScan 5: genome-scale protein function classification. *Bioinformatics* **30**: 1236-1240.

Jurkevitch, E. (2006) The Genus *Bdellovibrio*, In *The Prokaryotes: a Handbook on the Biology of Bacteria*, 3rd edn, vol. 7, pp. 12–30. (Ed.) M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrandt. New York: Springer.

Karp, P.D. and Paley, S. (1996) Integrated access to metabolic and genomic data. *J Comput Biol* **3**: 191-212.

Karp, P.D., Paley, S. and Romero, P. (2002) The Pathway Tools software. *Bioinformatics* **18**: S225-232.

Karunker, I., Rotem, O., Dori-Bachash, M., Jurkevitch, E. and Sorek, R. (2013) A global transcriptional switch between the attack and growth forms of *Bdellovibrio bacteriovorus*. *PLoS One* **8**: e61850.

Kessel, M. and Shilo, M. (1976) Relationship of *Bdellovibrio* elongation and fission to host cell size. *J Bacteriol* **128**: 477-480.

Kremer, J.R., Mastronarde, D.N. and McIntosh, J.R. (1996) Computer visualization of three-dimensional image data using IMOD. *J Struct Biol* **116**: 71-76.

Koval, S.F., Hynes, S.H., Flannagan, R.S., Pasternak, Z., Davidov, Y., and Jurkevitch, E. (2013) *Bdellovibrio exovorus* sp. nov., a novel predator of *Caulobacter crescentus*. *IJSEM* **63**: 146-151.

Lambert, C., Evans, K.J., Till, R., Hobley, L., Capeness, M., Rendulic, S., Schuster, S.C., Aizawa, S. and Sockett, R.E. (2006) Characterizing the flagellar filament and the role of motility in bacterial prey-penetration by *Bdellovibrio bacteriovorus*. *Mol Microbiol* **60**: 274-286.

Lambert, C., Chang, C.Y., Capeness, M.J. and Sockett, R.E. (2010) The first bite – Profiling the predatosome in the bacterial pathogen *Bdellovibrio*. *PLoS One* **5**: e8599.

Lerner, T.R., Lovering, A.L., Bui, N.K., Uchida, K., Aizawa, S.I., Vollmer, W. and Sockett, R.E. (2012) Specialized peptidoglycan hydrolases sculpt the intra-bacterial niche of predatory *Bdellovibrio* and increase population fitness. *PLOS Pathog* **8**: e1002524.

Mahmoud, K.K. and Koval, S.F. (2010) Characterization of type IV pili in the life cycle of the predator bacterium *Bdellovibrio*. *Microbiology* **156**: 1040-1051.

Marchler-Bauer, A., Lu, S., Anderson, J.B., Chitaz, F., Derbyshire, M.K., DeWeese-Scott, C., Fong, J.H., Geer, L.Y., Geer, R.C., Gonzales, N.R., Gwadz, M., Hurwitz, D.I., Jackson, J.D., Ke, Z., Lanczycki, C.J., Lu, F., Marchler, G.H., Mullokandov, M., Omelchenko, M.V., Robertson, C.L., Song, J.S., Thanki, N., Yamashita, R.A., Zhang, D., Zhang, N., Zheng, C. and Bryant, S.H. (2011) CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res* **39**: D225-D229.

Mastronarde, D.N. (1997) Dual-axis tomography: an approach with alignment methods that preserve resolution. *J Struct Biol* **120**: 343-352.

Meagher, R.B., Tait, R.C., Betlach, M. and Boyer, H.W. (1977) Protein expression in *E. coli* minicells by recombinant plasmids. *Cell* **10**: 521-536.

Milne, J.L., Borgnia, M.J., Bartesaqui, A., Tran, E.E., Earl, L.A., Schauder, D.M., Lenqyel, J., Pierson, J., Patwardhan, A. and Subramanian, S. (2013) Cryo-electron microscopy—a primer for the non-microscopist. *FEBS J* **280**: 28-45.

Rendulic, S., Jaqtap, P., Rosinus, A., Eppinger, M., Baar, C., Lanz, C., Keller, H., Lambert, C., Evans, K.J., Goesmann, A., Meyer, F., Sockett, R.E. and Schuster, S.C. (2004) A predator unmasked: life cycle of *Bdellovibrio bacteriovorus* from a genomic perspective. *Science*. **303**: 689-692.

Rittenberg, S.C. and Langley, D. (1975) Utilization of nucleoside monophosphates per se for intraperiplasmic growth of *Bdellovibrio bacteriovorus*. *J Bacteriol* **121**: 1137-1144.

- Ruby, E.G., McCabe, J.B. and Barke, J.I. (1985) Uptake of intact nucleoside monophosphates by *Bdellovibrio bacteriovorus* 109J. *J Bacteriol* **163**: 1087-1094.
- Shilo, M. (1969) Morphological and physiological aspects of the interaction of *Bdellovibrio* with host bacteria. *Curr Top Microbiol Immunol* **50**: 174-204.
- Simpson, J.T., Wong, K., Jackman, S.D., Schien, J.E., Jones, S.J.M. and Birol, I. (2009) ABySS: A parallel assembler for short read sequence data. *Genome Res* **19**: 1117-1123.
- Sommer, D.D., Delcher, A.L., Salzberg, S.L. and Pop, M. (2007) Minimus: a fast, lightweight genome assembler. *BMC Bioinformatics* **8**: 64.
- Stolp, H. (1967a) *Bdellovibrio bacteriovorus* (Pseudomonadaceae). Parasitischer befall und lysis von *Spirillum serpens* E. 1314. Institut für den wissenschaftlichen film, Göttingen.
- Stolp, H. (1967b) Lysis von bakterien durch den parasite *Bdellovibrio bacteriovorus*. Institut für den wissenschaftlichen film, Göttingen.
- Thomashow, L.S. and Rittenberg, S.C. (1985) Isolation and composition of sheathed flagella from *Bdellovibrio bacteriovorus* 109J. *J Bacteriol* **163**: 1047-1054.

Chapter 3

3. Characterization of PilT1 and PilT2 in the bacterial predator *Bdellovibrio bacteriovorus**

*A part of this chapter has been submitted to *PLoS One*, and is under revision

Chanyi, R.M. and Koval, S.F. (2014) Role of Type IV pili in predation by *Bdellovibrio bacteriovorus*.

3.1. Introduction

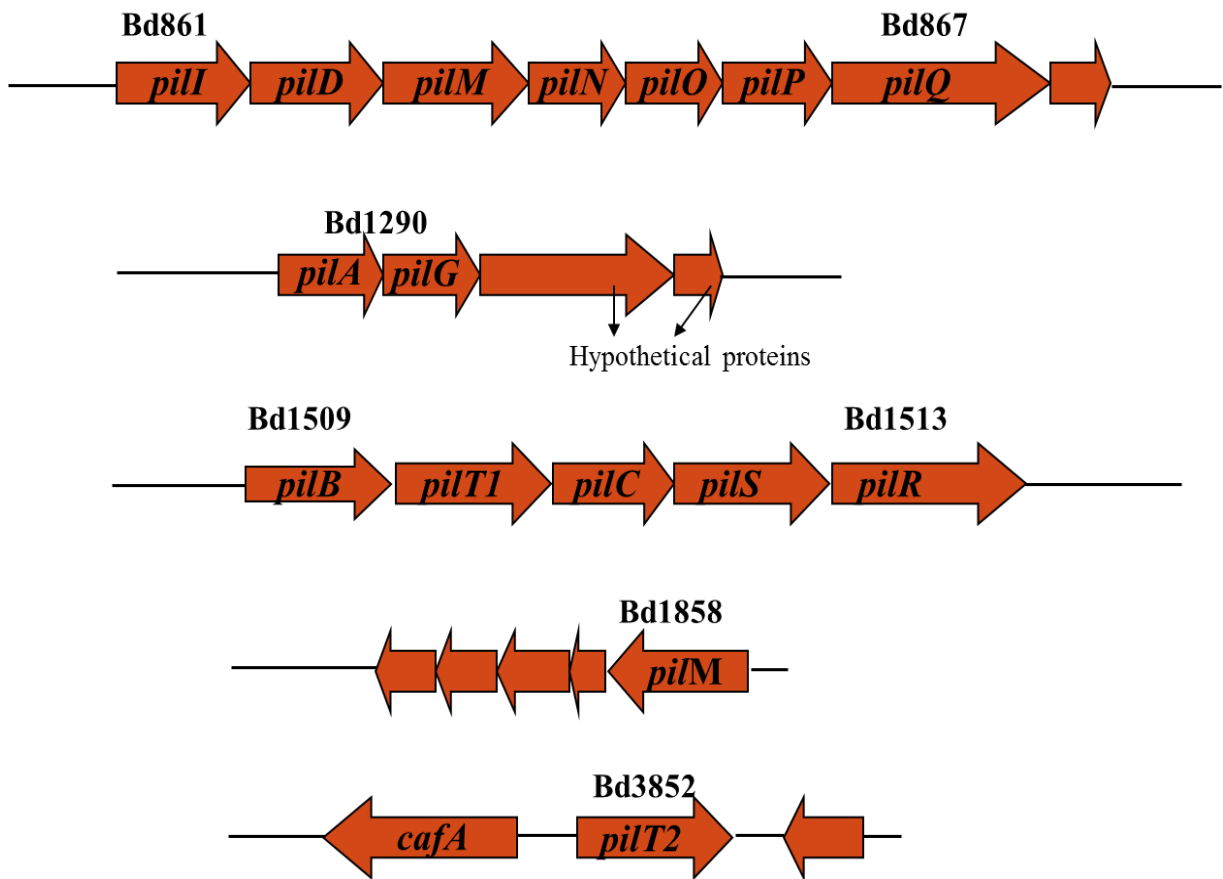
Bdellovibrio bacteriovorus is a Gram-negative obligate predator of other Gram-negative bacteria. The cells are small, vibroid in shape and highly motile via a single polar sheathed flagellum. Their life cycle consist of two stages: a motile attack phase and an intraperiplasmic growth phase. During the attack phase the cells will reversibly attach to potential prey cells for a short recognition period. If deemed suitable, *Bd. bacteriovorus* will irreversibly attach to the prey cell and begin to secrete hydrolytic enzymes to create a pore in the outer membrane and the peptidoglycan layer of the prey. *Bd. bacteriovorus* will squeeze through the pore into the periplasmic space. The pore is resealed and an osmotically stable 'bdelloplast' is formed. This signifies the end of the attack phase and the beginning of the growth phase.

Evans *et al.* (2007) showed the presence of polar pili on *Bd. bacteriovorus* and that the disruption of the *pilA* gene abolished predation. Mahmoud and Koval (2010) showed that these fibers were in fact TFP and that *Bd. bacteriovorus* in a coculture containing anti-PilA antibody was not able to prey. These results indicate that a direct

interaction between TFP and the prey cell is required for successful predation. However, it was not determined whether TFP are required for the attachment to or invasion of a prey cell. These small (8 nm diameter) polar fibers are incredibly strong and have the ability to extend, adhere to a surface and retract, as has been shown in *Pseudomonas aeruginosa* (Bradley, 1980) and *Myxococcus xanthus* (Wu and Kaiser, 1995). Successive cycles of extension, adherence and retraction is called twitching motility. The PilT protein, via ATP hydrolysis, powers the retraction of the pilus and can generate forces exceeding 100 pN per single fiber, making it one of the strongest biological motors (Maier *et al.*, 2002). Deletion of PilT in *P. aeruginosa* abolishes twitching motility and results in a hyperpiliated cell, unable to retract the pilus but still able to extend it. The number and chromosomal arrangement of TFP genes is the same in *Bd. bacteriovorus* strains HD100 and 109J (Fig. 13). *Bd. bacteriovorus* contains two annotated *pilT* genes, *pilT1* and *pilT2* (Rendulic *et al.*, 2004). Medina *et al.* (2008) reported that a transposon mutant of the *pilT2* gene, which encodes the motor that retracts the pilus, was unable to prey on a biofilm. Although not definitively demonstrated, this suggested that *Bd. bacteriovorus* uses the retraction of TFP to pull itself into the periplasmic space of the prey.

Koval *et al.* (2013) described an epibiotic predator, *Bdellovibrio exovorus*, which does not invade the periplasmic space of the prey but remains attached to the outer surface. Polar pili were demonstrated on the type strain *Bd. exovorus* JSS^T (Mahmoud and Koval, 2010) and the genome of this strain contains a full set of genes encoding TFP, including *pilT1* and *pilT2* (Pasternak *et al.*, 2014). *Bd. exovorus* does not require these two genes to invade the prey cell, and thus they must have some other function in the

Figure 13. Location of main type IV pili genes in *Bd. bacteriovorus*.



epibiotic life cycle. If *pilT1* and *pilT2* are essential for the epibiotic life cycle, mutants would need to be constructed in a prey-independent mutant. However, isolation of such mutants in *Bd. exovorus* JSS has not yet been successful (Pasternak *et al.*, 2014). Therefore, a study of the function of the *pilT1* and *pilT2* genes during the periplasmic life cycle of *Bd. bacteriovorus* 109J was undertaken.

Markerless in-frame deletion mutants of *pilT1*, *pilT2* as well as a *pilT1pilT2* double deletion mutant were constructed in the prey-independent mutant 109JA. All mutants produced bdelloplasts in cocultures with *E. coli* prey cells and thus the retraction of TFP is not required for successful invasion of a prey cell. This suggests TFP are required for initial attachment to the prey cell. However, *pilT2* is required for efficient predation on a biofilm. The results indicate that twitching motility may be required to penetrate and disrupt a biofilm.

3.2. Materials and Methods

3.2.1. Bacterial strains, media and culture conditions

E. coli strains (Table 2) were grown routinely in LB medium at 30°C overnight. When required, cells were grown in LB containing kanamycin (50 µg/ml) or chloramphenicol (25 µg/ml). *Bd. bacteriovorus* 109J was maintained in coculture with *E. coli* ML35. Cocultures were prepared by mixing a 1:3 ratio of predator to prey in HM buffer and incubated at 30°C overnight (Chanyi *et al.*, 2013). The facultative predator *Bd. bacteriovorus* 109JA was grown in PY medium overnight at 30°C (Mahmoud and Koval, 2010).

Table 2. Bacterial strains and plasmids used in this study.

Bacterial Strains	Source/Reference
<i>E. coli</i> ML35	Koval and Hynes (1991)
<i>E. coli</i> SY327 λ pir	Miller and Mekalanos (1988)
<i>E. coli</i> SM10 λ pir	Simon <i>et al.</i> (1983)
<i>E. coli</i> CO1	Peter Cadieux, University of Western Ontario, London, Canada
<i>Bd. bacteriovorus</i> 109J	Laboratory Strain
<i>Bd. bacteriovorus</i> 109JA	John Tudor, St. Joseph's University, Philadelphia, USA
<i>Bd. bacteriovorus</i> 109JA:: Δ <i>pilT1</i>	This study
<i>Bd. bacteriovorus</i> 109JA:: Δ <i>pilT2</i>	This study
<i>Bd. bacteriovorus</i> 109JA:: Δ <i>pilT1pilT2</i>	This study
<i>P. aeruginosa</i> PAK	Lori Burrows, McMaster University, Hamilton, Canada
<i>P. aeruginosa</i> PAK + pBADGr	This study
<i>P. aeruginosa</i> PAK + pBADGr:: <i>pilT1</i>	This study
<i>P. aeruginosa</i> PAK + pBADGr:: <i>pilT2</i>	This study
<i>P. aeruginosa</i> PAK:: Δ <i>pilT</i>	Lori Burrows, McMaster University, Hamilton, Canada
<i>P. aeruginosa</i> PAK:: Δ <i>pilT</i> + pBADGr	This study
<i>P. aeruginosa</i> PAK:: Δ <i>pilT</i> + pBADGr:: <i>pilT1</i>	This study
<i>P. aeruginosa</i> PAK:: Δ <i>pilT</i> + pBADGr:: <i>pilT2</i>	This study
Plasmids	
pSSK10	Silvia Piñeiro, University of Maryland, Baltimore, USA
pRCT1	This study
pRCT2	This study
pBADGr	Lori Burrows, McMaster University, Hamilton, Canada
pBADGr:: <i>pilT1</i>	This study
pBADGr:: <i>pilT2</i>	This study

3.2.2. DNA isolation

Chromosomal DNA was isolated by two methods. For large scale isolation of chromosomal DNA, a phenol/chloroform extraction was used. Briefly, 1 ml of a predator culture that had reached completed predation was centrifuged at 10 000 *g* for 5 min. The supernatant was discarded and another 1 ml of predator culture was added and concentrated as above. This was repeated until 3 ml of the predator culture had been concentrated. The pellet was resuspended in 467 μ l TE buffer (10 mM Tris-Cl, pH 8.0 and 1 mM EDTA, pH 8.0), and then 30 μ l of 10 % SDS and 3 μ l of proteinase K (20 mg/ml) were added. The mixture was incubated at 37°C for 1 h or until it was clear. One millilitre of phenol:chloroform:isoamyl-alcohol (25:24:1) was added. The mixture was briefly vortexed and then centrifuged for 10 min at 10 000 *g* in a microfuge. The upper aqueous layer was removed and placed in a sterile Eppendorf tube. This procedure was repeated once more. One millilitre of anhydrous ethanol and 50 μ l 3 M sodium acetate (pH 5.2) were added to the combined aqueous layers. The contents were mixed briefly before being placed at -20°C overnight.

The next day, the sample was centrifuged at 10 000 *g* for 10 min. The supernatant was discarded and resultant DNA pellet was rinsed in an ethanol series (100%, 90%, 2 \times 70%), centrifuged at 10 000 *g* for 5 min between each rinse. The pellet was dried by placing the Eppendorf at 37°C for 1 h. The pellet was resuspended in ddH₂O and concentration and purity were determined using the nanodrop machine. When large scale quantity was not required, the DNeasy® Blood & Tissue kit (Qiagen) was used following the manufacturer's directions.

3.2.3. Polymerase Chain Reaction (PCR)

Primers used in this study are listed in Table 3. Primers were designed using the flanking regions of the *pilT1* and *pilT2* genes using *Bd. bacteriovorus* HD100 as the reference genome. Gene products required for cloning or complementation studies in *P. aeruginosa* were amplified from genomic DNA isolated from *Bd. bacteriovorus* 109J using *i-pfu* polymerase (Frogga) under the following thermocycling conditions: 94°C for 2 min, 30 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 90 sec, and a final elongation at 72°C for 5 min. Colony PCR was performed using *Taq* polymerase (Fermentas) under the same thermocycling conditions as previously stated.

3.2.4. Construction of pRCT1 and pRCT2

Construction of the *pilT* mutants was performed in the facultative predator *Bd. bacteriovorus* 109JA because *pilT1* and *pilT2* gene deletions have the potential to be lethal. The *pilT1* gene (locus tag Bd1510) and the *pilT2* gene (locus tag Bd3852) were knocked out by allelic exchange using an in-frame deletion cloned on the suicide plasmid pSSK10 (Table 2). Deletion constructs were prepared as described in Steyert and Piñeiro (2007) using primers listed in Table 3 with modification of the restriction sites used. Construction of the *pilT1* knockout suicide vector pRCT1 was accomplished by triple ligation of the two flanking regions with the pSSK10 plasmid digested with *NdeI* and *XhoI*. One set of primers, *pilT1F1* and *pilT1R1*, was designed to include the first 46 amino acids of the PilT1 protein, and a second set of primers, *pilT1F2* and *pilT1R2*, was used to produce a PCR product including the 33 C-terminal amino acids. Collectively, the primers were designed such that, upon ligation of the two PCR products, the reading frame of the gene product is maintained, deleting the central 268 amino acid residues.

Table 3. PCR oligonucleotide primers used for amplification of *pilT1* and *pilT2*.

Primer	Sequence (5'–3')*	Target Gene
PilT1-F1	ATAGTAAC <u>CATATG</u> ACGTGAACATCTCCACCG	<i>pilT1</i>
PilT1-R1	AATAGCATGCATTCCGTCATCAGCGCTG	<i>pilT1</i>
PilT1-F2	ACATGCATGCAACATCTGATTCGTCGTCG	<i>pilT1</i>
PilT1-R2	ATACTCGAGACCGGAACCAGCGAAGAAT	<i>pilT1</i>
T1F	GTTGTTTTGCGTCTTTTGG	<i>pilT1</i>
T1R	CCATCACACCCAGGTACAGA	<i>pilT1</i>
PilT1-Ps-F	CCAACCGAATTCATGAGTCTGACCGAACTCCTTT T	<i>pilT1</i>
PilT1-Ps-R	TGGAAGA <u>AAGCTT</u> GGACGCAAACCTTCAGAC	<i>pilT1</i>
PilT2F1	CACACATATGGTCTTCGATACGACGGGAAA	<i>pilT2</i>
PilT2R1	AATAGCATGCGTTCAATGGAACCATGTTTC	<i>pilT2</i>
PilT2F2	AATCGCATGCACCAAAGTGGGCTAA	<i>pilT2</i>
PilT2R2	ATACTCGAGAAGTTCCGCGCAGGTCTT	<i>pilT2</i>
PilT2F _{com}	ATTCCCGGGCAAATCAGTCAGGATC	<i>pilT2</i>
PilT2R com	GCGGCAAGCTTCAGTATGAGTATTCCC	<i>pilT2</i>
PilT2-Ps-F	GCCGCCGAATTCATGGCAACAATTGATGAA	<i>pilT2</i>
PilT2-Ps-R	ATTGCTAAGCTTGGTGGCGATGGC	<i>pilT2</i>

*restriction sites are underlined

These two PCR products were 809 bp and 711 bp, respectively, and had an *SphI* site included on the primers. Similarly, construction of the *pilT2* knockout suicide vector pRCT2 was accomplished using the same triple ligation procedure with primers pilT2F1 and pilT2R1 (39 amino acids of *pilT2*), and pilT2F2 and pilT2R2 (5 C-terminal amino acids), deleting the central 323 amino acid residues. These two PCR products were 750 bp and 600 bp, respectively. Calcium chloride heat-shock transformation was used to transform competent *E. coli* SY327 λ pir as described in Section 3.2.5. The correct construction of each plasmid was confirmed by sequencing. However, to insert the plasmid into *Bd. bacteriovorus*, conjugation must be used. Therefore, pRCT1 and pRCT2 was electroporated into electrocompetent *E. coli* SM10 λ pir as described in Section 3.2.5.

3.2.5. Bacterial transformations

Preparation of competent *E. coli* SY327 λ pir was performed as follows: Two hundred microliters from an overnight culture were used to inoculate 20 ml of LB medium. These cells were grown at 37°C until an OD₆₀₀ of 0.4 was achieved (~2-3h). The culture was placed on ice for 30 min and then centrifuged at 3 000 g for 10 min at 4°C. The pellet was resuspended in 3 ml cold 0.1 M CaCl₂ and placed on ice again for 30 min.

The culture was centrifuged once more at 3 000 g for 10 min at 4°C. The pellet was resuspended in 1 ml cold 0.1 M CaCl₂ with 15% (v/v) glycerol added. Aliquots of 100 μ l were placed in sterile Eppendorf tubes and placed at -80°C. Aliquots were used for up to 6 months.

Heat shock transformation was used when transforming *E. coli* SY327 λ pir. An aliquot of competent cells was thawed. Isolated plasmid (5 μ l) or ligation product (15 μ l) was added to the competent cells and incubated on ice for 30 min. Cells were subjected to a heat shock at 42°C for 2 min then immediately placed back on ice for 5 min. One millilitre of LB medium was added and cultures were incubated at 37°C for 1 h. A 100 μ l aliquot of the suspension was plated on LB agar containing the appropriate antibiotic and incubated at 37°C overnight.

Transformation of *E. coli* SM10 λ pir required electroporation. Electrocompetent cells were prepared fresh from an overnight culture. A 1% (v/v) inoculum into 20 ml LB medium was incubated for 2 to 3 hours until an OD₆₀₀ of 0.4 to 0.5 was achieved. This culture was placed on ice for 20 min before being centrifuged at 6000 g for 10 min at 4°C. It was important from this point forward to keep cells as cold as possible. The pellet was resuspended in 20 ml of cold H₂O and centrifuged again at 6000 g for 10 min at 4°C. The pellet was resuspended with 3 ml cold 10% (v/v) glycerol, aliquoted (500 μ l) into sterile Eppendorf tubes and centrifuged at 8000 g for 2 min at 4°C. Each aliquot was resuspended in 50 μ l 10% (v/v) glycerol, and used for a single electroporation. The aliquot of cells was placed in a pre-sterilized electroporation cuvette (Molecular BioProducts) with a 2 mm gap and kept at -20°C. Five microliters of purified plasmid were added, mixed briefly and electroporated. Effective electroporation was achieved using 200 Ω resistance, 25 μ FD capacitance and a voltage of 1.8 kV. Directly after electroporation, the cells were added quickly to 1 ml of LB pre-warmed to 37°C and then incubated at 37°C for 2 h with shaking to recover. A 100 μ l aliquot was plated on 1.2%

LB agar containing chloramphenicol (25 µg/ml) and kanamycin (50 µg/ml) and incubated overnight at 37°C.

3.2.6. Conjugal matings

E. coli SM10 λ pir harboring either pRCT1 or pRCT2 was used as a donor for conjugation into the recipient *Bd. bacteriovorus* 109JA using a method modified from Cotter and Thomashow (1992). A sterile 0.45 µm nitrocellulose filter was placed on a 1% PY agar plate and let dry for 30 min. An overnight 20 ml coculture of *Bd. bacteriovorus* 109JA and *E. coli* ML35 was centrifuged at 2000 g for 5 minutes to remove residual prey cells. The supernatant was filtered through a 0.45 µm filter and then centrifuged at 10 000 g for 15 minutes. The pellet was resuspended in 2 ml of DNB and 100 µl placed on the nitrocellulose filter on the PY plate. This was allowed to dry for 30 min while the donor cells were being prepared. A 20 ml overnight culture of the donor *E. coli* strain was centrifuged at 6000 g for 15 min. The pellet was resuspended in 2 ml DNB. One hundred microliters were placed on the nitrocellulose filter with the *Bd. bacteriovorus* cells and incubated overnight at 30°C.

The *Bd. bacteriovorus* 109JA containing the recipient plasmid from the conjugal matings were harvested by placing the nitrocellulose filter into 5 ml HM buffer and vortexing vigorously. The cell suspension was used to make 10-fold serial dilutions, which were plated on 1% PY agar containing chloramphenicol (25 µg/ml) to select for cells containing the plasmid and streptomycin (50 µg/ml) to select for *Bd. bacteriovorus* 109JA. Integration of the plasmid into the genome was determined by the presence of merodiploid 109JA. Colony PCR was used to screen for merodiploid cells using the same outer flanking primers that had been used for cloning.

3.2.7. Counterselection

To promote excision of the plasmid from the *Bd. bacteriovorus* merodiploid genome, cells were cultured in PY medium without selection. This was repeated for three consecutive days. The presence of the plasmid still remaining in the genome was counter-selected against by growing the cells in PY medium containing 5% (v/v) sucrose at 30°C overnight. Dilutions of the overnight culture were plated for single colonies on 1% PY agar containing 5% (v/v) sucrose. Colony PCR was used to identify excisants that retained the deletion construct and removed the wild-type gene via a secondary homologous recombination event.

3.2.8. Western Blot

Total cellular PilA was used to assess the overall expression of the TFP system. *Bd. bacteriovorus* 109JA and the *pilT* mutants were grown axenically as described in Section 3.2.1. The OD₆₀₀ of an aliquot washed and resuspended in phosphate buffered saline (PBS) was used to standardize the number of cells in each sample. Samples were resuspended in 4× concentrated sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (125 mM Tris, pH 6.8, 2% [w/v] 2-mercaptoethanol, 20% [v/v] glycerol, 0.002% [w/v] bromophenol blue and 4% [w/v] SDS) and diluted with PBS. Samples were boiled for 15 min. Proteins (20 µl per sample) were separated by SDS-PAGE on 12% polyacrylamide gels using the XCell SureLock™ Mini-Cell system (Invitrogen) and electrophoretically transferred to nitrocellulose membranes with a XCell II™ blot module (Invitrogen) and the recommended buffer. Membranes were blocked with 5% non-fat milk in Tris buffered saline (TBS, 20 mM Tris, 500 mM NaCl, pH 7.5) with Tween (TTBS, 20 mM Tris, 500 mM NaCl, 0.05%

[v/v] Tween-20, pH 7.5) then incubated with 1:1000 anti-PilA (Mahmoud and Koval, 2010) in TTBS with 5% [w/v] non-fat milk. Membranes were washed three times in TTBS and antibody reactions detected using IRDye800 CW goat anti-rabbit IgG (Cedarlane Laboratories; 1:5000 in TTBS) secondary antibody and a Licor Odyssey system. For verification of *Bd. bacteriovorus* 109JA *pilT* mutants, the same protocol was followed using a Rabbit anti-PilT antibody produced against the PilT protein of *P. aeruginosa* (donated by Lori Burrows at McMaster University).

3.2.9. Predation assays

Samples were prepared by growing *Bd. bacteriovorus* 109JA and mutants axenically in PY medium (5% v/v inoculum) with shaking at 30°C. An aliquot of cells from each culture was washed and resuspended in 1 ml of HM buffer. The OD₆₀₀ was used to standardize the number of cells used for individual experiments.

An aliquot of each predator sample was standardized to have an OD₆₀₀ of 0.2. One millilitre was centrifuged at 2 500 g for 5 min and resuspended in HM buffer and added to a 20 ml coculture. A 16 h culture of *E. coli* ML35 was concentrated in HM buffer and added to obtain a coculture with an OD₆₀₀ reading of 1.0, with a multiplicity of infection of approximately 5:1.

For predation efficiency assays, cultures were incubated at 30°C for 48 h. Residual prey cell concentration was determined by serial plating onto LB agar. Plates were incubated overnight at 37°C.

For predation kinetic assays, 200 µl were added to each well of a 100-well BioScreen plate and incubated at 30°C for 48 h. OD₆₀₀ readings were taken every 30 min. Predation assays were performed in triplicate.

3.2.10. Biofilm predation assay

Using a protocol modified from Kadouri and O'Toole (2005), biofilms were formed in round-bottom microtiter dishes (BD Falcon). Microtiter wells were inoculated (200 μ l per well) from 18 h *E. coli* LB-grown cultures diluted 1:100 in LB. Cells were grown for 48 h at 30°C to allow the biofilm to form. Quantification of biofilm bacteria was performed as follows. The wells were washed 3 times with HM buffer in order to remove any planktonic cells and 200 μ l of crystal violet was then added for 15 min. Wells were washed with ddH₂O 4 times and allowed to dry. Crystal violet was solubilized by adding 200 μ l of 30% acetic acid for 20 min. An aliquot (125 μ l) of each well was transferred to a flat-bottom microtiter dish (BD Falcon) and analyzed using a microplate reader at OD₆₀₀.

To assess predation of *Bd. bacteriovorus* on *E. coli* CO1 biofilms, the preformed biofilms were grown as described in 3.2.10, washed three times with HM buffer to remove planktonic cells. *Bd. bacteriovorus* cultures grown in PY overnight were washed and diluted 1:100 in HM buffer and 200 μ l added to each well. As a control, 200 μ l of a filtered sterilized lysate was prepared by passing the *Bd. bacteriovorus*-containing lysate through a 0.22 μ m pore size filter. After filtering, no predator could be detected as judged by setting up a coculture as described in 3.2.1. The microtiter dish was incubated at 30°C for 24 h. Data presented were an average of 12-wells per replicate repeated three times. Statistical significance was measured using a 1-way ANOVA with a Bonferonni corrected post-hoc Students T-test, * $p < 0.005$.

3.2.11. Immunofluorescence Microscopy

Immunofluorescence microscopy was used to count the number of cells that had TFP. Cells of strain 109JA and the *pilT* mutants were grown as described earlier. An aliquot (200 μ l) was pre-fixed with 0.25% paraformaldehyde in Dulbecco's buffered saline (DPBS, 2.7 mM KCl, 1.5 mM KH_2PO_4 , 136.9 mM NaCl and 8.9 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$) and centrifuged at 5000 g for 5 min. Samples were resuspended in full strength fixative (2.5% paraformaldehyde in DPBS) and incubated at 37°C for 10 min before being washed twice in 2.5% bovine serum albumin in DPBS (BSA-DPBS). Samples were resuspended in 1:100 anti-pilA antibody (Mahmoud and Koval, 2010) in BSA-DPBS, incubated for 1 h at 37°C then washed twice in BSA-DPBS. Antibody detection was performed by resuspending samples in 1:100 sheep anti-rabbit IgG conjugated to Cy3 in BSA-DPBS, incubated for 1 h at 37°C and washed three times in BSA-DPBS. Samples were visualized by placing 10 μ l onto CELLSTAR (microscope slides) and viewed using an Axioskop II epifluorescence microscope (Zeiss) equipped with a QImaging Retiga 1300 cooled monochrome 12-bit camera and an HBO100/2 mercury lamp for epifluorescence illumination. Imaging was performed using Northern Eclipse software version 6.0 (Empix Imaging Inc.).

3.2.12. Electron Microscopy

To visualize TFP, cells were negatively stained and viewed by transmission electron microscopy (TEM). Cells were negatively stained on a 400-mesh grid that was inverted over a drop of cells for 1 min. The grid was then washed on 2 drops of water and the cells were stained with 1% uranyl acetate containing bacitracin (50 g/mL) as a

wetting agent. Negative stains were visualized using a Philips EM 410 transmission electron microscope operating at 60 kV.

To visualize biofilms, scanning electron microscopy (SEM) was used. *E. coli* CO1 biofilms were developed on a 12×22 mm PVC plastic coverslip (Fisher Scientific, Pittsburgh, PA). The coverslips were placed in a 6-well polystyrene cell culture plate (Corning, Inc., Corning, NY) and inoculated with a 1:100 culture of *E. coli* CO1 in LB. Plates were incubated at 30°C for 48 h with shaking. Preformed biofilms were rinsed three times in a buffer consisting of equal parts 0.07 M sodium phosphate dibasic and 0.07 M potassium phosphate monobasic (SEM buffer, pH 6.8) to remove any planktonic cells. *Bd. bacteriovorus* 109JA and the *pilT* mutants were prepared as described above for previous biofilm experiments and added to an appropriate well. The biofilms were incubated at 30°C with shaking for 24 h before being rinsed three times in SEM buffer. Residual biofilms were fixed for 30 min in SEM buffer containing 2% glutaraldehyde, followed by another three washes in SEM buffer and a 30 min secondary osmium fixation (2% osmium tetroxide). The samples were washed in sterile water and dehydrated step wise in an ethanol series (70%, 80%, 90%, 3×100%). Each step was left to sit for 10 min. The samples were critically point dried at the Biotron Integrated Microscopy Facility (University of Western Ontario, London, ON, Canada). The samples were platinum-coated and visualized using a Hitachi S-4500 field emission scanning electron microscope operating in high vacuum using Quartz XOne version 9.50 imaging software at the Surface Science Facility (University of Western Ontario, London, ON, Canada).

3.2.13. Construction of pBADGr::*pilT1* and pBADGr::*pilT2*

The *pilT1* and *pilT2* genes were amplified from the chromosomal DNA of *Bd. bacteriovorus* 109JA. The primers used are listed in Table 3. PCR conditions were the same as those listed in 3.2.3. An arabinose inducible *P. aeruginosa* expression plasmid, pBADGr (donated by Lori Burrows at McMaster University) and purified gene fragments were digested with *EcoRI* and *HindIII* and each gene fragment was directionally cloned into pBADGr. Electrocompetent *P. aeruginosa* were prepared as described in 3.2.5. for *E. coli* SM10 λ pir. Each plasmid (pBADGr, pBADGr::*pilT1* and pBADGr::*pilT2*) was electroporated as described above into electrocompetent *P. aeruginosa* PAK and a PilT mutant of this strain (donated by Lori Burrows at McMaster University). Cells were recovered for 1 h at 37°C in LB medium. Samples were plated onto 1.2% LB agar containing gentamicin (25 μ g/ml) and incubated overnight at 37°C. Colony PCR was used to detect the presence of either *pilT1* or *pilT2*. Plasmid purification was performed to verify the presence of pBADGr in control samples.

3.2.14. Subsurface twitching motility assay

An agar sub-surface twitching motility assay was used to assess whether PilT1 or PilT2 was able to restore twitching motility in the *P. aeruginosa* PAK PilT mutant. Cells were grown overnight in LB medium at 37°C. One millilitre of each sample was concentrated and resuspended in 100 μ l PBS. Plates containing 0.8% LB agar with 0.2% (v/v) arabinose to induce protein expression and plates with 0.8% LB agar with 0.1% (v/v) glucose to repress protein expression were stab inoculated. Plates were incubated overnight at 37°C. The agar was carefully removed from each plate and the bacteria adhered to the plate was stained with 0.1% (v/v) crystal violet for 10 min. The plates

were washed with ddH₂O. Twitching motility was measured as the diameter of the zone of adherent bacteria at the petri dish plastic-agar interface.

3.3. Results

3.3.1. Comparative analysis of active domains in *pilT* genes of *Bd. bacteriovorus*

As described by Mahmoud and Koval (2010), *pilT1* (Bd1510) is located within an operon containing *pilB* upstream and *pilC*, *pilS* and *pilR* directly downstream. It does not contain a Walker A box necessary for binding ATP. In contrast, *pilT2* (Bd3852) contains a Walker A (GPTGSGKS) box and the two turn amphipathic α -helical AIRNLIRE sequence important for its function (Aukema *et al.*, 2004). The *pilT2* gene is not found within an operon and is not close to any other genes involved in TFP assembly, regulation or function.

In this study, a closer analysis of PilT1 revealed that, although it does not contain a recognized nucleotide-binding site, it does contain a Walker B box. However, this would be insufficient for binding ATP. It does not contain any other domains recognized in the PilT proteins of *P. aeruginosa* or *Neisseria gonorrhoeae* that are important for function. Because of this, PilT1 may not be involved in the retraction process but may function in regulation or stability of the pilus, similar to PilU of *P. aeruginosa* (Chiang and Burrows, 2003).

Analysis of PilT2 revealed that it contains both the Walker A and the Walker B domains. It also contains many of the active domains found in PilT of *P. aeruginosa* with minor differences in amino acid composition. There was a minor variation in the AIRNLIRE sequence, with two amino acid differences (AISNLYRE). Aukema *et al.* (2004) believed this sequence may be involved in the regulation of PilT function and

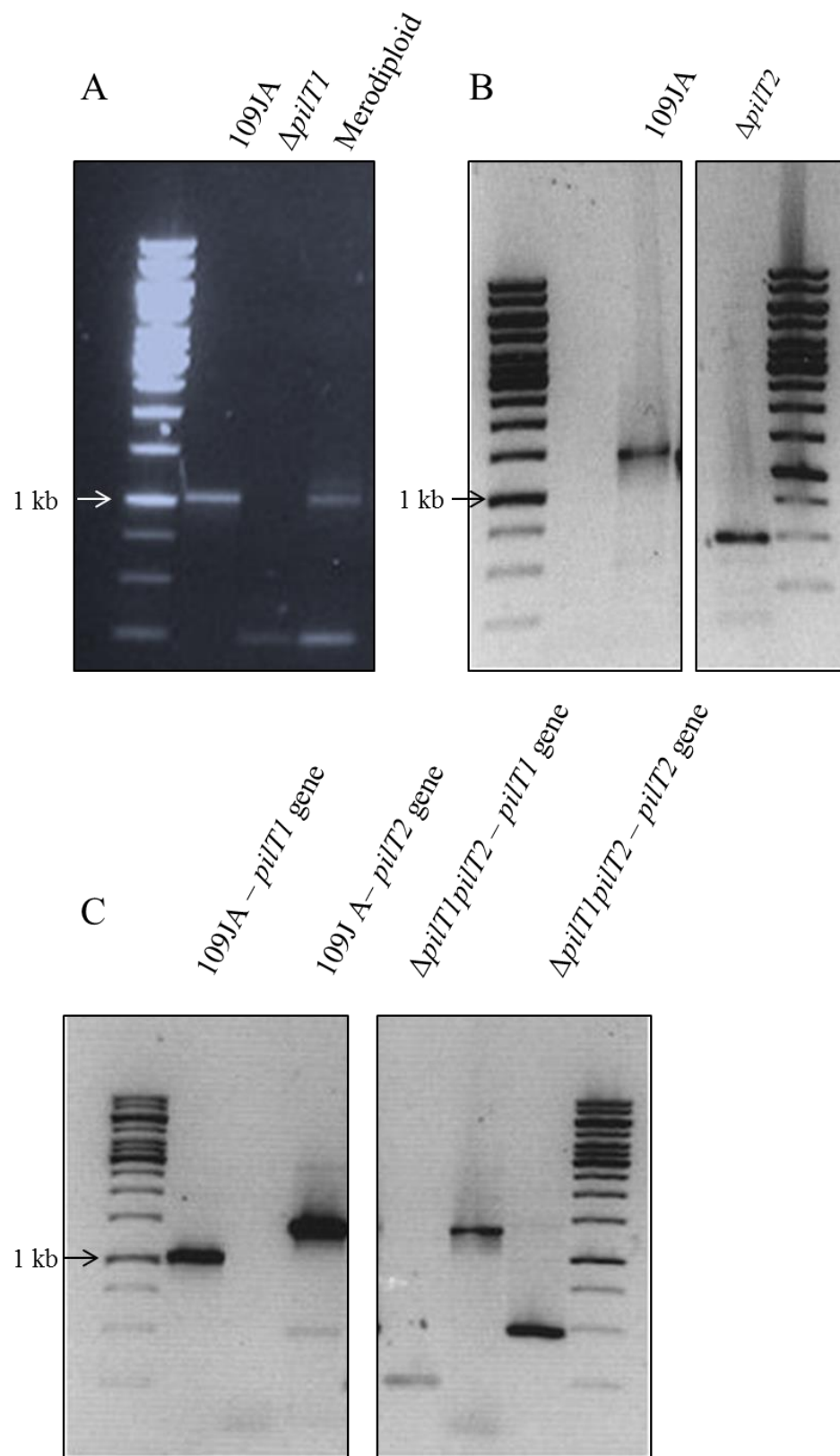
does not contain catalytic activity. Although both isoleucine residues are essential for PilT function (Aukema *et al.*, 2004), an isoleucine to valine substitution is generally regarded as a homologous substitution and should not inhibit proper function of PilT2. The Asp Box (EDPIE) is identical to that found in *P. aeruginosa*, while the His Box (HLVFGTVH) has a single leucine to isoleucine substitution; however, the histidine residues are present. Structural studies of the PilT gene in *Aquifex aeolicus* and *Vibrio cholerae* identified the ASP and His boxes as forming the catalytic pocket (Chiang *et al.*, 2008). Therefore, PilT2 contains the domains that bind ATP, form the catalytic pocket and is capable of regulating its own function. A mutant lacking this gene should not be able to retract its pilus and thus would help to determine if *Bd. bacteriovorus* uses this structure to generate the force necessary for the invasion process.

3.3.2. Verification of $\Delta pilT$ mutants

The mutants were confirmed through PCR (Fig. 14) and sequencing after multiple rounds of selection and counterselection. The deletion in both *pilT* genes can be observed by a shorter length fragment when amplified using flanking primers. The merodiploid culture containing both the wildtype *pilT* gene and the modified *pilT* gene in the genome show the intermediary steps in the mutation process (shown only for *pilT1*).

Currently there is not an anti-PilT antibody available for *Bd. bacteriovorus*. Instead, an antibody produced against the PilT protein of *P. aeruginosa* was used to verify the *Bd. bacteriovorus pilT* mutants. As *Bd. bacteriovorus* PilT2 shows a high level of sequence similarity to PilT of *P. aeruginosa*, possible cross-reactivity of the antibody would allow for verification of the *pilT2* mutant and demonstrate use in further experiments. There was a high degree of non-specific binding by the antibody, which

Figure 14. PCR confirmation of $\Delta pilT1$, $\Delta pilT2$ and $\Delta pilT1pilT2$ mutants. A) PCR products obtained using flanking primers *T1F* and *T1R* confirming the $\Delta pilT1$ mutant. The merodiploid intermediary step is shown; B) PCR products obtained using the flanking primers *PilT2Fcom* and *PilT2Rcom* confirming the $\Delta pilT2$ mutant. C) PCR products obtained using the flanking primers *T1F* and *T1R* confirming the absence of the *pilT1* gene and the flanking primers *PilT2Fcom* and *PilT2Rcom* confirming the absence of the *pilT2* gene. This confirms the absence of both genes in the $\Delta pilT1pilT2$ mutant.



was expected (Burrows lab, Personal Communication). Unfortunately, the antibody did not appear to bind to any protein in strain 109JA, which was absent in both the $\Delta pilT2$ and $\Delta pilT1pilT2$ mutants (Fig. 15).

3.3.3. Growth and predation assay of $\Delta pilT$ mutants

The facultative predator *Bd. bacteriovorus* 109JA was used to create *pilT* mutants because these genes were hypothesized to be essential for predation. The ability of the *pilT* mutant to grow axenically as well as a predator could then be tested.

In axenic growth mode, wild-type strain 109JA and the *pilT2* mutant grew in PY medium at the same rate reaching a final OD₆₀₀ of 0.4 after 36 h (Fig. 16). The *pilT1* mutant and the *pilT1pilT2* double mutant also grew at similar rates, reaching a final OD₆₀₀ of 0.5 after 36 h. After 30 h, growth of all strains began to plateau and there was no further increase in optical density. Examination by light microscopy did not reveal any variation in cellular morphology that would account for the increased optical density of the two mutants, such as elongated, undivided or corkscrew shaped cells, which are occasionally observed during axenic growth of these predators. In all three mutants and the wildtype, cells became elongated and occasionally formed a corkscrew shape if left in medium for longer than 48 h.

In predatory mode, *Bd. bacteriovorus* 109JA preyed efficiently and produced bdelloplasts on *E. coli* ML35 reaching maximum growth after 18 h as measured by a decrease in prey cell turbidity (Fig. 17). The *pilT1* mutant reached maximum growth after only 15 h. The *pilT2* mutant was still able to gain access into the periplasmic space of *E. coli* (as judged by phase contrast microscopy), albeit more slowly, reaching the maximal decrease in optical density after 30 h. The *pilT1pilT2* double mutant preyed

Figure 15. No cross reactivity of *P. aeruginosa* anti-PilT antibody with the PilT proteins of *Bd. bacteriovorus*. By Western blot analysis, the PilT protein was found in wildtype *P. aeruginosa* PAK but not the *pilT* mutant. There was no observable protein present in *Bd. bacteriovorus* 109JA, which is of similar size to the PilT protein in *P. aeruginosa*. Also, a protein that is present in *Bd. bacteriovorus* 109JA and not present in the *pilT2* or *pilT1pilT2* mutants was not observed. This indicates there was no cross-reactivity of the *P. aeruginosa* anti-PilT antibody with the PilT proteins of *Bd. bacteriovorus*.

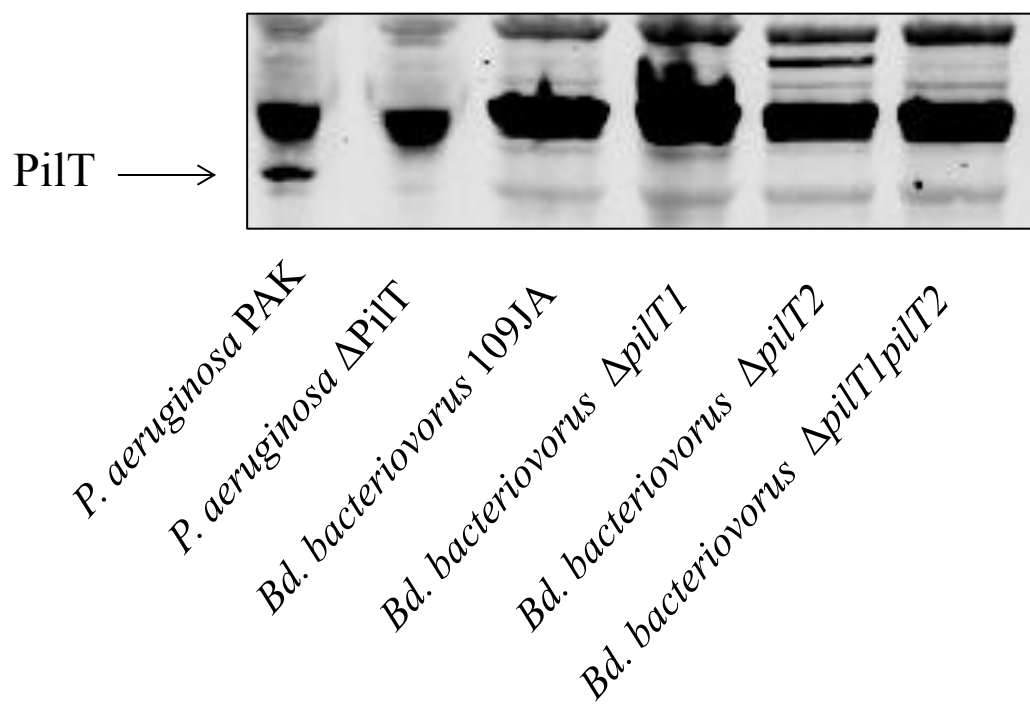


Figure 16. Axenic growth curve of *Bd. bacteriovorus* 109JA and *pilT* mutants.
Growth in PY medium over a 42 h period. Results shown are an average of 20 replicates repeated in triplicate. Error bars represent the standard error.

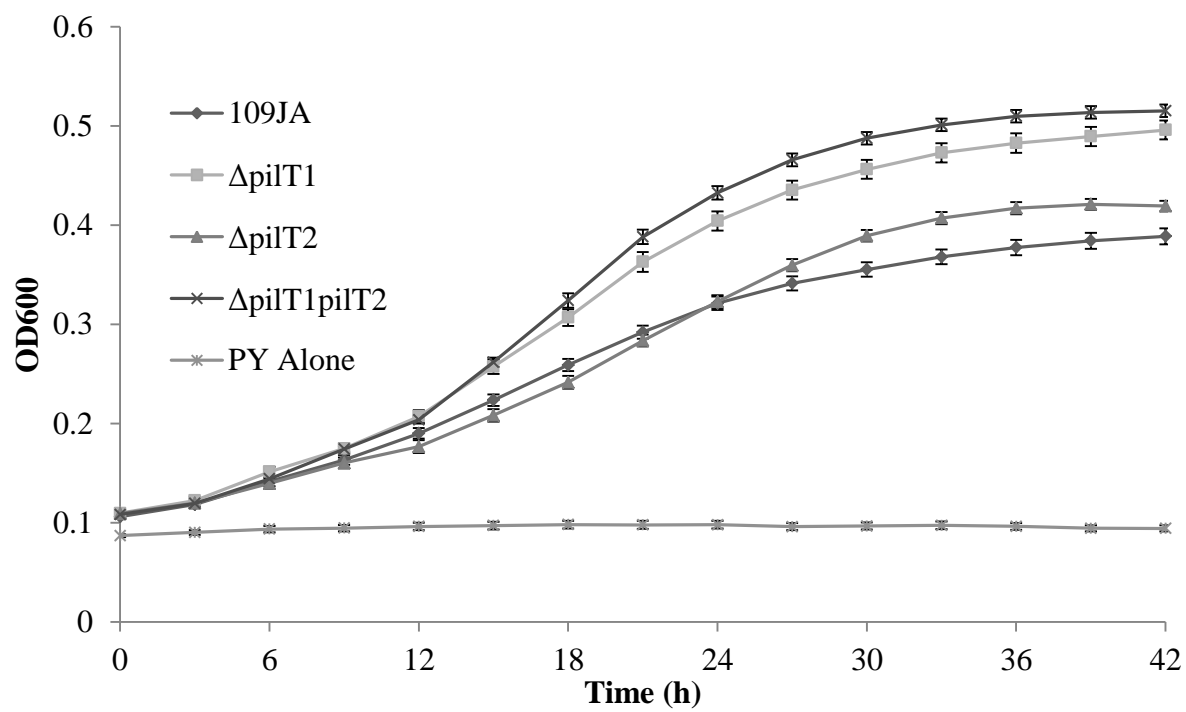
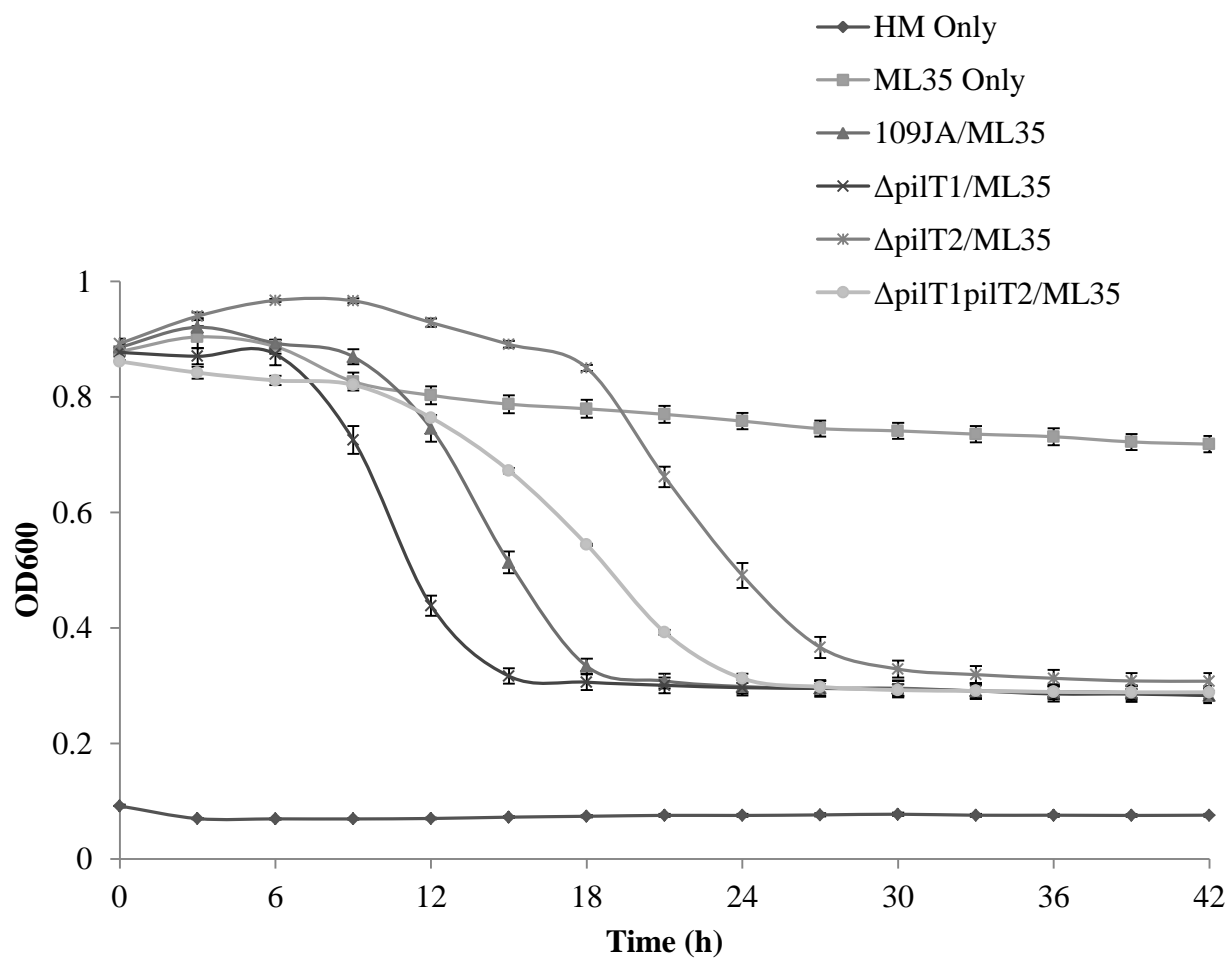


Figure 17. Effect of mutations in *pilT* genes on predation by *Bd. bacteriovorus* on *E. coli* prey cells. The decrease in prey cell optical density was used to assess predator growth. Results shown are an average of 20 replicates repeated in triplicate. Error bars represent the standard error.



within 24 h, which was slower than the *pilT1* mutant but quicker than the *pilT2* mutant, restoring it to a near wild-type predation pattern. All *pilT* mutants produced bdelloplasts of *E. coli* indicating that neither PilT1 nor PilT2 were required for the invasion process.

Another measure of growth is the rate at which the optical density decreases during the maximal growth period (Table 4). *Bd. bacteriovorus* 109JA decreased the optical density of a coculture at a rate of 0.061 OD units per hour (OD/h). The *pilT1* mutant was similar with an average decrease of 0.065 OD/h. Although the *pilT2* mutant took longer to reach stationary phase, once it began predation it was able to decrease the turbidity at 0.054 OD/h, which is slightly slower than wild type despite taking almost twice as long to reach the same final turbidity reading. The *pilT1pilT2* mutant was the slowest to prey, able to decrease the optical density at only 0.036 OD/h but did not have the same delay observed in the *pilT2* mutant.

There was no difference in the prey cell clearance of any of the *pilT* mutants when compared to wildtype after 48 h. Although minor variations in predation kinetics were observed, overall neither PilT1 nor PilT2 were required for invasion of prey cells.

3.3.4. Presence of type IV pili on $\Delta pilT$ mutants

Western blot analysis of whole cell lysates of axenically grown strain 109JA and the *pilT* mutants was used to assess the production of PilA, an indicator of overall TFP regulation. Both the unprocessed and processed (signal sequence cleaved) PilA proteins were observed. Both the levels of processed and unprocessed PilA protein were similar in the *pilT* mutants as compared to wildtype (Fig. 18). This is in direct contrast to what was observed in *P. aeruginosa*; that the absence of PilT greatly enhanced the level of PilA in the cell (Asikyan *et al.*, 2008). However, Western blot analysis cannot be used to

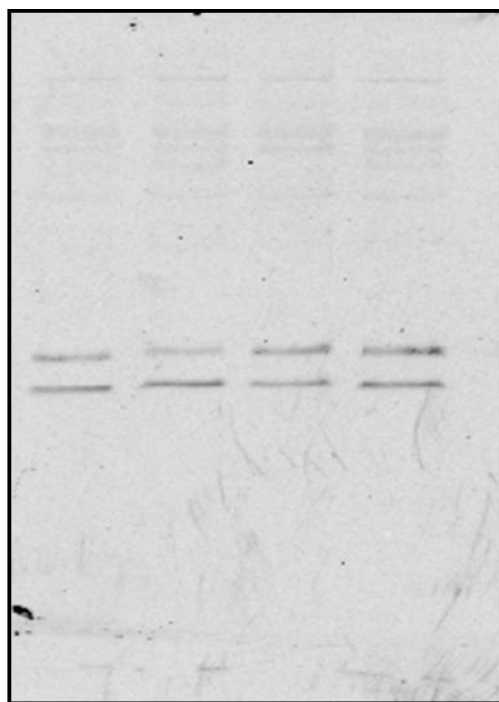
Table 4. Predation of *E. coli* ML35 by $\Delta pilT$ mutants. Co-cultures were prepared by adding prey cells to harvested predator cells ($\sim 1 \times 10^7$ PFU final concentration) or predator free control. Values represent the maximum Log_{10} change measured following 24 h incubation (compared to t_0). Each experiment was conducted in triplicate. The decrease in prey cell number was represented as the mean \log_{10} decrease and standard error.

Predator	Starting CFU	Final CFU	Log_{10} change	Percent Decrease
109JA	1.45×10^9	1.36×10^7	2.05 ± 0.18	99.06
$\Delta pilT1$	1.57×10^9	1.24×10^7	2.13 ± 0.11	99.21
$\Delta pilT2$	1.31×10^9	1.02×10^7	2.14 ± 0.14	99.22
$\Delta pilT1 pilT2$	1.56×10^9	1.15×10^7	2.14 ± 0.06	99.26

Figure 18. No difference in PilA levels in *pilT* mutants. The level of PilA in whole cell lysates of *Bd. bacteriovorus* 109JA and the *pilT* mutants grown prey-independently was assessed by a Western blot. Both the unprocessed (top band, ~23 kDa) and processed (bottom band, 19.6 kDa) PilA proteins could be observed. There was no difference in the level of PilA in the *pilT* mutants compared to wildtype.

Unprocessed PilA

Processed PilA



109JA

$\Delta pilT1$

$\Delta pilT2$

$\Delta pilT1pilT2$

assess the amount of surface piliation on each cell. To examine this, electron and immunofluorescence microscopy were used.

Although the average length was not determined, a distinct difference in length was observed when comparing TFP of *Bd. bacteriovorus* with the TFP of *P. aeruginosa* (Fig. 19).

Electron microscopy evaluation of strain 109JA showed that 22.3% (n = 336) of cells expressed TFP on their surface (Table 5). This was greater than in the *pilT1* mutant that showed only 8.1% (n = 346; p<0.005) of cells had TFP on their surface. Only 16.4% (n = 250; p<0.005) of the *pilT2* mutant cells were piliated. Surprisingly, the *pilT1pilT2* double mutant was able to restore the cells to near wildtype levels with 21.2% (n = 330) piliation. There was no statistical difference between strain 109JA and the *pilT1pilT2* mutant.

None of the mutants were hyperpiliated as was observed with *P. aeruginosa*. In this study, the location of the TFP did not appear to change; TFP were only on the non-flagellated pole. In the instance that the cell did not have a flagellum, TFP were never observed on both poles. The number of TFP on each cell did not appear to change across all mutants; however, this could be assessed only by electron microscopy. On occasion a cell was observed to have more than one pilus (Fig. 19A).

Due to the short length of TFP on *Bd. bacteriovorus*, staining artefacts and the laborious task of finding cells that were stained optimally for visualizing and thus counting TFP, electron microscopy was not an ideal method for determining the level of piliation. Therefore, immunofluorescence microscopy with antibodies to the PilA protein was used for a more accurate measurement. In all cases, the number of cells with TFP

Figure 19. Type IV pili on *Bd. bacteriovorus* are much shorter than those of *P. aeruginosa*. Electron micrographs of *Bd. bacteriovorus* 109J (A) and *P. aeruginosa* PAK (B). The pilus is visible on both cells extending from the pole of the bacterium. Cells were negatively stained with uranyl acetate. In (B), bar represents 500 nm.

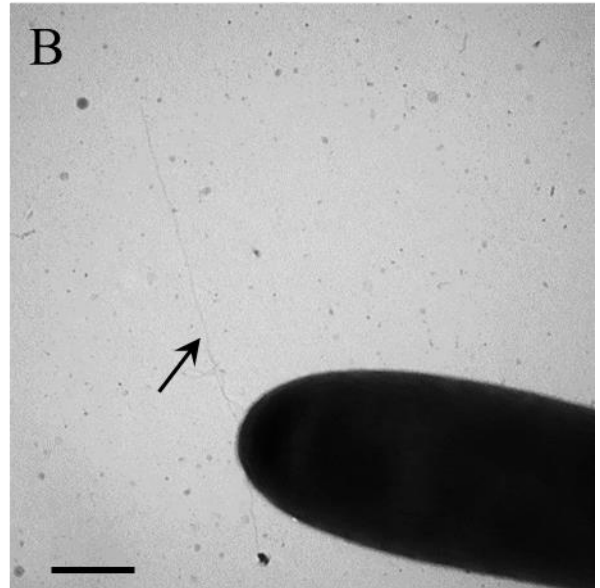
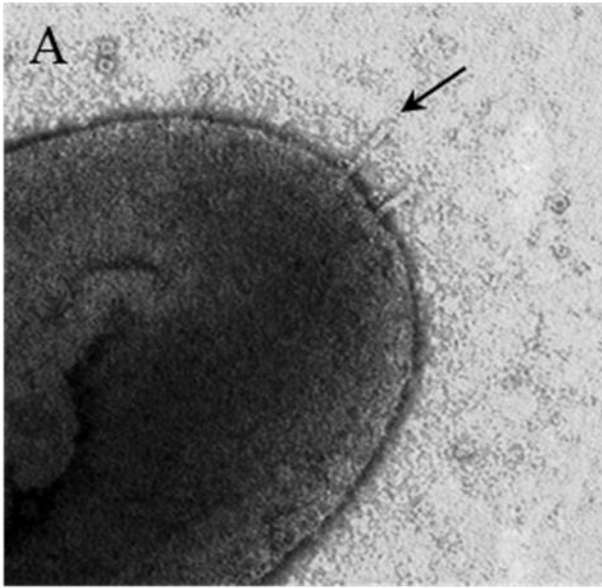


Table 5. Differences in surface piliation depending on quantitative method used.

Presence of type IV pili on the surface of cells as assessed by electron microscopy and immunofluorescence microscopy. Statistical significance determined using Chi-Square test, *p<0.005.

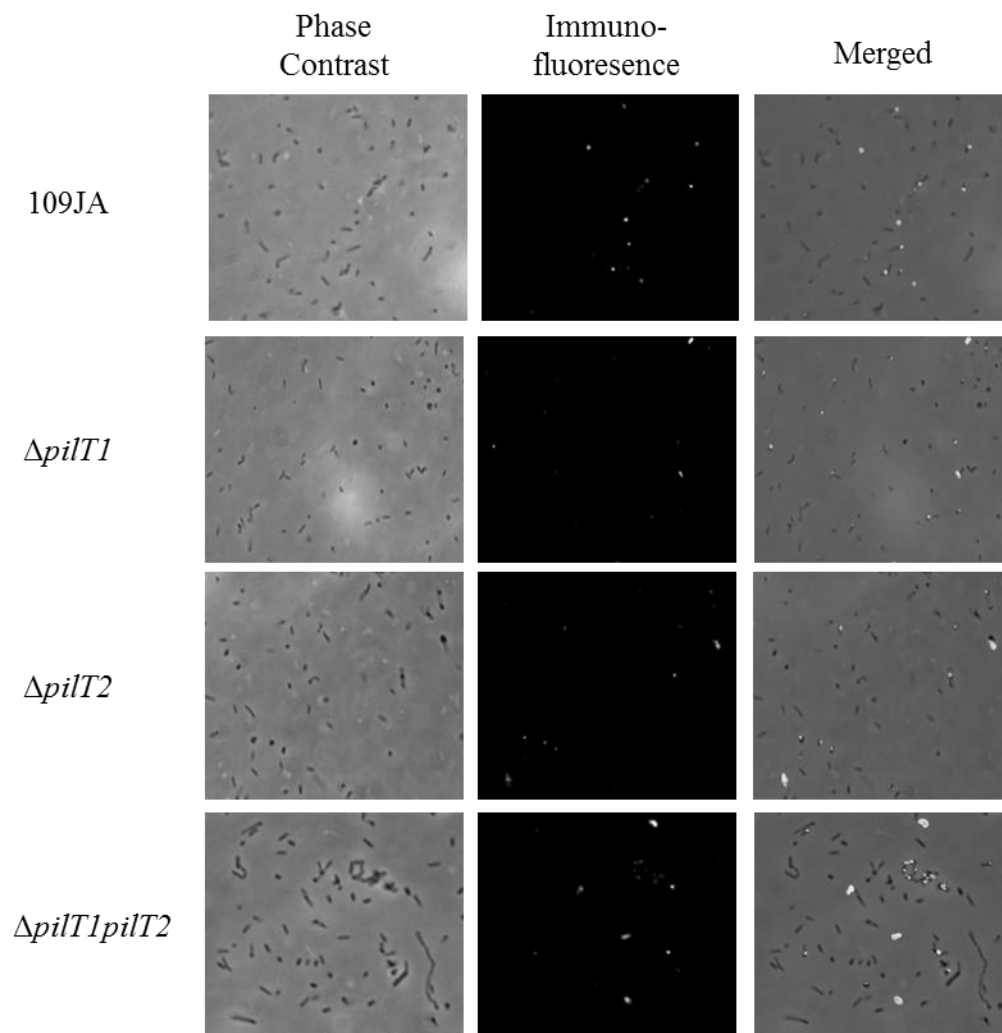
Strain	% Number of cells with TFP	
	Electron Microscopy	Immunofluorescence
109JA	22.3	27.0
$\Delta pilT1$	8.1*	33.4*
$\Delta pilT2$	16.4*	29.2*
$\Delta pilT1pilT2$	21.2	27.7

increased. Both the wildtype and the *pilT1pilT2* mutant had about 27% (n = 4308, n = 3103) of cells with TFP. Surprisingly, the *pilT1* mutant had 33.4% (n = 5252) piliation, a 4-fold increase from 8.2% measured by electron microscopy (p<0.005). The *pilT2* mutant had 29.2% (n = 3073) of cells with TFP, a small but significant increase above wildtype levels (p<0.005). Although the number of pili on the surface of each cell cannot be determined, one can observe the site of fluorescence as a single spot located on a single pole of the bacterium. This verifies what was observed by EM, that TFP are generally on a single pole (Fig. 20). As the fluorescent signal was sometimes weak, images were over-exposed, which made some cells appear to be covered in PilA. These cells were not included. It is also important to note that these cultures were grown prey-independently; therefore, there was no risk of counting pili on the prey cell which may cross-react with the antibody used.

3.3.5. Biofilm predation assay

A transposon *pilT2* mutant of *Bd. bacteriovorus* 109JA was unable to form plaques on a lawn of prey cells (Medina *et al.*, 2008). The results of this study confirm this, as the mutant with the markerless in-frame deletion of *pilT2* did not form plaques; however, the *pilT1* and *pilT1pilT2* mutants did (data not shown). Therefore, a more robust biofilm model was used to assess predation of these predators. *E. coli* CO1 was used as the prey cell as it is a known biofilm-producing strain isolated from the stool of a healthy woman. This strain showed an increased ability to attach to uroepithelial cells of the elderly and agglutinate P-type erythrocytes indicating it has potential to cause renal infections (Reid *et al.*, 1984). This strain was chosen over other known biofilm producers such as *P. aeruginosa* and *E. coli* strains GR12 and C1212. *E. coli* CO1

Figure 20. Immunofluorescence microscopy of *pilT* mutants with anti-PilA antibodies. Wildtype strain 109JA and the mutants are prey-independent isolates and therefore no prey cells are present. Any fluorescent signal is from a *Bd. bacteriovorus* cell. A representative image from each culture is shown. Cells that appear over exposed were excluded.



formed the best biofilm under the experimental conditions tested to allow for *Bdellovibrio* growth (Fig. 21). Wild type *Bd. bacteriovorus* 109JA reduced the mass of the biofilm by 65% while the *pilT2* mutant was unable to prey on the biofilm (Fig. 22). The *pilT1* mutant reduced the biofilm mass by 72%, slightly more than wildtype, which coincides with results observed in liquid cocultures where the *pilT1* mutant was slightly more aggressive in predation. Surprisingly, the *pilT1pilT2* double mutant was able to prey on the biofilm, but able to reduce the mass by only 49%. This indicates that although *pilT2* is required for predation on the biofilm, the *pilT1* deletion is able to partially restore function.

3.3.6. Scanning electron microscopy

Biofilm formation is a complex process that involves the secretion of many factors to create the extracellular polymeric substance (EPS). The EPS secreted by *E. coli* CO1 was seen as a dense, fibrous matrix that protects the cells beneath (Fig. 23A). Cells on the surface were not as protected as those buried beneath the EPS. *Bd. bacteriovorus* 109JA was able to penetrate the EPS layer and disrupt the biofilm. An overall loss in surface biofilm combined with the observation of bdelloplasts and many attack phase *Bd. bacteriovorus* (Fig. 23B) suggests that the EPS is not a barrier to predation. The *pilT1* mutant was also able to disrupt the biofilm (Fig. 23C). Less predation was observed with the *pilT2* and the double mutant (Fig. 23D and E). However, there was less EPS on the biofilm exposed to the *pilT1pilT2* mutant, indicating predation is continuing. Small structures thought to be outer membrane vesicles were observed in all samples tested, especially in the samples exposed to *Bd. bacteriovorus*.

Figure 21. Biofilm formation by *E. coli* strains. Variation in biofilm formation of *E. coli* strains was assessed by staining residual biofilm cells with crystal violet. *E. coli* CO1 shows the best biofilm formation under the experimental conditions used. Results shown are an average of 12 replicates repeated in triplicate.

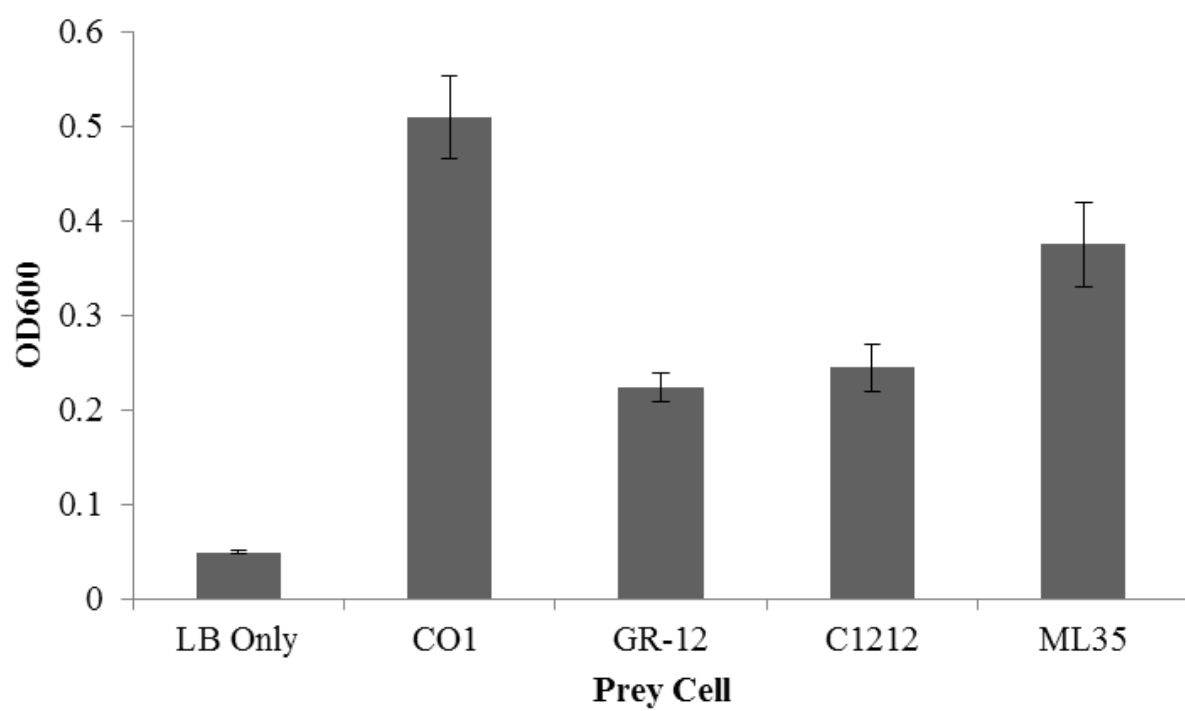


Figure 22. Disruption of the preformed *E. coli* CO1 biofilm by predation by *Bd. bacteriovorus* and the *pilT* mutants. Biofilms of *E. coli* CO1 were pre-formed for 48 h in 96-well microtiter plates. Predator cultures containing either *Bd. bacteriovorus* 109JA or a *pilT* mutant were added and the plates incubated for a further 24 h. Residual biofilm cells were stained with crystal violet and the optical density at 600nm (OD600) determined. To exclude secreted factors contributing to the decrease in remaining biofilm, a 0.45 μ m filtrate of *Bd. bacteriovorus* 109JA was used as a control. The percent biofilm remaining relative to the 109JA filtrate is shown on the secondary y-axis. Results shown are an average of 12 replicates repeated in triplicate. Statistical significance was measured using a 1-way ANOVA with a Bonferonni corrected post-hoc Students T-test, *p<0.005.

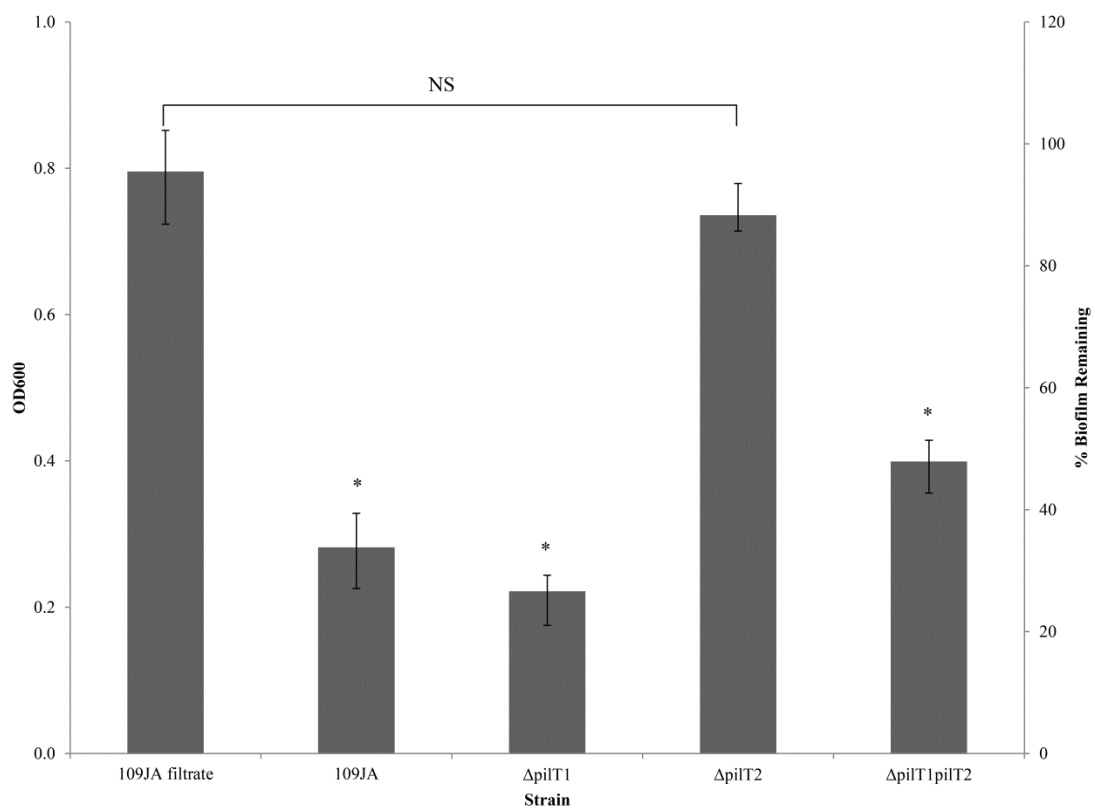
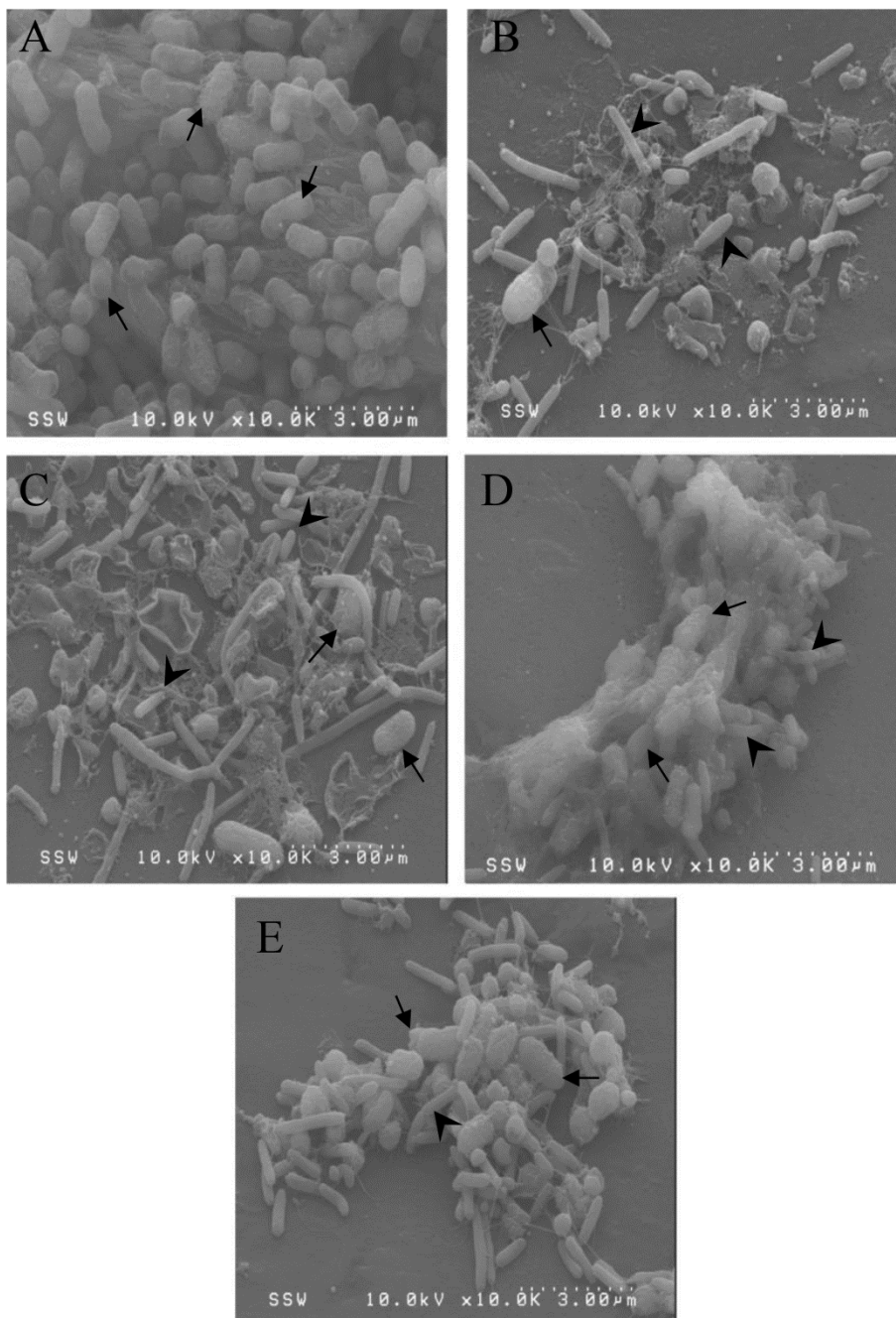


Figure 23. Scanning electron microscopy of predation on preformed biofilms of *E. coli* CO1. Biofilms of *E. coli* CO1 (arrows) were formed for 48 h on polyvinyl chloride plastic coverslips (A). Predator cultures (arrowheads) of *Bd. bacteriovorus* 109JA (B), $\Delta pilT1$ (C), $\Delta pilT2$ (D) or $\Delta pilT1pilT2$ (E) were added for a further 24 h.



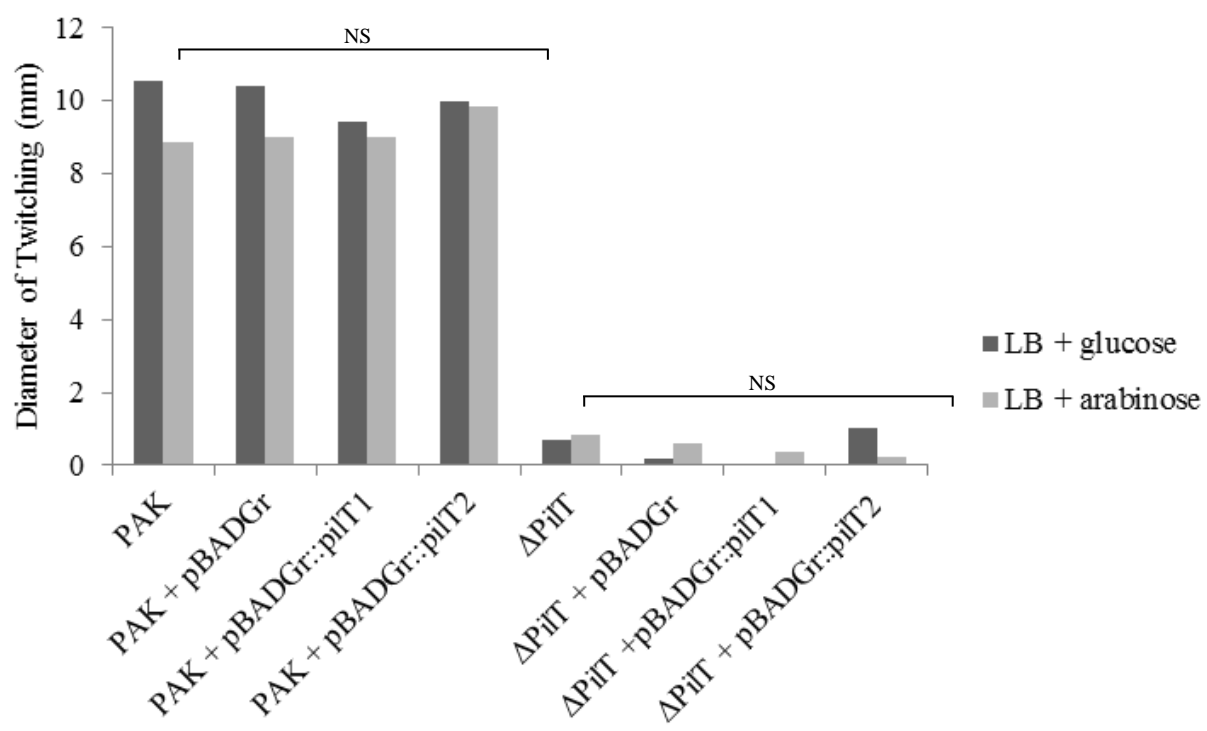
3.3.7. Expression of *Bd. bacteriovorus* PilT1 or PilT2 in *P. aeruginosa*

To determine whether PilT1 or PilT2 of *Bd. bacteriovorus* is able to restore twitching motility in a PilT mutant of *P. aeruginosa*, the genes were cloned into an arabinose inducible expression vector for *P. aeruginosa*. Overexpression of PilT1 or PilT2 at different arabinose concentrations could not be detected by SDS-PAGE (data not shown). The wild type *P. aeruginosa* PAK exhibited normal levels of twitching motility (Fig. 24). Assuming these genes are expressed, PilT1 or PilT2 did not inhibit the zone of motility in the wildtype strain. Unfortunately neither PilT1 nor PilT2 were able to restore motility in the PilT mutant of *P. aeruginosa*. These assays were repeated at different agar concentrations but yielded similar results.

3.4. Discussion

The mechanism that facilitates invasion of the prey cell periplasmic space by *Bd. bacteriovorus* is beginning to be understood. Publication of the genome sequence in 2004 allowed us to speculate which genes may be important for this process. Evans *et al.* (2007) and Mahmoud and Koval (2010) demonstrated that TFP are essential for predation to occur. Deletion of the gene for the major pilin protein, PilA, resulted in non-predacious *Bd. bacteriovorus*. Mahmoud and Koval (2010) raised antibodies against the PilA protein and showed that the presence of anti-PilA in cocultures was enough to delay and inhibit predation. No attachment between *Bd. bacteriovorus* and the prey cell was observed for 16 h, suggesting that TFP are important for attachment to prey cells. However, because these fibers are able to extend and retract, it was necessary to ask the question: Is retraction of TFP the driving force behind prey cell entry? This study has

Figure 24. Neither PilT1 nor PilT2 are able to complement twitching motility in a *P. aeruginosa* PilT mutant. *P. aeruginosa* PAK and a PilT mutant expressing PilT1 or PilT2 from *Bd. bacteriovorus* were used to assess twitching motility. PilT proteins were expressed on an arabinose inducible plasmid (pSSK10) and the *P. aeruginosa* cultures plated on LB agar containing 0.1% arabinose. As a control, 0.2% glucose was added to repress expression. The zone of motility was visualized using crystal violet and diameter of each zone measured.



demonstrated that PilT1 and PilT2 are not required for predation in liquid cocultures and, therefore, TFP must be necessary just for recognition of or attachment to a prey cell.

Bioinformatic analysis of the active domains present in PilT1 of *Bd. bacteriovorus* revealed that although it was annotated as a homologue of a PilT protein, it does not appear to function in this regard. It may be more similar to PilU in *P. aeruginosa*, which is thought to increase pilus strength or stability (Chiang and Burrows, 2003). This may account for the decrease in number of TFP assessed by negative staining of the *pilT1* mutant; however, there is very limited sequence similarity between the two proteins. This staining method may possibly shear the pilus during the preparation process, giving the appearance of decreased surface-associated TFP.

Bioinformatic analysis of PilT2 revealed high conservation within the active domains among other PilT proteins known to retract TFP, such as those encoded within the genomes of *P. aeruginosa* and *Neisseria gonorrhoeae*. Twitching motility could not be demonstrated in *Bd. bacteriovorus* 109JA or the *pilT* mutants, by use of a conventional sub-surface twitching motility assay (Asikyan *et al.*, 2008). Expression of *Bd. bacteriovorus* PilT1 or PilT2 in a *P. aeruginosa* PilT deletion mutant was unable to restore the twitching phenotype. Lambert *et al.* (2011) described *Bdellovibrio* gliding motility, a type of surface motility not flagellar or pilus-mediated. In this study, the *pilT* mutants were still able to use gliding motility, confirming this is independent of TFP and PilT1 or PilT2.

It is an unresolved question as to why so few cells of *Bd. bacteriovorus* express TFP on their surface in *in vitro* cocultures, yet TFP are essential for survival. It is not known what percentage of *Bd. bacteriovorus* cells in a batch culture are capable of

predation. It is possible that only a minority of the population are able to prey and therefore only this minority would express TFP. This could explain the overall lack of a hyperpiliated phenotype of the *pilT* mutants, which is observed in *P. aeruginosa* but does not occur in *Bd. bacteriovorus*. Both the *pilT1* and *pilT2* mutants show an increased (but small) level of TFP on the cell surface as assessed by immunofluorescence microscopy. This result suggests that these genes are involved in TFP retraction but does not elucidate what role each gene is playing, whether it be regulation or direct involvement.

The ability of *Bd. bacteriovorus* to disrupt and kill the cells within a biofilm has been studied previously (Dwidar *et al.*, 2012; Kadouri and O'Toole, 2005; Kadouri and Tran, 2013). This study has shown that *pilT2* is important for predation on a biofilm, confirming the results of Medina *et al.* (2008). However, the ability of the *pilT2* mutant to be predacious in liquid cocultures demonstrates that the retraction of TFP is not the driving force that allows prey cell invasion. SEM analysis revealed that an EPS layer is produced by *E. coli* cells during biofilm formation. This layer adds a level of protection from the outer environment but not to *Bd. bacteriovorus* predation, as predators were capable of penetrating and disrupting the biofilm. However, the *pilT2* and *pilT1pilT2* mutants did not do this as efficiently. It appears predation occurs on the cells at the surface of the biofilm. Eventually other cells would be preyed upon, but this would require a higher concentration of *Bdellovibrio* or more time. Taken together, the delay in predation on biofilms by *pilT2* and *pilT1pilT2* mutants is possibly due to the inability of these mutants to retract their TFP, and thus, unable to use twitching motility. The *pilT1* mutant still disrupted the biofilm and also partially restored the ability of the *pilT1pilT2*

mutant to prey on the biofilm; however, the mechanism by which this occurs requires further study.

These results argue against what is written in some *Bdellovibrio* literature: the retraction of TFP pulls the predator into the prey cell. The genome sequence of the epibiotic predator, *Bd. exovor* JSS, contains a fully annotated set of TFP genes, including both *pilT1* and *pilT2* (Pasternak *et al.*, 2014). In this species invasion does not occur, an observation that supports the results of this study that retraction of TFP is not the driving force behind the invasion process. A *PilT2* deletion in *Bd. exovor* could not be isolated as it would not form plaques on an agar surface. To avoid this problem prey-independent strains are generally used. However, the Koval laboratory and that of Jurkevitch (Pasternak *et al.*, 2014) have been unable to produce a prey independent derivative of *Bd. exovor* JSS.

The ability of most BALOs to penetrate the outer membrane of a Gram-negative cell without membrane fusion, in direct opposition of the internal turgor pressure of the prey cell, still remains an enigma. Other mechanisms must be at play to allow this event to occur. Pasternak *et al.* (2014) hypothesized that the epibiotic predator *Bd. exovor* evolved from a periplasmic predator by gene loss, including the loss of the genes that enable prey invasion. The identification of such genes would be step forward in understanding the invasion process.

3.5. References

- Asikyan, M.L., Kus, J.V. and Burrows, L.L. (2008) Novel proteins that modulate type IV pilus retraction dynamics in *Pseudomonas aeruginosa*. *J Bacteriol.* **190**: 7022-7034.
- Aukema, K.G., Kron, E.M., Herdendorf, T.J. and Forest, K.T. (2004) Functional dissection of a conserved motif within the pilus retraction protein PilT. *J Bacteriol.* **187**: 611-618.

- Bradley, D.E. (1980) A function of *Pseudomonas aeruginosa* PAO pili: twitching motility. *Can J Microbiol* **26**: 146–154.
- Chanyi, R.M., Ward, C., Pechey, A. and Koval, S.F. (2013) To invade or not to invade: two approaches to a prokaryotic predatory life cycle. *Can J Microbiol* **59**: 273-279.
- Chiang, P. and Burrows, L.L. (2003) Biofilm formation by hyperpiliated mutants of *Pseudomonas aeruginosa*. *J Bacteriol* **185**: 2374-2378.
- Chiang, P., Sampaleanu, L.M., Ayers, M., Pahuta, M., Howell PL, and Burrows, L.L. (2008) Functional role of conserved residues in the characteristic secretion NTPase motifs of the *Pseudomonas aeruginosa* type IV pilus motor proteins PilB, PilT and PilU. *Microbiology* **154**: 114-126.
- Cotter, T.W. and Thomashow, M.F. (1992) A conjugation procedure for *Bdellovibrio bacteriovorus* and its use to identify DNA sequences that enhance the plaque-forming ability of a spontaneous host-independent mutant. *J Bacteriol* **174**: 6011-6017.
- Dwidar, M., Hong, S., Cha, M., Jang, J. and Mitchell, R.J. (2012) Combined application of bacterial predation and carbon dioxide aerosols to effectively remove biofilms. *Biofouling* **28**: 671-680.
- Evans, K.J., Lambert, C. and Sockett, R.E. (2007) Predation by *Bdellovibrio bacteriovorus* HD100 requires type IV pili. *J Bacteriol* **189**: 4850-4859.
- Kadouri, D. and O'Toole, G.A. (2005) Susceptibility of biofilms to *Bdellovibrio bacteriovorus* attack. *Appl Environ Microbiol* **71**: 4044-4051.
- Kadouri, D.E. and Tran, A. (2013) Measurement of predation and biofilm formation under different ambient oxygen conditions using a simple gasbag-based system. *Appl Environ Microbiol* **79**: 5264-5271.
- Koval, S.F. and Hynes, S.H. (1991) Effect of paracrystalline protein surface layers on predation by *Bdellovibrio bacteriovorus*. *J Bacteriol* **17**: 2244-2249.
- Koval, S.F., Hynes, S.H., Flannagan, R.S., Pasternak, Z., Davidov, Y., and Jurkevitch, E. (2013) *Bdellovibrio exovorus* sp. nov., a novel predator of *Caulobacter crescentus*. *IJSEM* **63**: 146-151.
- Lambert, C., Fenton, A.K., Hobley, L. and Sockett, R.E. (2011) Predatory *Bdellovibrio* bacteria use gliding motility to scout for prey on surfaces. *J Bacteriol* **193**: 3139-3141.
- Mahmoud, K.K. and Koval, S.F. (2010) Characterization of type IV pili in the life cycle of the predator bacterium *Bdellovibrio*. *Microbiology* **156**: 1040-1051.

Maier, B., Potter, L., So, M., Long, C.D., Seifert, H.S. and Sheetz, M.P. (2002) Single pilus motor forces exceed 100 pN. *Proc Natl Acad Sci USA* **99**:16012-16017.

Medina, A.A., Shanks, R.M. and Kadouri, D.E. (2008) Development of a novel system for isolating genes involved in predator-prey interactions using host independent derivatives of *Bdellovibrio bacteriovorus* 109J. *BMC Microbiol* **8**: 33.

Miller, V.L. and Mekalanos, J.J. (1988) A novel suicide vector and its use in construction of insertion mutations - osmoregulation of outer-membrane proteins and virulence determinants in *Vibrio cholerae* requires ToxR. *J Bacteriol* **170**: 2575-2583.

Pasternak, Z., Njagi, M., Shani, Y., Chanyi, R., Rotem, O., Lurie-Weinberger, M.N., Koval, S., Pietrokovski, S., Gophna, U. and Jurkevitch, E. (2014) In and out: an analysis of epi-biotic vs periplasmic bacterial predators. *ISME J* **8**: 625-635.

Reid, G., Zorzitto, M.L., Bruce, A.W., Jewett, M.A.S., Chan, R.C.Y. and Costerton, J.W. (1984) Pathogenesis of urinary tract infection in the elderly: the role of bacterial adherence to uroepithelial cells. *Curr Microbiol* **11**: 67-72.

Rendulic, S., Jaqtap, P., Rosinus, A., Eppinger, M., Baar, C., Lanz, C., Keller, H., Lambert, C., Evans, K.J., Goesmann, A., Meyer, F., Sockett, R.E. and Schuster, S.C. (2004) A predator unmasked: life cycle of *Bdellovibrio bacteriovorus* from a genomic perspective. *Science*. **303**: 689-692.

Simon, R., Priefer, U. and Pühler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Nat Biotechnol* **1**: 784-791.

Steyert, S.R. and Piñeiro, S.A. (2007) Development of a novel genetic system to create markerless deletion mutants of *Bdellovibrio bacteriovorus*. *Appl Environ Microbiol* **73**: 4717-4724.

Wu, S.S. and Kaiser, D. (1995) Genetic and functional evidence that type IV pili are required for social gliding motility in *Myxococcus xanthus*. *Mol Microbiol* **18**: 547-558.

Chapter 4

4. Dual use of *Bdellovibrio exovorus* and antibiotics against the opportunistic cystic fibrosis pathogen *Stenotrophomonas maltophilia**

4.1. Introduction

Stenotrophomonas maltophilia is a Gram-negative, rod shaped bacterium that can be isolated from the root nodules of many plants. The presence of *S. maltophilia* in the soil has been linked to providing a growth advantage to many plants using a variety of mechanisms (Berg, 2009). The ability to colonize the surface of the nodule and the secretion of lytic enzymes that kill plant pathogens are of greatest interest for agricultural biocontrol practices. *S. maltophilia* has also been studied in its capacity for bioremediation. It is highly resistant to many metals, including cadmium, lead, cobalt, zinc and arsenic. It also has the ability to degrade polycyclic aromatic hydrocarbons such as *p*-nitrophenol and 4-chlorophenol, selenium compounds, benzene, toluene and ethylbenzene. Unfortunately these attributes that make *S. maltophilia* an attractive tool also aid in its ability to survive within an immunocompromised patient.

S. maltophilia is now considered an emerging Gram-negative multidrug-resistant opportunistic pathogen. Infections usually occur in immunocompromised individuals and have been associated with pneumonia (Fujita *et al.*, 1996), bacteremia (Kremery *et al.*, 2004; Lai *et al.*, 2004), urinary tract and soft tissue infections (Bin Abdulhak *et al.*, 2009; Landrum *et al.*, 2005; Vartivarian *et al.*, 1994), eye infections (Lin *et al.*, 2011; Mauger *et al.*, 2010), endocarditis (Aydin *et al.*, 2000; Gutiérrez Rodero *et al.*, 1995) and meningitis

(Nguyen and Muder, 1994; Rojas *et al.*, 2004). In contrast to many other well studied pathogens, *S. maltophilia* is not considered highly virulent as its genome does not encode for many virulence genes. It relies almost entirely on its ability to colonize surfaces, form biofilms and adapt quickly to harsh environments to establish infections. *S. maltophilia* has been isolated along with *P. aeruginosa* from respiratory samples of cystic fibrosis patients and this prevalence is increasing (Razvi *et al.*, 2009). However, it is controversial whether this disease severity can be linked to the presence of *S. maltophilia* (Goss *et al.*, 2002; Goss *et al.*, 2004; Karpati *et al.*, 1994).

Similar to *P. aeruginosa*, *S. maltophilia* is highly antibiotic resistant. Although there is a wide degree of strain variability, resistance has been observed against trimethoprim-sulphamethoxazole, β -lactam antibiotics, macrolides, cephalosporins, fluoroquinolones, aminoglycosides, carbapenems, chloramphenicol, tetracyclines and polymyxins. The use of antibiotics in a clinical setting is not to blame for this resistance as many environmental isolates are intrinsically resistant (Martinez, 2008).

As traditional methods are becoming less effective at controlling infections, we must develop novel therapeutics. One such example is the use of phage therapy. Phages have been isolated from sputum samples, catheters and pleural effusions that target *S. maltophilia* (Chang *et al.*, 2005). Phages tested against a panel of 87 *S. maltophilia* hospital isolates were found to be strain-specific (Donlan, 2009). Therefore, their use as a therapeutic would not be effective as the host range is far too narrow. There are also reservations about using phage therapy as phages have the ability to transfer resistance genes to their host, thereby making the target bacterium more resistant than it originally

was. Therefore, the use of a broad acting lysogenic agent with less risk to the patient would be ideal.

Another novel therapeutic agent would be the use of a bacterial predator. *Bdellovibrio* and like organisms (BALOs) are a group of Gram-negative obligate predators of other Gram-negative bacteria. These predators are ubiquitous in the environment and have been isolated from soil, compost, raw sewage, activated sludge and marine and terrestrial waters. These bacteria typically have a broad prey range; however, they are unable to attack Gram-positive or eukaryotic cells, making them candidates for use as a live antimicrobial against Gram-negative pathogens (Dashiff and Kadouri, 2011; Dashiff *et al.*, 2011; Harini *et al.*, 2013). There have been studies on the ability of *Bd. bacteriovorus* to disrupt biofilms (Dwidar *et al.*, 2012; Kadouri and O'Toole, 2005; Kadouri and Tran, 2013; Van Essche *et al.* 2009), even ones formed by the Gram-positive pathogen *Staphylococcus aureus* (Monappa *et al.*, 2014).

In a survey of susceptibility of human pathogens to predation by *Bd. bacteriovorus* 109J, Dashiff *et al.* (2011) reported that 6 clinical isolates of *S. maltophilia* were not used as a prey cells. Therefore, the Koval lab sought to isolate a new predator using enrichment cultures with *S. maltophilia* as a prey cell. This study reports the successful isolation of a BALO on a clinical isolate of *S. maltophilia*. Interestingly this new predator had the highest 16S rRNA gene sequence similarity to *Bd. exovorus*, known for its unique epibiotic life cycle (Koval *et al.*, 2013).

Annotation of the genome of *Bd. exovorus* shows a high level of antibiotic resistance genes (Pasternak *et al.*, 2014). As *S. maltophilia* is also resistant to many antimicrobials, in a large part due to its ability to colonize and form biofilms, the question

arises whether the addition of the environmental predator would disrupt biofilms of *S. maltophilia*. Also, would this disruption occur in the presence of antibiotics and could this have a synergistic effect on eliminating *S. maltophilia*?

This study demonstrates that *Bd. exovorus* alone is capable of disrupting biofilms of both clinical and environmental isolates of *S. maltophilia*. There was a synergistic effect in biofilm disruption of a few *S. maltophilia* isolates when using ciprofloxacin and *Bd. exovorus* together.

4.2. Materials and Methods

4.2.1. Bacterial strains, media and culture conditions

Bacterial strains used in this study are listed in Table 6. *Escherichia coli* ML35, *Pseudomonas aeruginosa* PAK and *Burkholderia cenocepacia* K56-2 were grown overnight in LB medium at 37°C. Isolates of *S. maltophilia* were obtained from J.S. Brooke (DePaul University) and grown in LB Lennox medium overnight at 37°C. Cocultures were prepared by mixing 3 ml of prey cells with 1 ml of an active predator culture in 20 ml of HM buffer (3 mM HEPES, pH 7.6, with 1 mM CaCl₂ and 0.1 mM MgSO₄). Prey cells were washed in HM buffer and concentrated 10-fold. Cocultures were incubated at 37°C overnight with vigorous shaking (Chanyi *et al.*, 2013). Predation was assessed by light microscopy.

4.2.2. Isolation of environmental predators

Environmental BALOs were isolated from raw sewage collected from the Adelaide Street Pollution Control Plant, London, Ontario, using *Stenotrophomonas maltophilia* X26332 as a prey cell in enrichment cultures (Chanyi *et al.*, 2013). An overnight culture of prey cells was washed and resuspended in dilute nutrient broth

Table 6. Bacterial strains used in this study.

Bacterial Strains	Source/Reference
Predators	
<i>Bdellovibrio bacteriovorus</i> 109J	Chanyi <i>et al.</i> (2013)
<i>Bdellovibrio exovorus</i> JSS	Koval <i>et al.</i> (2013)
<i>Bdellovibrio exovorus</i> FFRS-5	This Study
Prey Cells	
<i>Burkholderia cenocepacia</i> K56-2	Chanyi <i>et al.</i> (2013)
<i>Caulobacter crescentus</i> KSAC	John Smit, University of British Columbia
<i>Escherichia coli</i> ML35	Koval and Hynes (1991)
<i>Pseudomonas aeruginosa</i> PAK	Lori Burrows, McMaster University
<i>Stenotrophomonas maltophilia</i>	
H59246	Infection
X26332	Infection
C3 3:00(76)2	Hospital Environment
C3 3:00(72)1	Hospital Environment
F69694	Infection
H2138	Infection
S18202	Infection
C1 3:00(78)	Hospital Environment
D2 6:15(19)1	Hospital Environment
D3 6:00(79)	Hospital Environment
H43306	Infection
D1 6:15(51)2	Hospital Environment
D1 1:15(76)1	Hospital Environment
C2 7:30(74)2	Hospital Environment
F28889	Infection
C2 7:30(75)1	Hospital Environment
D3 5:00(77)	Hospital Environment
F7221	Infection
JB12-23	LPS Mutant

(DNB). DNB was added to the sewage sample (1:4 v/v) and incubated at 30°C for 1 h. The suspension was then centrifuged at 3 000 g and an equal volume of raw sewage supernatant and washed prey cells were combined. The enrichment coculture was incubated at 30°C for 48 h and monitored by light microscopy. After incubation, serial ten-fold dilutions of the enrichment culture were prepared in HM buffer and plated using the double layer agar technique (Koval and Hynes, 1991; Koval, 2007; Chanyi *et al.*, 2013) with DNB medium. Plates were incubated at 30°C for 5 days. A pasteur pipette was used to remove an agar 'plug' from a well isolated plaque. The plug was put into 5 ml HM buffer and vortexed vigorously before 1 ml of washed *S. maltophilia* X26332 cells was added. This culture was incubated at 30°C and monitored by light microscopy for signs of predation. All BALOs were plaque purified 3 times.

4.2.3. Identification of environmental isolates

All environmental isolates were identified by 16S rRNA gene sequencing. Cocultures were incubated until predation was complete, generally 48 h. To remove residual prey cells, the coculture was centrifuged at 2 000 g for 10 min. The supernatant was filtered once through a 0.45 µm filter and the filtrate was centrifuged at 7 000 g to concentrate the BALOs. Cell suspensions were boiled for 10 min before being added to the PCR reaction. The universal primers 63F (CAGGCCTAACACATGCAAGTC) and 1387R (GGCGGTGTGTACAAGGC) were used to amplify approximately 1300 bp of the 16S rRNA gene using *i-pfu* polymerase (FroggaBio). The thermocycle conditions used were 94°C for 2 min, 30 cycles of 94°C for 30 sec, 52°C for 30 sec and 72°C for 90 sec. A final 5 min elongation at 72°C ensured all DNA was amplified.

PCR products were purified using the Illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare) according to manufacturer's directions. For DNA sequencing, purified samples were sent to the Robarts Research Institute DNA Sequencing Facility at the University of Western Ontario (London, Ontario, Canada). Sequencing results were compared for alignment with known 16S rRNA gene sequences using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) for nucleotide sequences.

4.2.4. Prey range assessment

Two methods were used to assess predation on other prey cells: an agar predation assay and liquid cocultures.

4.2.4.1. Agar predation assay: *B. cenocepacia* K56-2, *E. coli* ML35, *P. aeruginosa* PAK and *S. maltophilia* X26332 were grown as described in 4.2.1. Prey cells were centrifuged at 5 000 g and resuspended in HM buffer to 1/10 or 1/20 of the original culture volume in order to maximize lawn density. Two hundred microliters were added to 3.5 mL of 0.6% DNB agar and poured over a 1.5% DNB agar plate. The agar lawn was allowed to solidify for approximately 30 min before plates were divided into sectors and 10 µl of each BALO sample was spotted onto the lawn. Clearing of the prey cell lawn was assessed as complete or partial clearing, or plaques within the area of the aliquot. An equal aliquot of HM buffer was used on each plate as a negative control. *Bd. bacteriovorus* 109J was used as a positive control on *E. coli* ML35.

4.2.4.2. Liquid cocultures: A fresh coculture of *Bd. exovorus* FFRS-5 incubated for 48 h was used for all assays. Cocultures were filtered through 0.45 µm nitrocellulose

filters to remove excess prey cells prior to being used in liquid coculture predation assessments. All predator-prey combinations were tested on at least two different occasions and were incubated at 37°C for up to 5 days. Predation was monitored by phase contrast light microscopy. For predation kinetic assays, 6 ml of prey cells were concentrated and resuspended in 1 ml HM buffer. Cocultures were prepared as described in 4.2.1. and aliquoted (200 µl per well) into 5 wells of a 100-well BioScreen plate. Identical cultures were prepared without the addition of the predator and aliquoted into another 5 wells of a BioScreen plate. The OD₆₀₀ was measured every 15 min for 3 days with continuous shaking at 37°C. An average of the 5 wells at each time point.

4.2.5. *Bd. exovorus* FFRS-5 predation efficiency on *S. maltophilia* isolates

All isolates of *S. maltophilia* were grown overnight in LB Lennox medium at 37°C. Prey cultures were prepared by centrifuging 6 ml at 7 000 *g* for 5 min. The pellet was resuspended in 1 ml HM buffer. The predator was prepared by filtering a 48 h coculture of *S. maltophilia* X26332 and *Bd. exovorus* FFRS-5 through a 0.45 µm nitrocellulose filter. The prey cells were added to 20 ml HM buffer and 1 ml of the predator in the filtrate was then added. Pure prey cell cultures were also prepared by adding 1 ml HM buffer instead of the predator. To obtain the initial concentration of prey cells, 10-fold serial dilutions were made of each culture. Ten microliters of each dilution was spot-plated onto 1.2% LB Lennox agar and incubated overnight at 37°C. Liquid cultures were then incubated for 48 h to allow predation to continue to completion. Spot plating was repeated to obtain the final concentration (CFU) of prey cells. Each dilution was plated in triplicate and the number of colonies were averaged across the different dilutions and replicates.

4.2.6. Predation assay with antibiotics

S. maltophilia X26332 was prepared for cocultures as described in Section 4.2.1. A 48 h coculture of *Bd. exovorus* FFRS-5 and *S. maltophilia* was filtered through a 0.45 µm filter. Twenty millilitre cocultures and prey-only cultures were prepared and prey cell concentration determined by spot plating 10-fold serial dilutions on 1.2% LB Lennox agar. Antibiotics were added at the following concentrations: ampicillin (1, 4, 8, 16 and 50 µg/ml), ceftazidime (1, 4, 8, 16, 32, 64 and 128 µg/ml), ciprofloxacin (1, 5, 10 and 20 µg/ml), chloramphenicol (16, 32, 64 and 128 µg/ml), colicin (32, 64, 128 and 256 µg/ml), kanamycin (16, 32, 64 and 128 µg/ml) and polymyxin B (1, 4, 8, 16 µg/ml). Cocultures were incubated for 48 h at 37°C with vigorous shaking. Spot-plating was repeated after 48 h and final prey cell concentration determined. Cultures were monitored by phase contrast light microscopy to observe any alteration in predator or prey cell morphology, relative prey cell concentration and signs of predation. Ciprofloxacin and kanamycin were chosen for further study to determine the maximal concentration of antibiotic to use without inhibiting predation. Cultures were prepared as above, using ciprofloxacin (1, 5, 10 and 20 µg/ml) or kanamycin (1, 5, 10 and 25 µg/ml).

4.2.7. Biofilm disruption assay

Preformed biofilms were used to assess predation of *Bd. exovorus* FFRS-5 on *S. maltophilia* in the presence and absence of antibiotics [ciprofloxacin (10 µg/ml), kanamycin (5µg/ml) and both together]. Biofilms of *S. maltophilia* were formed in flat-bottom 96-well plates using the method of Di Bonaventura *et al.* (2004) with modifications. Overnight cultures of *S. maltophilia* in 5 ml of LB Lennox were diluted with fresh LB Lennox to achieve a culture with an OD₆₀₀ of 1.0. Twenty microliters were

used to inoculate 5 ml of fresh LB Lennox medium and then incubated at 37°C for 2 h. Aliquots (20 µl) of the standardized inoculum were added to the wells of sterile flat-bottom polystyrene 96-well plate (Cellstar), and incubated at 37°C overnight. Each well was washed three times with HM buffer in order to remove planktonic cells. *Bd. exovor* was prepared as described earlier (2.2.1.) and diluted 3-fold with HM buffer. Each *S. maltophilia* biofilm was tested under the following conditions: HM buffer alone, HM buffer with ciprofloxacin (10 µg/ml), HM buffer with kanamycin (5 µg/ml), HM buffer with ciprofloxacin (10 µg/ml) and kanamycin (5 µg/ml), *Bd. exovor* FFRS-5 alone, *Bd. exovor* FFRS-5 with ciprofloxacin (10 µg/ml), *Bd. exovor* FFRS-5 with kanamycin (5 µg/ml) and *Bd. exovor* FFRS-5 with ciprofloxacin (10 µg/ml) and kanamycin (5 µg/ml). Each 96-well plate was incubated for another 48 h at 37°C.

Quantification of biofilm mass was performed as follows. The wells were washed 3 times with HM buffer in order to remove planktonic cells and 200 µl crystal violet was added and incubated at room temperature for 15 min. Wells were washed with ddH₂O 4 times and allowed to dry overnight. Crystal violet was solubilized by adding 200 µl of 30% acetic acid for 20 min. An aliquot (125µl) of each well was transferred to a flat-bottom microtiter dish and analyzed using a microplate reader at 600 nm.

Enumeration of *S. maltophilia* isolates remaining in the biofilm was performed by preparing biofilm samples as described above. After biofilms were incubated in the presence of antibiotics and *Bd. exovor* FFRS-5, remaining biofilms were washed three times with PBS. Then 200 µl of PBS was added to each well and the biofilm was solubilized by sonication for 5 min in a sonicating water bath (VWR). Serial dilutions of the contents of each well were performed and 10 µl of each dilution was spot-plated onto

LB Lennox agar and incubated overnight at 37°C. Statistical significance was measured using a 1-way ANOVA with a Bonferonni corrected post-hoc Students T-test, * $p < 0.05$, ** $p < 0.005$.

4.2.8. Electron microscopy

For transmission electron microscopy, 5 ml samples of co-cultures were centrifuged, resuspended in 1.5 ml fixative (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) and left overnight at 4°C. Cells were then fixed with 1% (w/v) osmium tetroxide and 1% (w/v) uranyl acetate and enrobed in agar. Samples were dehydrated in an ethanol series and embedded in LR White resin. Thin sections were cut and stained with 2% uranyl acetate and lead citrate. Negative stains were prepared with uranyl acetate. All preparations were visualized using a Philips CM10 transmission electron microscope operating at 80 kV. Digital micrographs were obtained using an SIS/Olympus Morada 11 megapixel CCD camera.

4.3. Results

4.3.1. Isolation and identification of environmental predators

Bd. bacteriovorus 109J was unable to prey on any of the *S. maltophilia* isolates listed in Table 6. Therefore, a predator was isolated from the environment. Enrichment cultures were prepared from raw sewage collected at the Adelaide Street Pollution Control Plant (London, ON) during the summer months using *S. maltophilia* X26332 as a prey. This strain was chosen as it is the parent strain to the LPS mutant, strain JB12-23, which was being used in other studies. Multiple predators were isolated, and 10 were chosen for further study. All cocultures revealed the presence of ghost prey cells and an absence of bdelloplasts, observations that are typical of an epibiotic predator. Isolate

FFRS-5 appeared to be the most active predator and was therefore used in further studies. The sequence of the 16S rRNA gene of strain FFRS-5 showed 100% sequence similarity to that of *Bd. exovorus* JSS^T. Thus the environmental isolate was denoted, *Bd. exovorus* FFRS-5.

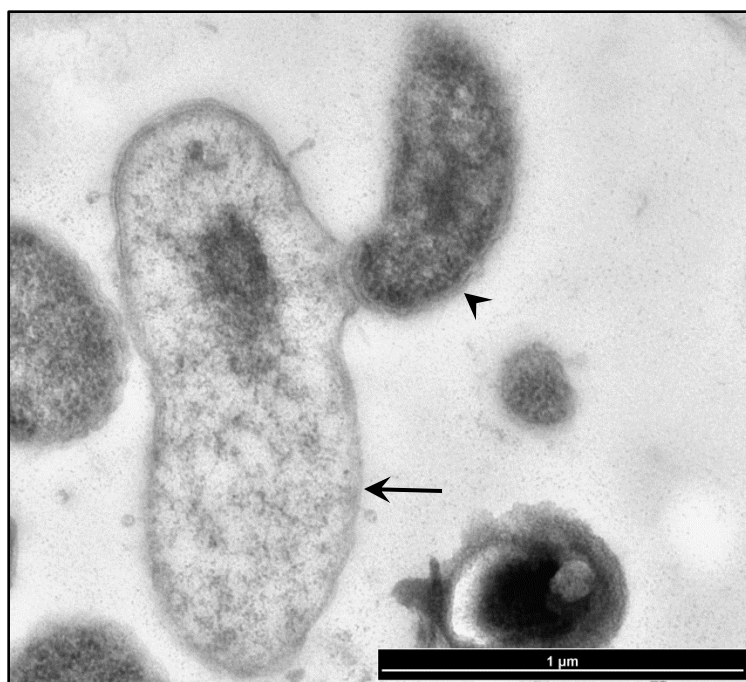
4.3.2. Prey range assessment of *Bd. exovorus* FFRS-5

Predation by *Bd. exovorus* FFRS-5 on *E. coli*, *P. aeruginosa*, *B. cenocepacia* and *C. crescentus* was assessed using liquid cocultures and an agar predation assay. Similar to *Bd. exovorus* JSS, strain FFRS-5 was able to prey on *C. crescentus* while unable to prey on *E. coli*, *P. aeruginosa* and *B. cenocepacia*. Strain JSS was also capable of preying on *S. maltophilia*. A previous report identified other potential prey cells for the type strain *Bd. exovorus* JSS (Chanyi *et al.*, 2013); however, none were as susceptible as the prey *S. maltophilia*.

4.3.3. Electron microscopy

Both negative staining and thin sections of cocultures of *S. maltophilia* and *Bd. exovorus* FFRS-5 confirmed the presence of ghost cells but, more importantly, they confirmed the absence of bdelloplasts. This is the first characterization of an epibiotic predator isolated on a prey cell other than *C. crescentus*. Negative staining confirmed that strain FFRS-5 was a small, vibroid shaped cell with a polar sheathed flagellum (data not shown). Growth of strain FFRS-5 followed similar steps as did strain JSS. Predation involved attachment to the prey cell with no membrane fusion occurring. The predator remained at the surface of the prey cell (Fig. 25), where the pole appears to penetrate into the periplasmic space, which is also observed with strain JSS. Division by binary fission

Figure 25. Epibiotic life cycle of *Bd. exovorus* FFRS-5 confirmed by thin section transmission electron microscopy. Thin section of *Bd. exovorus* FFRS-5 (black arrowhead) at the surface of *S. maltophilia* (black arrow) cell during predation. A small amount of cytoplasmic contents remains in the *S. maltophilia* prey cell.



was observed, coincidental with a loss of cytoplasmic content in the prey cell. These observations confirm that strain FFRS-5 utilizes an epibiotic life cycle.

4.3.4. Predation efficiency of *Bd. exovorus* FFRS-5 on *S. maltophilia* isolates

Isolates of *S. maltophilia* were obtained from both clinical samples and from hospital surfaces (such as bathroom sinks). Predation by *Bd. exovorus* FFRS-5 was observed on all isolates of *S. maltophilia* regardless of their source of isolation (Table 7).

Environmental isolate D3 6:00(79) was the most susceptible to predation as there was almost a 3-log reduction in bacterial concentration when exposed to *Bd. exovorus* FFRS-5 while the concentration of isolate D1 1:15 (76) 1 decreased only 1-log, corresponding to approximately 93% killing by *Bd. exovorus* FFRS-5 (Fig. 26). Prey cell lysis did not occur in cultures without *Bd. exovorus* FFRS-5, with the exception of prey cell isolate F28889 (Fig. 26). Although prey cell lysis did occur, with the addition of *Bd. exovorus* FFRS-5 there was a further decrease in prey cell numbers indicating that predation was occurring.

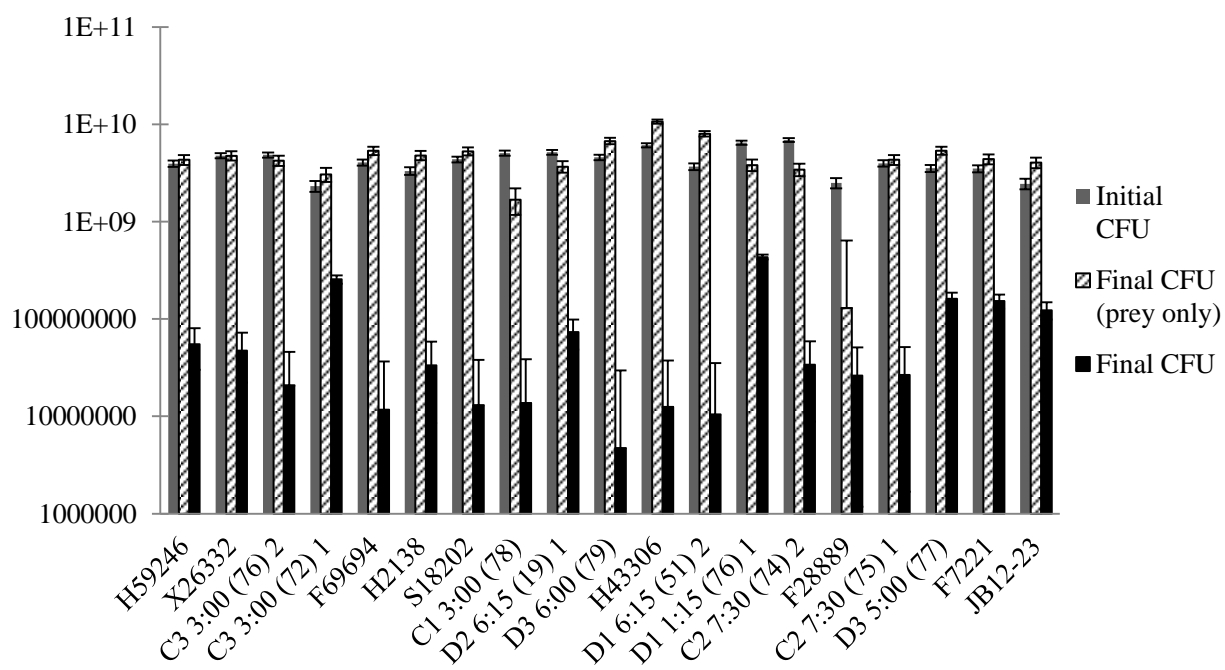
Overall, there was an average of a 2-log decrease in prey cell concentration. Cocultures were left for up to 5 days with no additional decrease in prey cell concentration. Minor variations in predation efficiency were detected amongst different isolates; however, the ability for *Bd. exovorus* FFRS-5 to prey on *S. maltophilia* is fairly conserved and no isolate was resistant to predation (Fig. 26). There was no preference for a clinical or an environmental isolate of *S. maltophilia* to be used as a prey cell by *Bd. exovorus* FFRS-5.

Table 7. *Bd. exovorus* FFRS-5 preys on all *S. maltophilia* isolates. Predation efficiency of *Bd. exovorus* FFRS-5 on 19 *S. maltophilia* isolates in liquid cocultures. Cocultures were incubated for 48 h at 37°C. Colony forming units (CFU) indicate the prey cell concentration in cocultures. Decrease in bacterial concentration expressed as a \log_{10} decrease and percent decrease.

Prey Isolate*	Initial CFU	Final CFU	Log Decrease	Percent Decrease
H59246	3.93 x 10 ⁹	5.52 x 10 ⁷	1.85	98.6
X26332	4.77 x 10 ⁹	4.73 x 10 ⁷	2.00	99.0
C3 3:00 (76) 2	4.85 x 10 ⁹	2.08 x 10 ⁷	2.37	99.6
C3 3:00 (72) 1	2.32 x 10 ⁹	2.56 x 10 ⁸	0.96	88.9
F69694	4.05 x 10 ⁹	1.17 x 10 ⁷	2.54	99.7
H2138	3.32 x 10 ⁹	3.35 x 10 ⁷	2.0	99.0
S18202	4.37 x 10 ⁹	1.30 x 10 ⁷	2.53	99.7
C1 3:00 (78)	5.07 x 10 ⁹	1.37 x 10 ⁷	2.57	99.7
D2 6:15 (19) 1	5.17 x 10 ⁹	7.33 x 10 ⁷	1.85	98.6
D3 6:00 (79)	4.58 x 10 ⁹	4.70 x 10 ⁶	2.99	99.9
H43306	6.10 x 10 ⁹	1.25 x 10 ⁷	2.69	99.8
D1 6:15 (51) 2	3.68 x 10 ⁹	1.04 x 10 ⁷	2.55	99.7
D1 1:15 (76) 1	6.47 x 10 ⁹	4.33 x 10 ⁸	1.17	93.3
C2 7:30 (74) 2	6.90 x 10 ⁹	3.38 x 10 ⁷	2.31	99.5
F28889	2.50 x 10 ⁹	2.62 x 10 ⁷	1.98	99.0
C2 7:30 (75) 1	4.0 x 10 ⁹	2.67 x 10 ⁷	2.17	99.3
D3 5:00 (77)	3.53 x 10 ⁹	1.62 x 10 ⁸	1.34	95.4
F7221	3.50 x 10 ⁹	1.52 x 10 ⁸	1.36	95.6
JB12-23	2.45 x 10 ⁹	1.23 x 10 ⁸	1.30	95.0

**Stenotrophomonas maltophilia* isolate

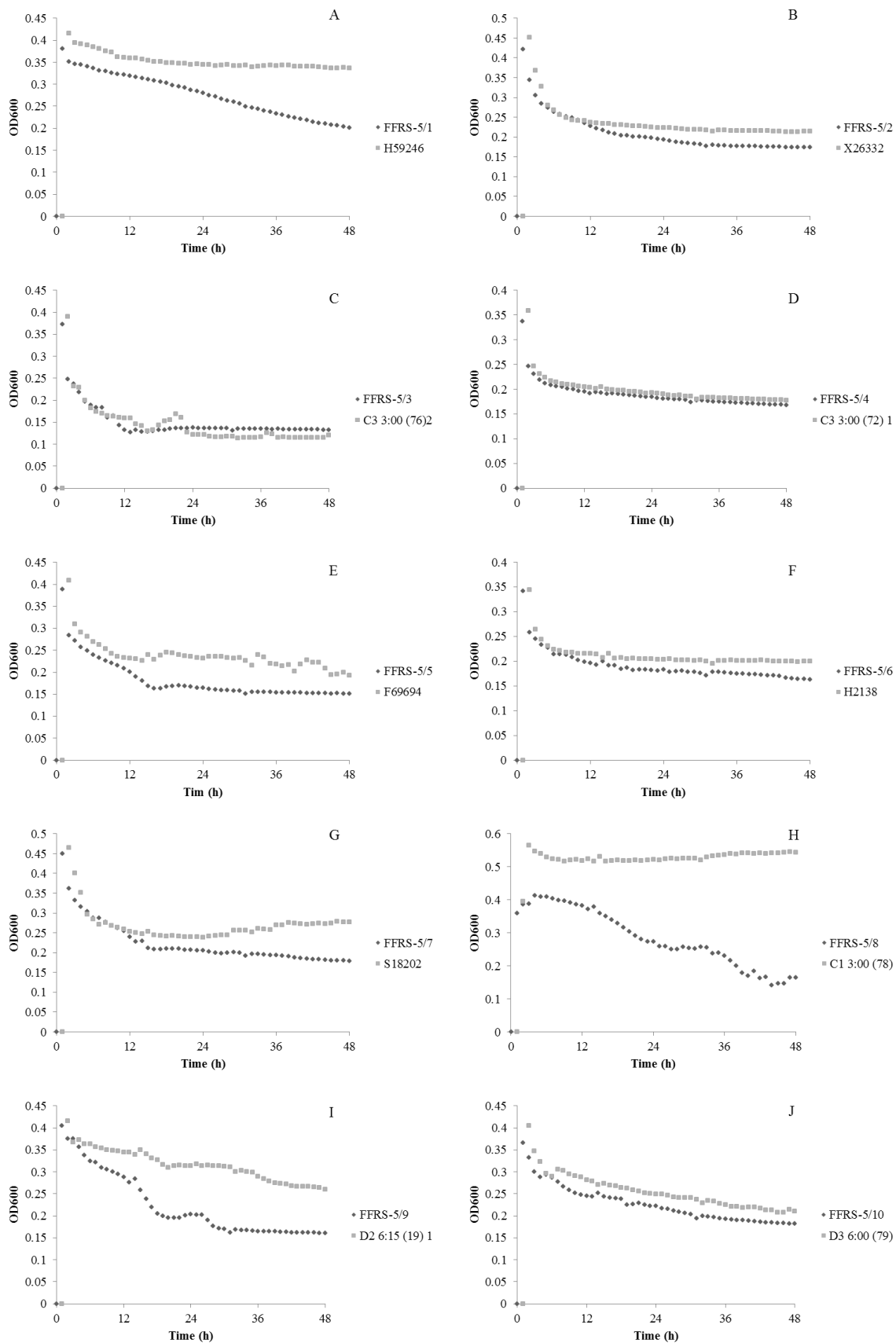
Figure 26. *Bd. exovorus* FFRS-5 is able to prey on all *S. maltophilia* isolates. *Bd. exovorus* FFRS-5 is able to prey on all environmental isolates of *S. maltophilia* to varying degrees, as assessed by a liquid predation assay. The initial CFU (grey) was determined prior to the addition of *Bd. exovorus* FFRS-5. The final CFU (black) was determined after 48 h of predation. A prey only culture was subjected to the same conditions to control for prey cell lysis in the coculture buffer (striped). Experiment was repeated in duplicate, value representing the mean and standard error from one representative experiment.

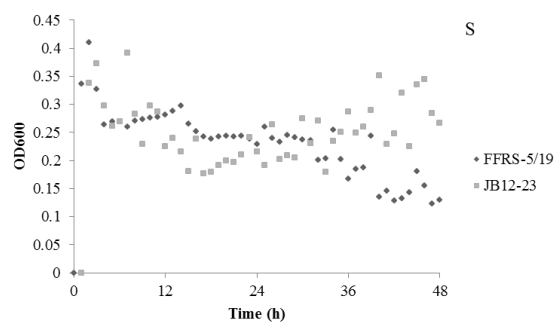
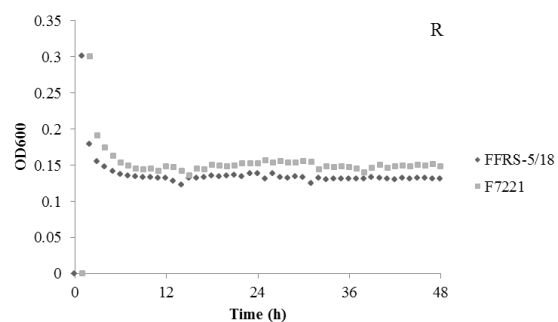
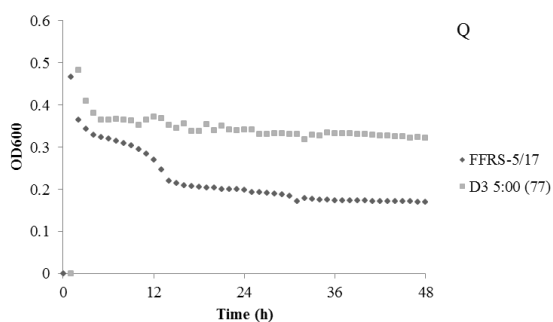
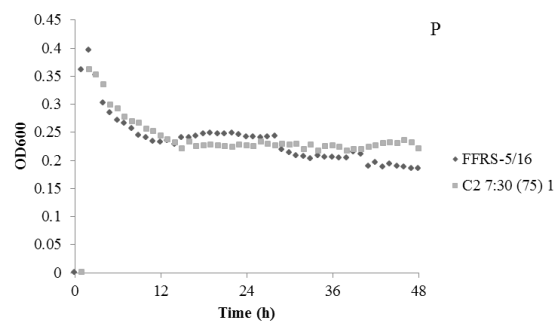
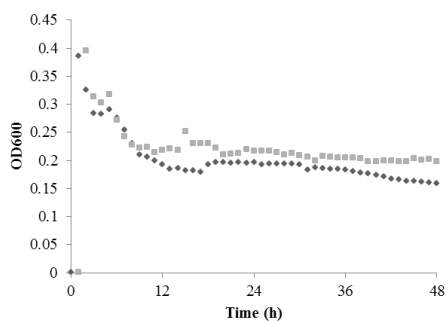
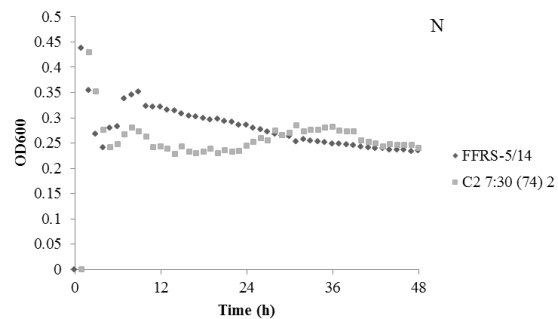
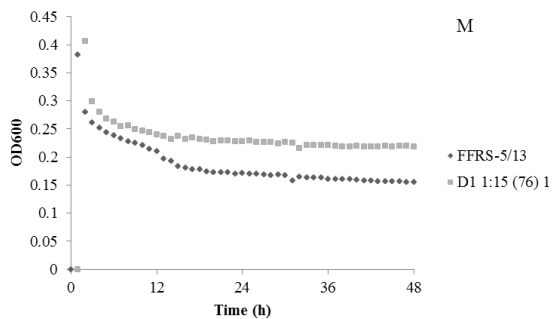
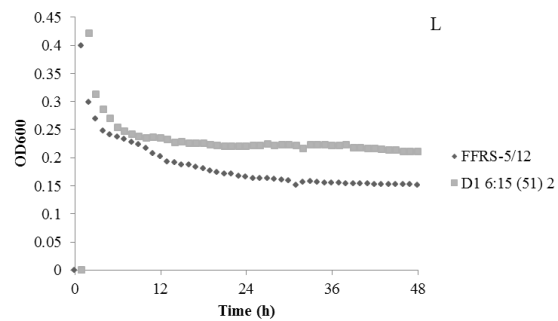
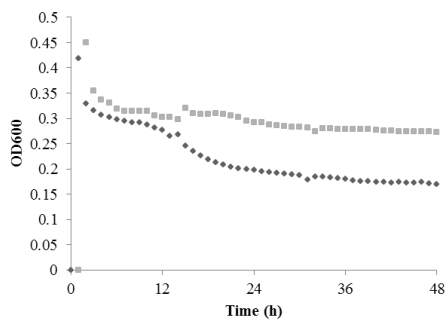


4.3.5. Predation kinetics of *Bd. exovorus* FFRS-5 on *S. maltophilia* isolates

A standard predation growth curve was used to determine variations in predation kinetics between *Bd. exovorus* FFRS-5 and individual *S. maltophilia* isolates. Measuring predation kinetics using the BioScreen apparatus yields results similar to those of larger cocultures (20 mL) measured over an identical time course. Unfortunately the optical density readings obtained using *S. maltophilia* isolates as a prey cell could not be used to accurately measure predation (Fig. 27). This was due to excessive clumping of the prey cells in the cocultures. For most isolates tested, there was a decrease in optical density of the prey-only control cultures, which did not contain *Bd. exovorus* FFRS-5. Therefore, a decrease in optical density of the coculture is not indicative of predation occurring and could not be used as an accurate measure for comparison among prey cell isolates. The predation assay using isolate C1 3:00 (78) demonstrated that predation occurred slowly compared to other BALO isolates (Fig. 27H). Typically, BALOs with a periplasmic life cycle such as strains 109J or HD100 reach stationary phase within 12 to 18 h; however, *Bd. exovorus* required 36 h. This result is consistent with observations made by phase contrast microscopy for all isolates tested. A previous report has suggested using different buffers for cocultures depending on the prey cell used (Chanyi *et al.*, 2013). Cocultures prepared using *Bd. exovorus* FFRS-5 and prey cell strain X26332 in Tris Buffer, YP/10 or DNB all displayed the same level of prey cell clumping (data not shown).

Figure 27. Predation kinetic assays of *Bd. exovorus* preying on *S. maltophilia* isolates. The rate at which the OD₆₀₀ of the prey cell decreases in a coculture was used to measure *Bd. exovorus* growth. Due to excessive clumping of many of the *S. maltophilia* isolates, the prey-only cultures decreased in optical density. Thus, a comparative analysis was unable to be performed. Predation kinetic assays were performed using the BioScreen apparatus. Each prey-only and coculture was performed in quintuplet and the average used to determine each data point. The prey cell in each culture was (A) H59246; (B) X26332; (C) C3 3:00(76)2; (D) C3 3:00(72)1; (E) F69694; (F) H2138; (G) S18202; (H) C1 3:00(78); (I) D2 6:15(19)1; (J) D3 6:00(79); (K) H43306; (L) D1 6:15(51)2; (M) D1 1:15(76)1; (N) C2 7:30(74)2; (O) F28889; (P) C2 7:30(75)1; (Q) D3 5:00(77); (R) F7221; (S) JB12-23.





4.3.6. Predation of *S. maltophilia* isolates by *Bd. exovorus* FFRS-5 in the presence of antibiotics

To determine whether *Bd. exovorus* is able to prey on *S. maltophilia* in the presence of antibiotics, cocultures using prey strain X26332 were prepared using a wide array of antibiotics at different concentrations (Table 8). As an initial screen, cocultures were monitored for 48 h by phase contrast microscopy to observe for signs of predation, such as: motility of predators, attachment, a decrease in prey cell numbers and the presence of ghost cells. Cocultures prepared with ampicillin or polymyxin B did not show any signs of predation occurring even at 1 µg/ml, the lowest concentration tested for both antibiotics. Cocultures prepared with chloramphenicol above 16 µg/ml also did not show any indications of predation occurring. Ceftazidime, the lowest chloramphenicol concentration (16 µg/ml), colicin, kanamycin and ciprofloxacin were chosen for further analysis.

A decrease in colony forming units (CFU) of the prey cell was observed with increasing concentrations of ceftazidime (16, 32, and 64 µg/ml) (Table 8). However, this decrease was not due to predation. It appears the higher concentration of the antibiotic was killing the prey cell. At lower concentrations of ceftazidime (1, 4 and 8 µg/ml), predation was not observed (data not shown). Similarly, chloramphenicol present at 16 µg/ml was enough to inhibit predation.

There was large variability in the results obtained using colicin (Table 8). At 32 µg/ml, predation was inhibited. A decrease in prey cell CFU at higher concentrations of colicin (64, 128, and 256 µg/ml) was more pronounced when *Bd. exovorus* FFRS-5 was not present. This may be due to the antibiotic being taken up by *Bd. exovorus*

Table 8. Predation by *Bd. exovorus* FFRS-5 on *S. maltophilia* X26332 in the presence of antibiotics. Efficiency of predation by *Bd. exovorus* FFRS-5 on *S. maltophilia* X26332 in the presence of antibiotics at different concentrations. Cocultures were incubated for 48 h at 37°C and serial plated onto LB Lennox agar. Colony forming units (CFU) were used to determine residual *S. maltophilia* remaining in culture. Cultures without the addition of predator were used to control for the reduction of prey cells due to the antibiotics.

Antibiotic	Concentration (µg/ml)	CFU no FF ¹	CFU + FF	Percent Decrease
None	N/A	1.29 x 10 ⁹	6.12 x 10 ⁸	52.5
Ceftazidime	16	4.00 x 10 ⁸	1.08 x 10 ⁹	-168.7
	32	8.00 x 10 ⁸	3.60 x 10 ⁸	55.0
	64	5.70 x 10 ⁸	3.20 x 10 ⁸	43.9
	128	6.00 x 10 ⁸	1.90 x 10 ⁸	68.3
Chloramphenicol	16	2.45 x 10 ⁸	2.74 x 10 ⁸	-11.8
Colicin	32	1.00 x 10 ⁹	9.60 x 10 ⁸	4.0
	64	2.30 x 10 ⁸	1.28 x 10 ⁸	44.3
	128	1.02 x 10 ⁹	1.95 x 10 ⁹	-91.2
	256	3.62 x 10 ⁷	8.67 x 10 ⁷	-139.2
Kanamycin²	16	2.52 x 10 ⁸	2.52 x 10 ⁸	0
	32	1.62 x 10 ⁸	2.30 x 10 ⁸	-41.5
	64	1.00 x 10 ⁸	1.82 x 10 ⁸	-82.5
	128	1.45 x 10 ⁷	1.22 x 10 ⁸	-744.8
Ciprofloxacin	1	6.60 x 10 ⁸	2.45 x 10 ⁷	96.3
	5	7.75 x 10 ⁸	1.98 x 10 ⁷	97.4
	10	7.00 x 10 ⁸	5.20 x 10 ⁷	92.6
	20	5.88 x 10 ⁸	7.75 x 10 ⁷	86.8

¹*Bdellovibrio exovorus* FFRS-5

²At lower concentrations (5µg/ml), predation happened slowly

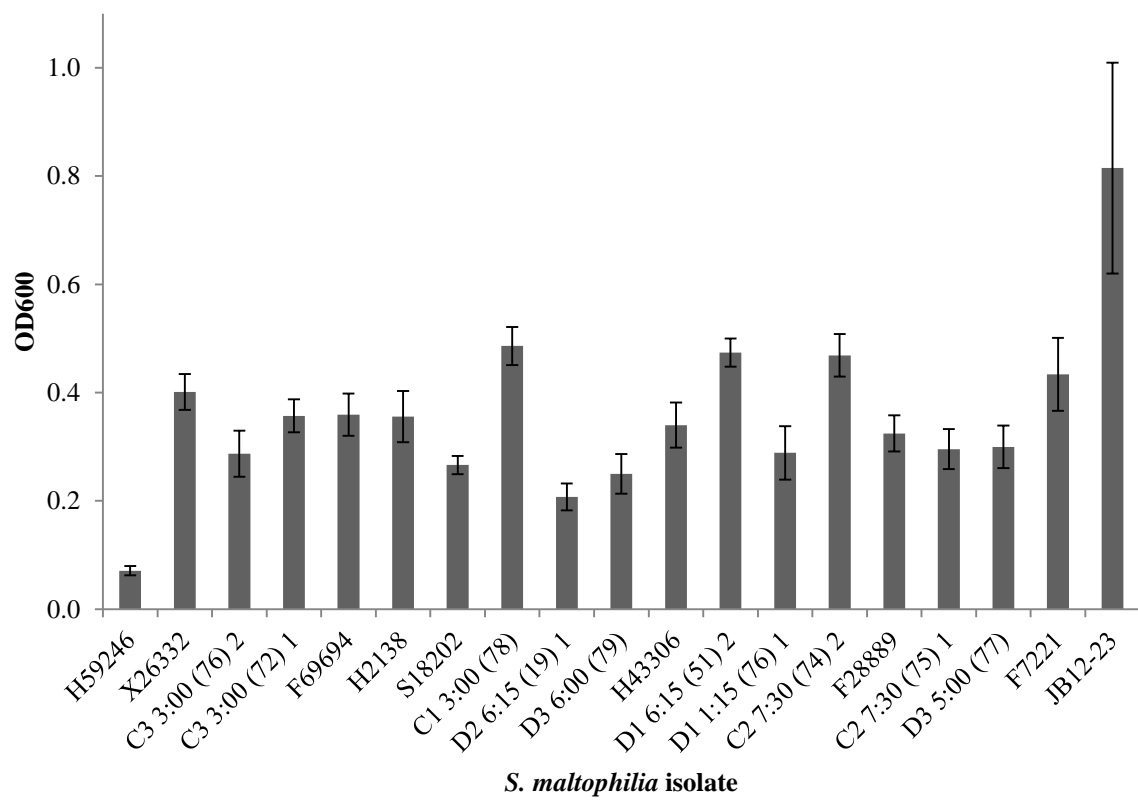
FFRS-5, effectively reducing the effective dose, increasing survival of the prey cell in the presence of *Bd. exovorus*. The same result was observed using higher concentrations of kanamycin (32, 64, and 128 $\mu\text{g/ml}$) (Table 8). Although predation was not observed when kanamycin was present, attachment was observed by light microscopy. Using lower concentrations of kanamycin (1, 5, 10 and 25 $\mu\text{g/ml}$), predation was observed to occur at 1, 5 and 10 $\mu\text{g/ml}$ (Table 3). Using 5 $\mu\text{g/ml}$ kanamycin was the highest effective dose with the least inhibition to predation.

Bd. exovorus FFRS-5 was able to prey in the presence of all concentrations of ciprofloxacin tested (1, 5, 10, and 20 $\mu\text{g/ml}$), decreasing prey cell concentration on average 40 % more than the no antibiotic control (Table 8). Based on these results, kanamycin and ciprofloxacin were chosen for further study. The highest concentration dose for ciprofloxacin and kanamycin without negatively affecting predation was 10 $\mu\text{g/ml}$ and 5 $\mu\text{g/ml}$, respectively.

4.3.7. Biofilm formation of *S. maltophilia* isolates

S. maltophilia isolates form very dense biofilms. The original protocol tested formed biofilms so dense that quantification was not possible (data not shown). Therefore, the protocol was altered to decrease biofilm formation such that quantification and comparison was possible. Under the experimental conditions used, the panel of isolates of *S. maltophilia* had varying abilities to form biofilms (Fig. 28). Isolate H59246 did not form a strong biofilm. In contrast, isolate JB12-23, an LPS mutant of isolate X26332, formed a dense biofilm (Fig. 28). Surprisingly this mutant formed a more robust biofilm than did its parent strain, X26332.

Figure 28. Biofilm formation by *S. maltophilia* isolates. Biofilms of *S. maltophilia* isolates were formed overnight in 96-well plates. Residual biofilm was quantified by OD₆₀₀ after crystal violet staining. There was no correlation between the ability to form biofilms and the source of isolation. Each replicate is an average of 3 repeated five times.



4.3.8. Exposure of preformed biofilms of *S. maltophilia* to ciprofloxacin and/or kanamycin

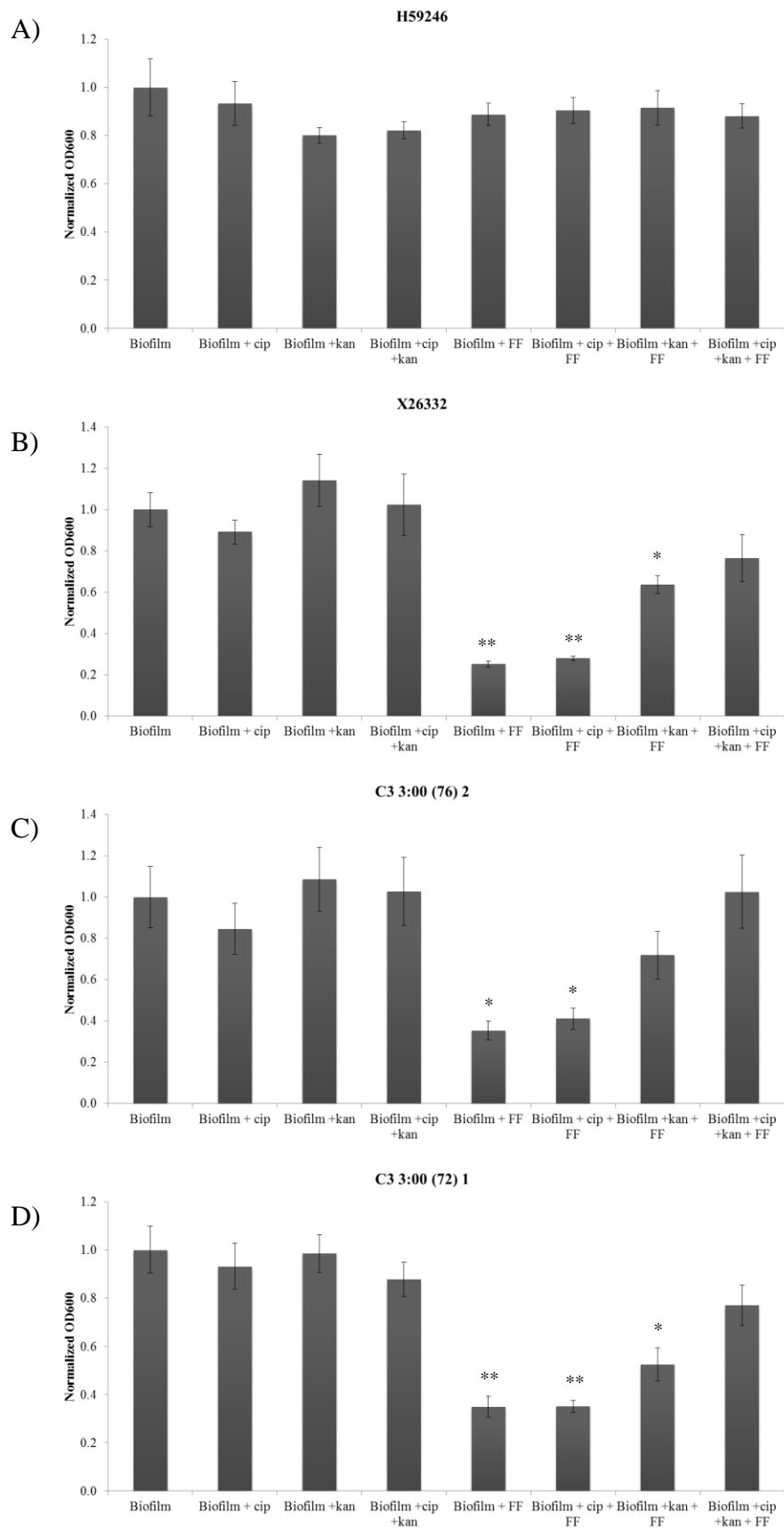
Exposure of preformed biofilms of *S. maltophilia* isolates to ciprofloxacin, kanamycin or both ciprofloxacin and kanamycin did not show a statistically significant decrease in biofilm mass for any of the *S. maltophilia* isolates tested (Fig. 29). Overall, there was a 13.3% and 14.6% decrease in biofilm mass when exposed to ciprofloxacin and ciprofloxacin and kanamycin, respectively (Table 9). However, strain JB12-23 showed growth sensitivity to ciprofloxacin (Fig. 29S), decreasing biofilm mass by almost 50% whenever ciprofloxacin was present (Table 9). Interestingly, eight *S. maltophilia* isolates had an increase in biofilm mass when exposed to kanamycin. Of these, only isolate C2 7:30 (75) 1 had an increase when both ciprofloxacin and kanamycin are added.

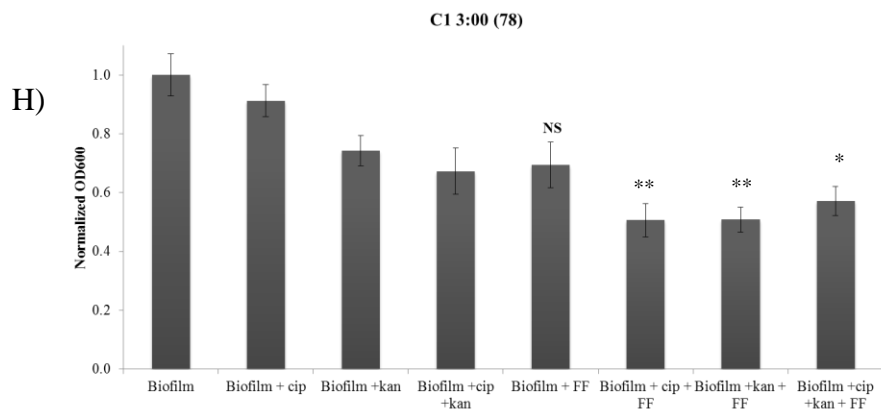
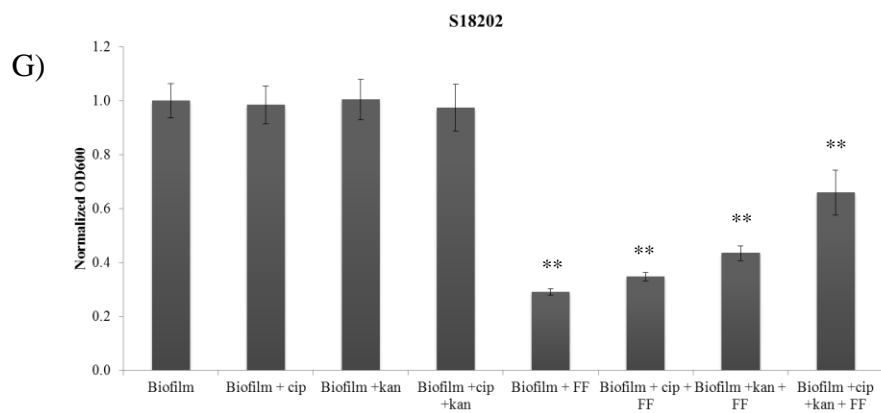
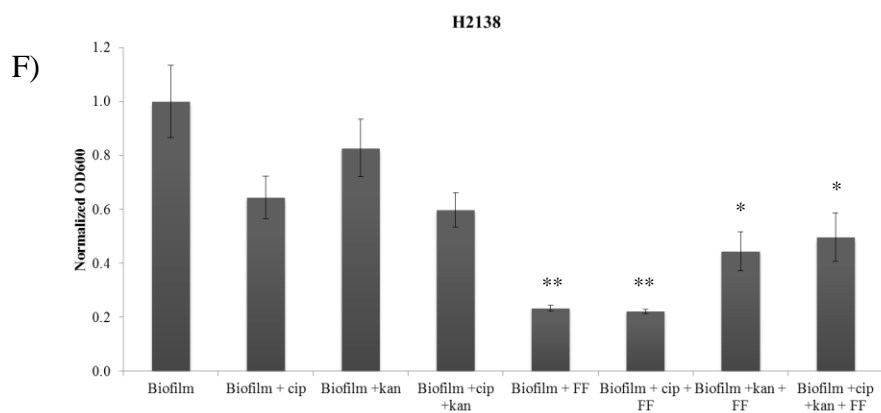
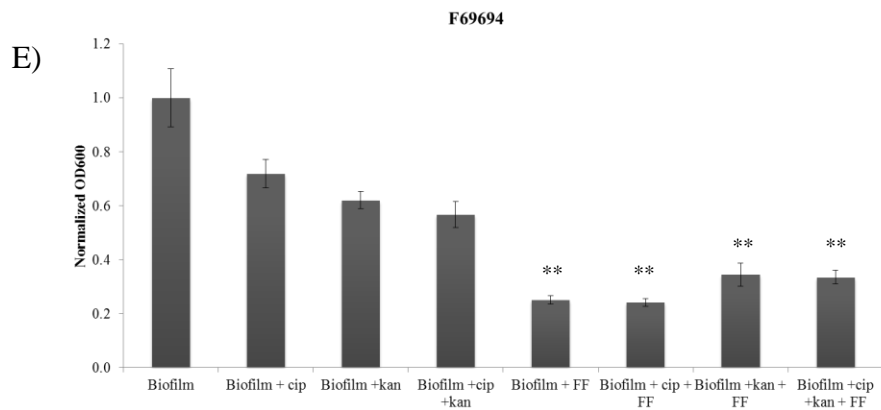
4.3.9. Disruption of biofilms by *Bd. exovorus* FFRS-5

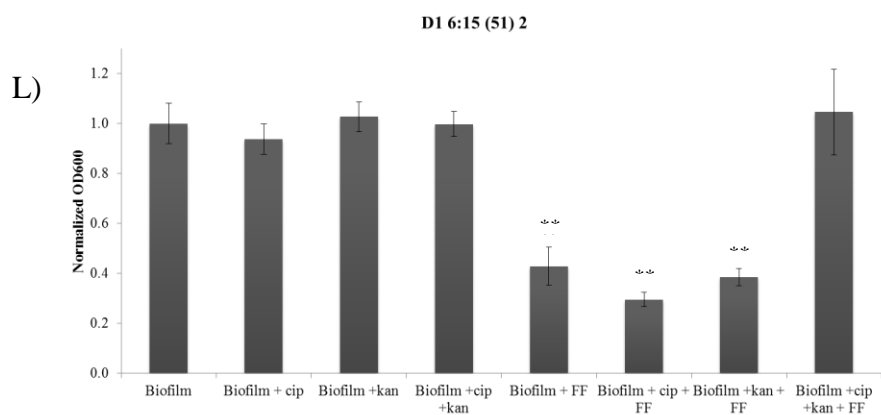
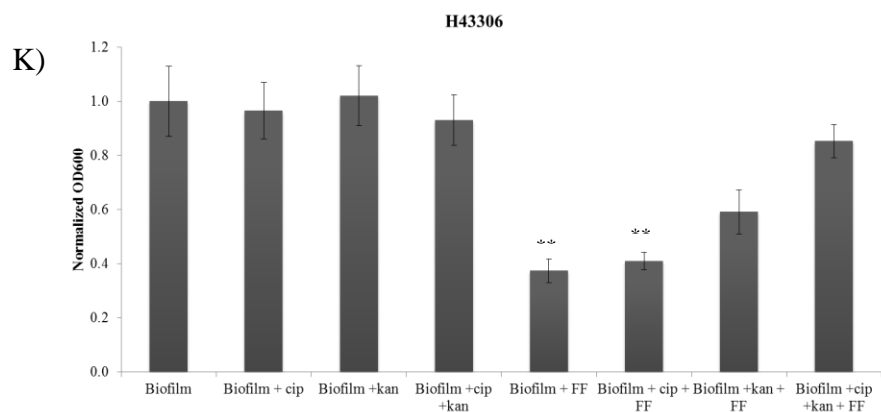
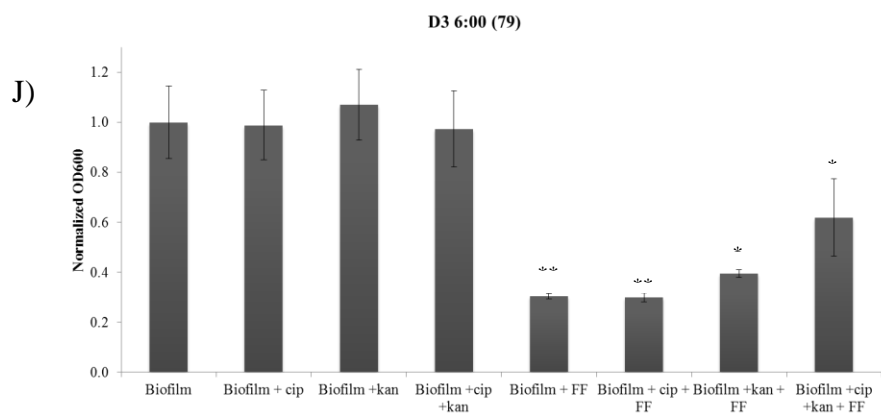
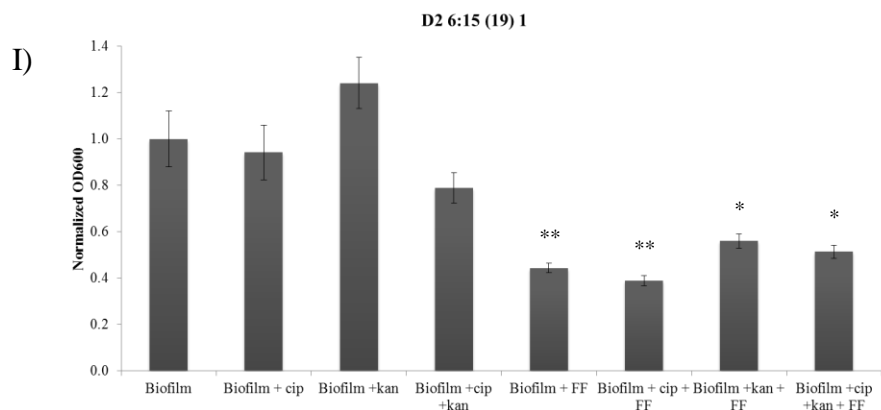
When exposed to *Bd. exovorus* FFRS-5, almost all biofilms were significantly removed ($p < 0.005$). Due to the inability of isolate H59246 (Fig. 29A) to form a biofilm under the experimental conditions, *Bd. exovorus* FFRS-5 did not have a significant effect. *Bd. exovorus* FFRS-5 was able to decrease the mass of the biofilms formed by isolates C1 3:00 (78) (Fig. 29H) and JB12-23 (Fig. 29S); however, this decrease was not statistically significant. All other *S. maltophilia* biofilms were susceptible to predation to varying degrees (Fig. 29; Table 9). Across all *S. maltophilia* isolates, on average there was a 62.4% decrease in biofilm mass when exposed to *Bd. bacteriovorus* FFRS-5 (Table 9). Predation on isolate H2138 (Fig. 29F) was the best, removing almost 77% of the biofilm.

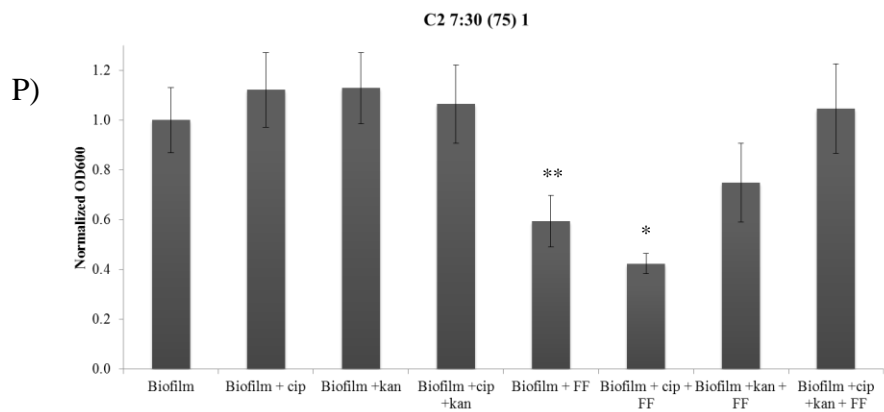
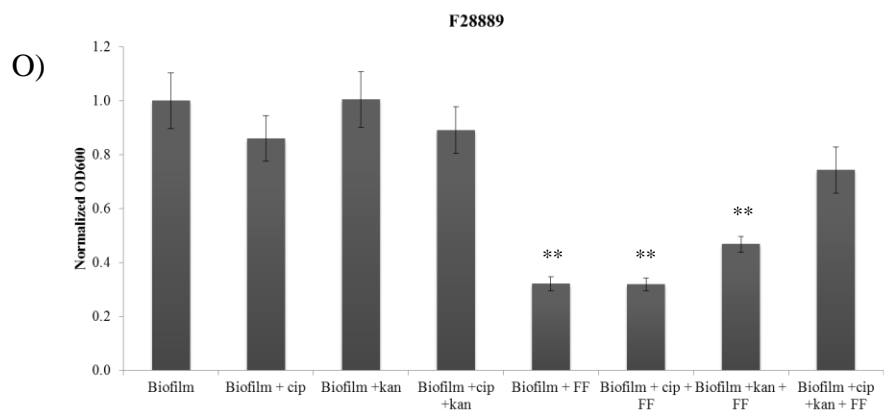
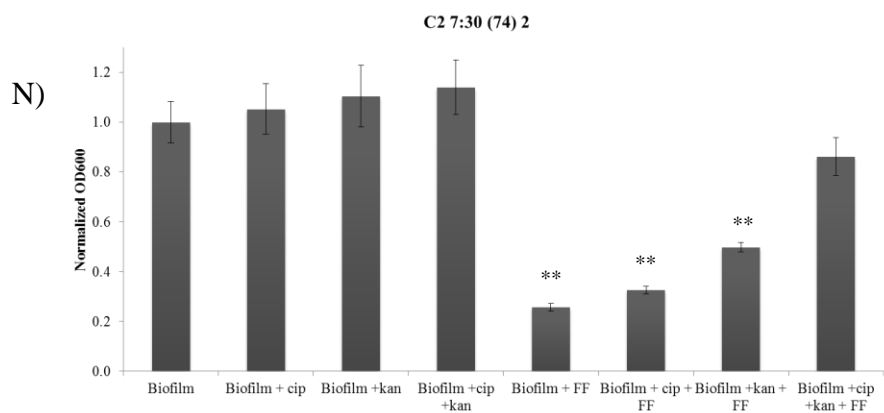
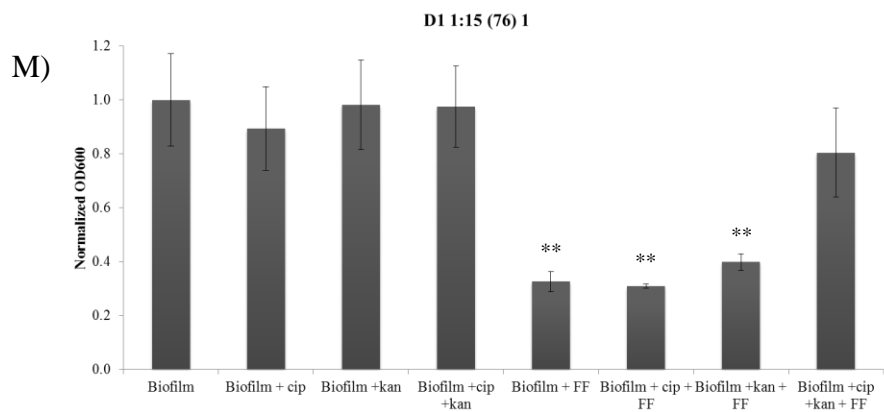
Adding ciprofloxacin with *Bd. exovorus* FFRS-5 increased the average removal of the biofilm to a 64.5% total decrease (Table 9). Overall the addition of ciprofloxacin did

Figure 29. Predation by *Bd. exovorus* FFRS-5 on preformed biofilms of *S. maltophilia* isolates with and without antibiotics. Biofilms were formed overnight in 96-well plates before being exposed to one of the following with and without the addition of *Bd. exovorus* FFRS-5; HM buffer, HM and ciprofloxacin (20 µg/ml), HM and kanamycin (5 µg/ml), HM, ciprofloxacin (10 µg/ml) and kanamycin (5 µg/ml). *Bd. exovorus* FFRS-5 reduced the mass of preformed biofilms of all *S. maltophilia* isolates tested. Biofilm mass was quantified using OD₆₀₀ after crystal violet staining. Each individual group was standardized to its prey-only control. Each replicate is an average of 3 wells per each condition repeated five times. The prey cell in each culture was (A) H59246; (B) X26332; (C) C3 3:00(76)2; (D) C3 3:00(72)1; (E) F69694; (F) H2138; (G) S18202; (H) C1 3:00(78); (I) D2 6:15(19)1; (J) D3 6:00(79); (K) H43306; (L) D1 6:15(51)2; (M) D1 1:15(76)1; (N) C2 7:30(74)2; (O) F28889; (P) C2 7:30(75)1; (Q) D3 5:00(77); (R) F7221; (S) JB12-23. Statistical significance was measured using a 1-way ANOVA with a Bonferonni corrected post-hoc Students T-test, *p<0.05, **p<0.005.









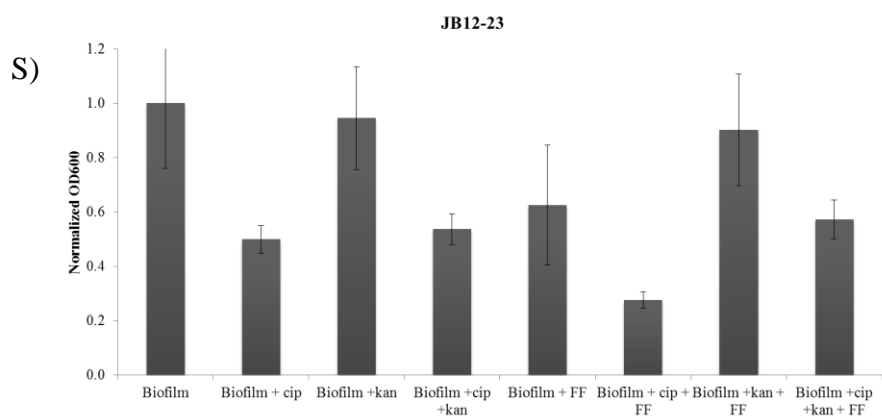
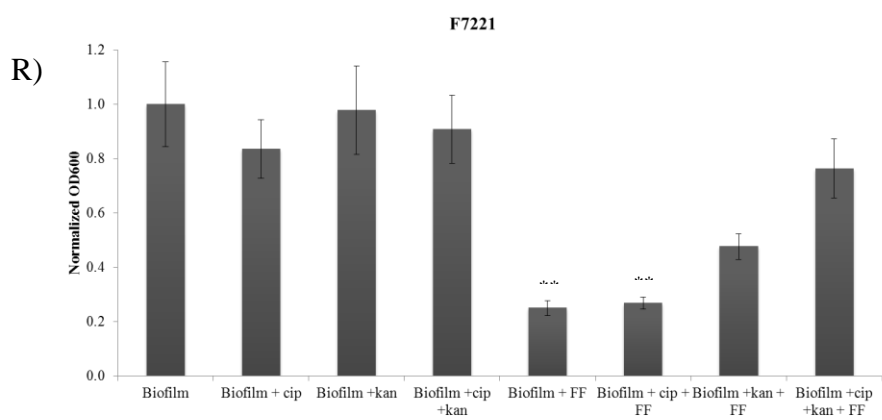
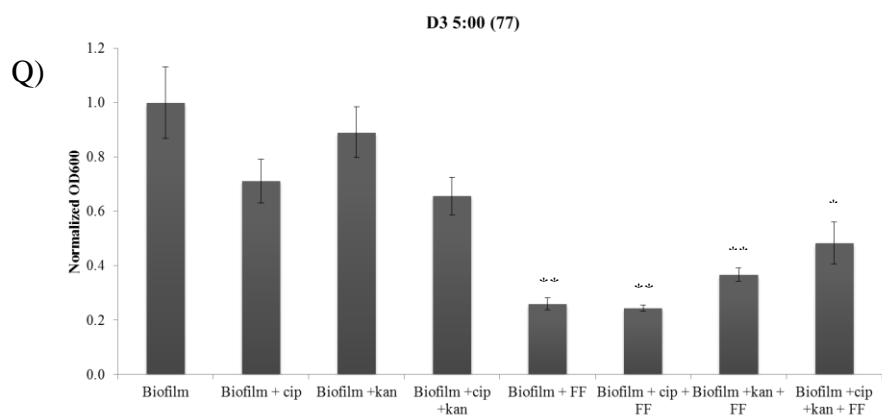


Table 9. *Bd. exovorus* FFRS-5 is able to prey on preformed biofilms of all *S. maltophilia* isolates in the presence of ciprofloxacin and/or kanamycin. Remaining biofilm was quantified by crystal violet staining after being exposed to ciprofloxacin, kanamycin, both ciprofloxacin and kanamycin and under these conditions when *Bd. exovorus* FFRS-5 is present for 48 h. Results shown are the percent difference compared to the biofilm only control of each prey cell. Each replicate is an average of 3 wells per each condition repeated five times.

Prey Isolate ¹	Cip ²	Kan ³	Cip + Kan	Bd ⁴	Bd + Cip	Bd + Kan	Bd + Cip + Kan
H59246	6.7	19.9	17.8	11.2	9.5	8.4	11.8
X26332	10.8	-14.3	-2.4	74.9	72.0	36.3	23.5
C3 3:00 (76) 2	15.4	-8.6	-2.7	64.6	58.9	28.2	-2.6
C3 3:00 (72) 1	11.0	5.9	16.1	70.4	66.4	55.5	29.1
F69694	28.0	37.9	43.2	74.9	75.8	65.6	66.5
H2138	35.6	17.3	40.1	76.7	77.8	55.7	50.3
S18202	1.6	-0.5	2.5	70.8	65.3	56.5	34.1
C1 3:00 (78)	8.8	25.7	32.7	33.3	49.4	49.2	42.9
D2 6:15 (19) 1	5.8	-24.0	21.2	55.6	61.1	44.0	48.7
D3 6:00 (79)	1.1	-7.1	2.5	69.5	70.1	60.5	38.1
H43306	7.1	1.8	10.5	64.0	60.6	43.1	18.0
D1 6:15 (51) 2	11.4	2.9	5.7	67.6	72.1	63.6	17.8
D1 1:15 (76) 1	10.7	1.8	2.5	67.4	69.1	60.1	19.6
C2 7:30 (74) 2	-5.3	-10.5	-14.0	74.4	67.4	50.2	13.8
F28889	13.9	-0.5	10.9	68.0	68.1	53.2	25.7
C2 7:30 (75) 1	-5.6	-6.3	-0.2	56.4	60.1	40.7	3.0
D3 5:00 (77)	28.8	10.9	34.4	74.0	75.6	63.3	51.7
F7221	16.4	2.2	9.2	74.9	73.1	52.3	23.7
JB12-23	50.0	5.4	46.4	37.5	72.4	9.8	42.8
Average	13.3	3.2	14.6	62.4	64.5	47.2	29.4

¹*Stenotrophomonas maltophilia* prey cell isolate

²Ciprofloxacin, 10 µg/ml

³Kanamycin, 5 µg/ml

⁴*Bdellovibrio exovorus* FFRS-5

not have a significant impact on the decrease of biofilm mass when compared to adding *Bd. exovorus* alone. However, the addition of ciprofloxacin and *Bd. exovorus* to prey cell C1 3:00 (78) significantly decreased biofilm mass, a result not achieved with *Bd. exovorus* alone (Fig. 29H).

Fourteen of the nineteen isolates showed a significant decrease in biofilm mass in the presence of both kanamycin and *Bd. exovorus* FFRS-5 (Fig. 29). Even strains D2 6:15 (19) 1 and C2 7:30 (74) 2 that showed an increase in biofilm mass when exposed to kanamycin alone and both ciprofloxacin and kanamycin, respectively, showed a significant reduction in the amount of biofilm remaining when *Bd. exovorus* was added (Fig. 29I and N). However, the majority of isolates had more biofilm remaining than when compared to adding *Bd. exovorus* alone. With the addition of ciprofloxacin, kanamycin and *Bd. exovorus*, ten isolates showed a decrease in residual biofilm; however, as a general trend, there was more biofilm remaining than when compared with *Bd. exovorus* alone. This indicates that although *Bd. exovorus* is capable of significantly killing *S. maltophilia* isolates in the presence of antibiotics, disruption of the biofilm occurs better with *Bd. exovorus* alone. The data suggests the best course of action for efficient removal of a biofilm would be to use *Bd. exovorus* FFRS-5 in the presence of ciprofloxacin.

4.3.10. Enumeration of bacteria within residual biofilm

To determine the number of bacteria remaining in the biofilm under each condition tested, colony forming units were determined after brief sonication of the microtiter plate. Although this was repeated twice, the CFU values obtained did not match with the decrease in biofilm mass observed previously. The concentration of

residual bacteria appeared to be very similar even in the samples that contained *Bd. exovor* FFRS-5 (Fig. 30). Early in colony growth, it was observed that two colony types were present. One formed a larger colony without a distinct edge while the other colony was smaller in size and had a distinct edge. Both were slightly brown, fairly round and present at similar quantities within the same sample. It is not known if this was a contaminant caused by the aerosols created during sonication of the microtiter plates. This colony type was present in a second replicate after each prey cell was isolated for single colonies and the assay repeated. Statistical analysis was not performed as the CFU values obtained were inconsistent and did not coincide with the results obtained by crystal violet staining.

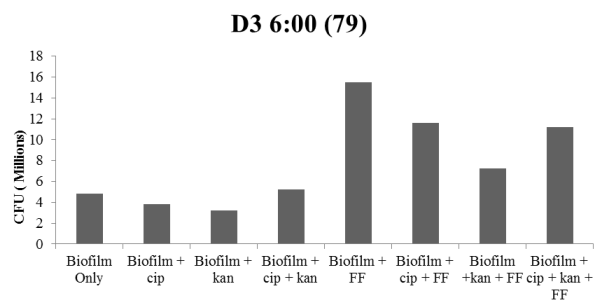
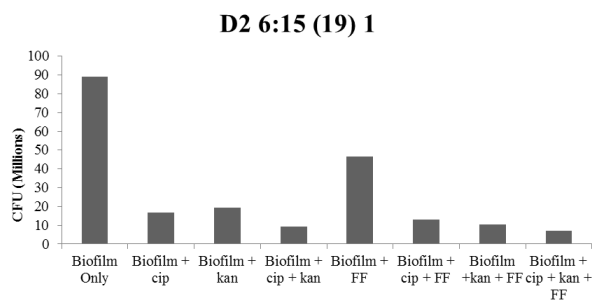
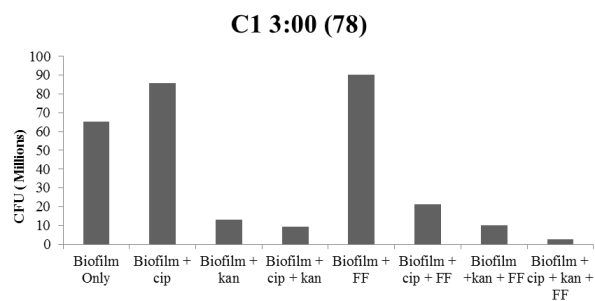
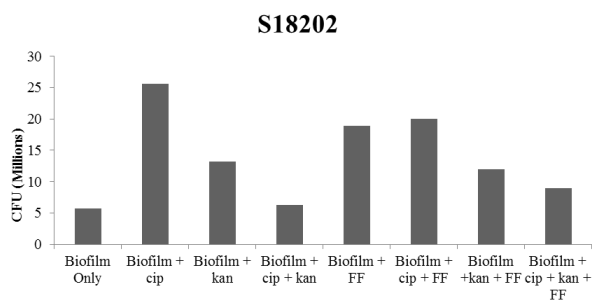
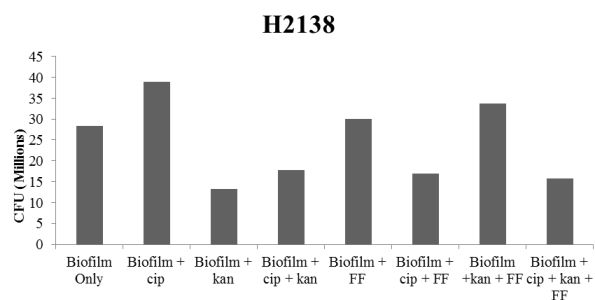
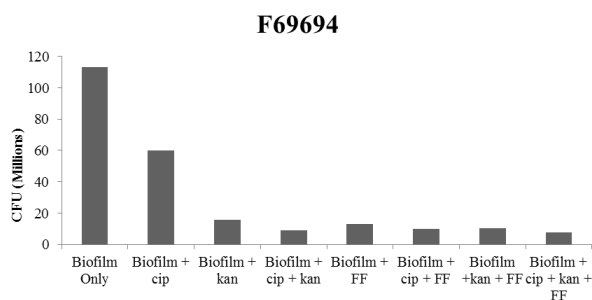
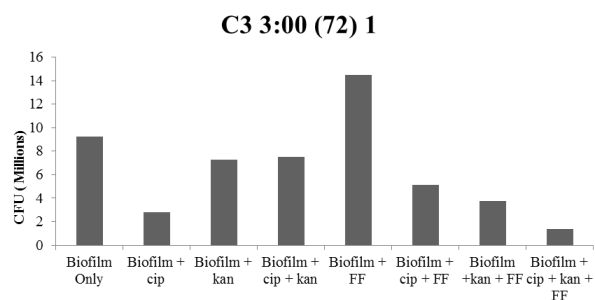
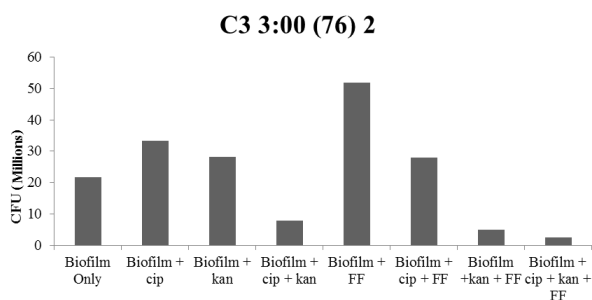
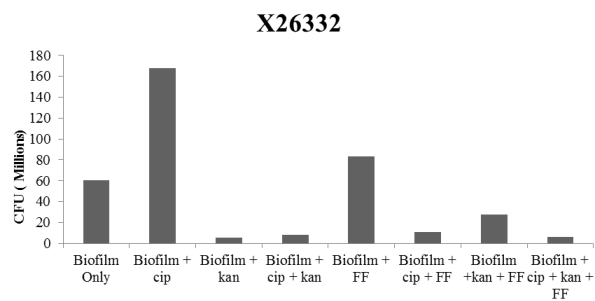
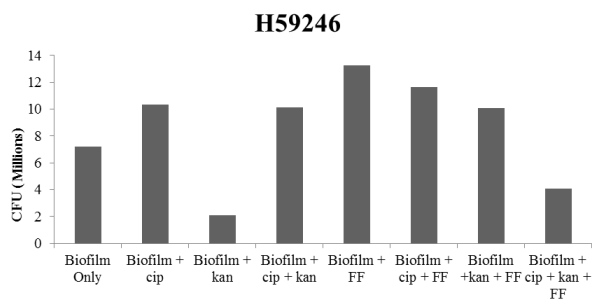
4.4. Discussion

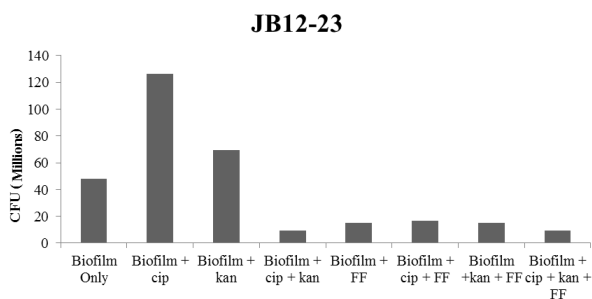
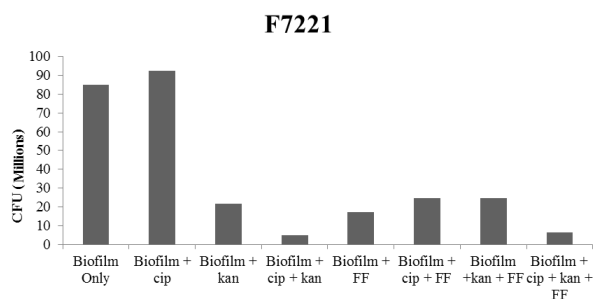
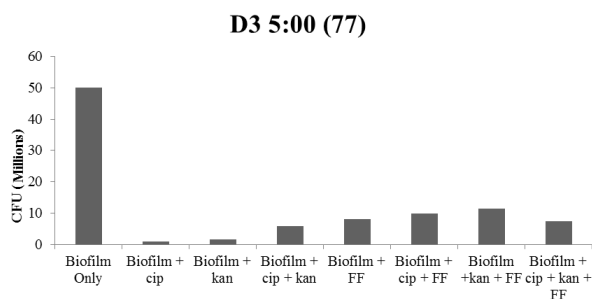
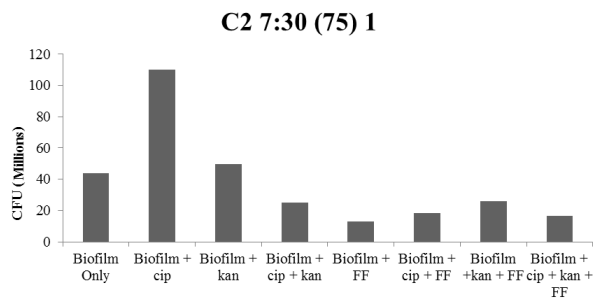
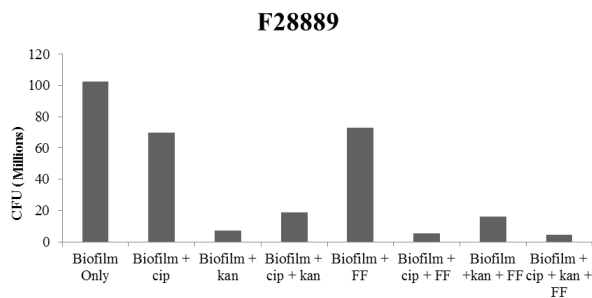
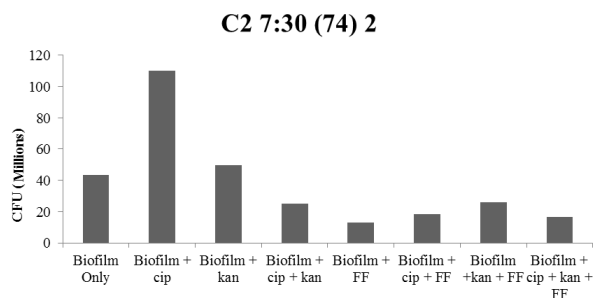
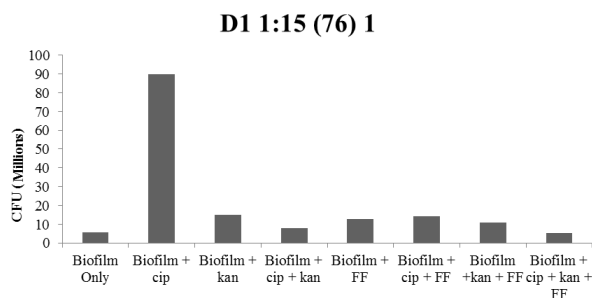
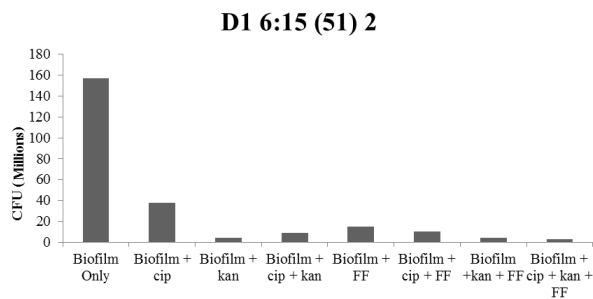
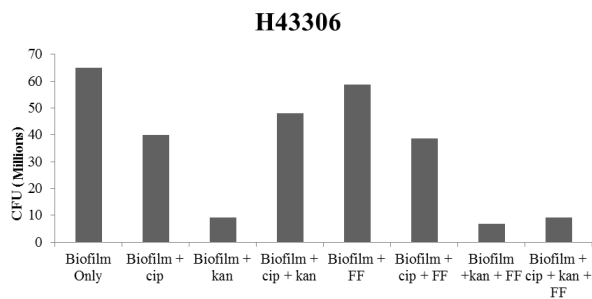
In this study the bacterial predator *Bd. exovor* was used to disrupt pre-formed biofilms of 19 clinical *S. maltophilia* isolates (hospital-environment and clinical samples). A combined approach with antibiotics and *Bd. exovor* was used to determine if there was a potential synergistic effect.

Lambina *et al.* (1982) used *S. maltophilia* (*Pseudomonas maltophilia*) in enrichment cultures for prokaryotic predators. Although a BALO was not isolated, a novel bacterial predator was, *Micavibrio admirandus* (an α -proteobacterium). Dashiff *et al.* (2011) tested the prey range of two predators, *Bd. bacteriovorus* 109J and *Micavibrio aeruginosavorus* ARL-13, and found that neither were capable of preying on 6 clinical isolates of *S. maltophilia*. Thus this study is the first characterization of a BALO predator capable of killing *S. maltophilia*.

16 S rRNA gene sequence analysis of strain FFRS-5 revealed an identical match

Figure 30. Enumeration of residual *S. maltophilia* in biofilms. The concentration of residual bacteria remaining in the biofilm was determined by sonicating the 96-well plate and serial plating the contents of each well onto LB Lennox agar. Data shown was a median of two replicates.





to *Bd. exovorus*, a novel epibiotic predator with a fairly limited prey range (Chanyi *et al.*, 2013; Koval *et al.*, 2013). Electron microscopic study of this predator showed it utilizes an epibiotic life style for survival. These results confirm the identity of the predator as *Bd. exovorus* FFRS-5, the first epibiotic BALO isolated on a prey cell other than *Caulobacter crescentus*. A limited prey range analysis confirmed that *Bd. exovorus* FFRS-5 was able to prey on *C. crescentus* and was unable to prey on *B. cenocepacia*, *E. coli* or *P. aeruginosa*, in liquid coculture and on an agar surface. As *C. crescentus* has its own developmental life cycle, using *S. maltophilia* as a prey cell may simplify future experiments when looking at predation by *Bd. exovorus*. Previous analysis determined that phage therapy was strain-specific and ineffective against many similar strains of *S. maltophilia* (Donlan, 2009). *Bd. exovorus* FFRS-5 did not show this same specificity and was able to prey on all isolates of *S. maltophilia*, regardless of the source of isolation. The decrease in prey cell concentration was similar to those previously reported for *Bd. bacteriovorus* predation on human pathogens (Dashiff and Kadouri, 2011; Dashiff *et al.*, 2011).

All *S. maltophilia* isolates had variable abilities to form biofilms under the experimental conditions used. Biofilm formation was so dense in some isolates that the protocol had to be modified such that less biofilm was formed to allow for accurate quantification. Increased aeration of *S. maltophilia* cultures increased biofilm mass but was not necessary to form a very dense mat. Cultures were, therefore, incubated without shaking. Strain H59246 was unable to form a biofilm under these conditions. As strain H59246 is a clinical isolate, a strong biofilm is not the only contributing factor to pathogenesis, although further study must be conducted. There was no correlation in the

ability to form biofilms between the hospital-environment isolates and the clinical isolates, a result that agrees with previous studies (Pompilio *et al.*, 2011). This result highlights that biofilm formation is not a pathogenic trait that is selected for in an infection but is required to colonize their natural niche, the rhizosphere and root nodules of plants (Ryan *et al.*, 2009). Interestingly, the LPS-deficient mutant, strain JB12-23 formed a stronger biofilm than did the wildtype strain X26332. This suggests that modifications of the outer membrane play an important role in the formation of a biofilm.

The genome sequence of *Bd. exovor* JSS^T was recently published and implied that it is resistant to many antibiotics, which include ampicillin, ceftazidime, chloramphenicol, ciprofloxacin, colicin, kanamycin and polymyxin B (Pasternak *et al.*, 2014). Ciprofloxacin and kanamycin were the only antibiotics in the presence of which *Bd. exovor* FFRS-5 could prey on *S. maltophilia* X26332. These differences may be attributed to strain variation between the type strain *Bd. exovor* JSS and *Bd. exovor* FFRS-5, which were isolated separately over 25 years apart on different prey cells. At 20 µg/ml ciprofloxacin, predation was delayed but did occur. The same result was observed for kanamycin concentrations greater than 10 µg/ml. Although these concentrations are low, they represent concentrations bacterial predators would be exposed to within a biofilm.

In most cases, the addition of either ciprofloxacin and/or kanamycin did not have an appreciable effect on the decrease of biofilm mass. However, there were strains that were more impacted by the antibiotics than others, demonstrating strain variability in antibiotic resistance among *S. maltophilia* isolates. The addition of kanamycin and kanamycin with ciprofloxacin increased residual biofilm formation by some

S. maltophilia isolates. This may be due to a stress response triggered in the bacterium when exposed to antibiotics, and in some cases, like *E. coli*, upregulating adhesins (Boehm *et al.*, 2009).

Bd. exovorus FFRS-5 was able to prey on all biofilms of *S. maltophilia*. On average, *Bd. exovorus* alone removed 62.4% of the biofilm mass. Generally, ciprofloxacin did not improve the disruption of the biofilm but it did not prevent *Bd. exovorus* from removing it. Unfortunately, a synergistic effect between *Bd. exovorus* and antibiotics was not observed in any of the prey cell isolates tested.

Overall, these results demonstrate that *Bd. exovorus* removes biofilms of *S. maltophilia* even in the presence of ciprofloxacin. Currently there are studies to develop materials that decrease the formation of biofilms in general; however, when a biofilm does form, preventative therapies will not be effective. The use of *Bd. exovorus* is a good candidate for further study as it shows a wide prey range on *S. maltophilia* isolates. An antimicrobial cocktail of multiple BALO isolates could be produced that combine predators of, but not limited to, *P. aeruginosa*, *B. cenocepacia*, *E. coli* and *Acinetobacter* spp. This approach may be useful to prevent infection of open wounds, disrupt biofilms on catheters and stents and possibly be used as an aerosol to help eliminate pathogenic bacteria in CF patients and upper respiratory infections.

The increasing emergence of multidrug-resistant bacteria creates the need to develop smarter tools to combat future infections. Soon it will no longer be enough to combat simple infections with a single antibiotic. Multidrug cocktails are currently being studied to look into the synergistic effects a combinatorial administration may provide, such as trimethoprim-sulfamethoxazole. However, it will not be long before these

therapies will also become of limited use. The ability to form biofilms within an infection exacerbates this problem as this makes the bacteria inherently more resistant to antimicrobials than their planktonic counterparts. This has been attributed to the formation of slow-growing and dormant persister cells that will not be affected by antibiotics (Goneau *et al.*, 2014). Unfortunately, enumeration of residual persister cells in the biofilms exposed to *Bd. exovorus* was not possible in this study. The physical barrier of the biofilm itself provides protection as the antibiotic is not able to penetrate the outer layers. This exposes bacteria to sub-inhibitory concentrations that can promote resistance and, in some cases, more biofilm formation to occur. Novel methods to fight infections must take this into account.

4.5. References

Aydin, K., Köksal, I., Kaygusuz, S., Kaklikkaya, I., Caylan, R. and Ozdemir, R. (2000) Endocarditis caused by *Stenotrophomonas maltophilia*. *Scand J Infect Dis* **32**: 427-430.

Berg, G. (2009) Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl Microbiol Biotechnol* **84**: 11-18.

Bin Abdulhak, A.A., Zimmerman, V., Al Beirouti, B.T., Baddour, L.M. and Tleyjeh, I.M. (2009) *Stenotrophomonas maltophilia* infections of intact skin: a systematic review of the literature. *Diagn Microbiol Infect Dis* **63**: 330-333.

Boehm, A., Steiner, S., Zaehring, F., Casanova, A., Hamburger, F., Ritz, D., Keck, W., Ackermann, M., Schirmer, T and Jenal, U. (2009) Second messenger signalling governs *Escherichia coli* biofilm induction upon ribosomal stress. *Mol Microbiol* **72**: 1500-1516.

Chang, H.C., Chen, C.R., Lin, J.W., Shen, G.H., Chang, K.M., Tseng, Y.H. and Weng, S.F. (2005) Isolation and characterization of novel giant *Stenotrophomonas maltophilia* phage ϕ SMA5. *Appl Environ Microbiol* **71**: 1387-1393.

Chanyi, R.M., Ward, C., Pechey, A. and Koval, S.F. (2013) To invade or not to invade: two approaches to a prokaryotic predatory life cycle. *Can J Microbiol* **59**: 273-279.

Dashiff, A. and Kadouri, D.E. (2011) Predation of oral pathogens by *Bdellovibrio bacteriovorus* 109J. *Mol Oral Microbiol* **26**: 19-34.

Dashiff, A., Junka, R.A., Libera, M. and Kadouri, D.E. (2011) Predation of human pathogens by the predatory bacteria *Micavibrio aeruginosavorus* and *Bdellovibrio bacteriovorus* **110**: 431-444.

Di Bonaventura, G., Spedicato, I., D'Antonio, D., Robuffo, I. and Piccolomini, R. (2004) Biofilm formation by *Stenotrophomonas maltophilia*: modulation by quinolones, trimethoprim-sulphamethoxazole, and ceftazidime. *Antimicrob Agents Chemother* **48**: 151-160.

Donlan, R.M. (2009) Preventing biofilms of clinically relevant organisms using bacteriophage. *Trends Microbiol* **17**: 66-72.

Dwidar, M., Hong, S., Cha, M., Jang, J. and Mitchell, R.J. (2012) Combined application of bacterial predation and carbon dioxide aerosols to effectively remove biofilms. *Biofouling* **28**: 671-680.

Fujita, J., Yamadori, I., Xu, G., Hojo, S., Negayama, K., Miyawaki, H., Yamaji, Y. and Takahara, J. (1996) Clinical features of *Stenotrophomonas maltophilia* pneumonia in immunocompromised patients. *Respir Med* **90**: 35-38.

Goneau, L.W., Yeoh, N.S., MacDonald, K.W., Cadieux, P.A., Burton, J.P., Razvi, H. and Reid, G. (2014) Selective target inactivation rather than global metabolic dormancy causes antibiotic tolerance in uropathogens. *Antimicrob Agents Chemother* **58**: 2089-2097.

Goss, C.H., Otto, K., Aitken, M.L. and Rubenfeld, G.D. (2002) Detecting *Stenotrophomonas maltophilia* does not reduce survival of patients with cystic fibrosis. *Am J Respir Crit Care Med* **166**: 356-361.

Goss, C.H., Mayer-Hamblett, N., Aitken, M.L., Rubenfeld, G.D. and Ramsey, B.W. (2004) Association between *Stenotrophomonas maltophilia* and lung function in cystic fibrosis. *Thorax* **59**: 955-959.

Gutiérrez Rodero, F., Masiá, M.M., Cortés, J., Ortiz de la Tabla, V., Mainar, V. and Vilar, A. (1996) Endocarditis caused by *Stenotrophomonas maltophilia*: case report and review. *Clin Infect Dis* **23**: 1261-1265.

Harini, K., Ajila, V. and Hegde, S. (2013) *Bdellovibrio bacteriovorus*: A future antimicrobial agent? *J Indian Soc Periodontol* **17**: 823-825.

Kadouri, D. and O'Toole, G.A. (2005) Susceptibility of biofilms to *Bdellovibrio bacteriovorus* attack. *Appl Environ Microbiol* **71**: 4044-4051.

Kadouri, D.E. and Tran, A. (2013) Measurement of predation and biofilm formation under different ambient oxygen conditions using a simple gasbag-based system. *Appl Environ Microbiol* **79**: 5264-5271.

Karpati, F., Malmborg, A.S., Alfredsson, H., Hjelte, L. and Strandvik, B. (1994) Bacterial colonisation with *Xanthomonas maltophilia*—a retrospective study in a cystic fibrosis patient population. *Infection* **22**: 258-263.

Koval, S.F. (2007) The search for hunters: culture-dependent and -independent methods for analysis of *Bdellovibrio* and like organisms. In: *Predatory prokaryotes – Biology, ecology and evolution*. Jurkevitch, E. (ed.) Springer Microbiology Monographs. pp. 191-211.

Koval, S.F. and Hynes, S.H. (1991) Effect of paracrystalline protein surface layers on predation by *Bdellovibrio bacteriovorus*. *J Bacteriol* **17**: 2244-2249.

Koval, S.F., Hynes, S.H., Flannagan, R.S., Pasternak, Z., Davidov, Y. and Jurkevitch, E. (2013) *Bdellovibrio exovorus* sp. nov., a novel predator of *Caulobacter crescentus*. *Int J Syst Evol Microbiol* **63**: 146-151.

Kremery, V. Jr., Koprnova, J. and Harniciarova, A. (2004) *Stenotrophomonas maltophilia* bacteremia. *Scand J Infect Dis* **36**: 400

Lai, C.H., Chi, C.Y., Chen, H.P., Chen, T.L., Lai, C.J., Fung, C.P., Yu, K.W., Wong, W.W. and Liu, C.Y. (2004) Clinical characteristics and prognostic factors of patients with *Stenotrophomonas maltophilia* bacteremia. *J Microbiol Immunol Infect* **37**: 350-358.

Lambina, V.A., Afinogenova, A.V., Romai Penabad, S., Konovalova, S.M. and Pushkareva, A.P. (1982) *Micavibrio admirandus* gen. et sp. nov. *Mikrobiologiya* **51**: 114-117.

Landrum, M.L., Conger, N.G. and Forgione, M.A. (2005) Trimethoprim-sulfamethoxazole in the treatment of *Stenotrophomonas maltophilia* osteomyelitis. *Clin Infect Dis* **40**: 1551-1552.

Lin, H.C., Ma, D.H., Chen, Y.F., Yeh, L.K. and Hsiao, C.H. (2011) Late-onset intrascleral dissemination of *Stenotrophomonas maltophilia* scleritis after pterygium excision. *Cornea* **30**: 712-715.

Martinez, J.L. (2008) Antibiotics and antibiotic resistance genes in natural environments. *Science* **321**: 365-367.

Mauger, T.F., Kuennen, R.A., Smith, R.H. and Sawyer, W. (2010) *Acanthamoeba* and *Stenotrophomonas maltophilia* keratitis with fungal keratitis in the contralateral eye. *Clin Ophthalmol* **4**: 1207-1209.

Monnappa, A.K., Dwidar, M., Seo, J.K., Hur, J.H. and Mitchell, R.J. (2014) *Bdellovibrio bacteriovorus* inhibits *Staphylococcus aureus* biofilm formation and invasion into human epithelial cells. *Sci Rep* **22**: 3811.

Nguyen, M.H. and Muder, R.R. (1994) Meningitis due to *Xanthomonas maltophilia*: case report and review. *Clin Infect Dis* **19**: 325-326.

Pasternak, Z., Njagi, M., Shani, Y., Chanyi, R., Rotem, O., Lurie-Weinberger, M.N., Koval, S., Pietrokovski, S., Gophna, U. and Jurkevitch, E. (2014) In and out: an analysis of epibiotic vs periplasmic bacterial predators. *ISME J* **8**: 625-635.

Pompilio, A., Pomponio, S., Crocetta, V., Gherardi, G., Verginelli, F., Fiscarelli, E., Dicuonzo, G., Savini, V., D'Antonio, D. and Di Bonaventura, G. (2011) Phenotypic and genotypic characterization of *Stenotrophomonas maltophilia* isolates from patients with cystic fibrosis: Genome diversity, biofilm formation and virulence. *BMC Microbiol* **11**: 159.

Razvi, S., Quittell, L., Sewall, A., Quinton, H., Marshall, B. and Saiman, L. (2009) Respiratory microbiology of patients with cystic fibrosis in the United States, 1995 to 2005. *Chest* **136**: 1554-1560.

Rojas, P., Garcia, E., Calderón, G.M., Ferreira, F. and Rossa, M. (2009) Successful treatment of *Stenotrophomonas maltophilia* meningitis in a preterm baby boy: a case report. *J Med Case Rep* **3**: 7389.

Ryan, R.P., Monchy, S., Cardinale, M., Taghavi, S., Crossman, L., Avison, M.B., Berg, G., van der Lelie, D. and Dow, J.M. (2009) The versatility and adaptation of bacteria from the genus *Stenotrophomonas*. *Nat Rev Microbiol* **7**: 514-525.

Van Essche, M., Quirynen, M., Slipen, I., Van Eldere, J. and Teughels, W. (2009) *Bdellovibrio bacteriovorus* attacks *Aggregatibacter actinomycetemcomitans*. *J Dent Res* **88**: 182-186.

Vartivarian, S.E., Papadakis, K.A., Palacios, J.A., Manning, J.T. Jr and Anaissie, E.J. (1994) Mucocutaneous and soft tissue infections caused by *Xanthomonas maltophilia*. *Ann Intern Med* **121**: 969-973.

Chapter 5

5. General Discussion

5.1. Cryoelectron microscopy

Bdellovibrio and like organisms arguably spend the majority of their life cycle enclosed within the bdelloplast. The laboratory cocultures are an artificial system in which nutrients (prey cells) are provided and all bdelloplasts release the attack phase cells after the life cycle is complete. Sánchez-Amat and Torrella (1990) demonstrated that marine BALOs will form stable bdelloplasts in nutrient-deprived conditions, increasing long-term viability when compared to attack phase cells under the same conditions. In the natural environment, it is not likely that a predator will have an endless supply of prey. Therefore, previous CEM studies focussing only on the attack phase BALOs (Borgnia *et al.*, 2008; Butan *et al.*, 2011) fail to address the main physiological and morphological changes that occur during attachment, invasion and bdelloplast formation.

Cryoelectron microscopy and tomography were used in the current study as these are the most advanced tools currently available to image bacterial cells. CEM provides projection images of vitreous samples in a near-native state without the artefacts associated with other methods. Using CEM, morphological changes in the prey cells were identified, which researchers in the field believed were necessary for invasion but had never before been observed. Early upon attachment, the periplasmic space of the prey cell ‘makes room’ for the invading BALO. The periplasmic space and the cytoplasm are iso-osmotic, therefore it is difficult to understand how the periplasm expands. One theory involves influxes of water caused by an imbalance in the osmolarity of the periplasmic space (Abram *et al.*, 1974). This idea is supported by Lambert *et al.*

(2010) who showed that an *E. coli* prey cell upregulates many genes involved in the stress response within the initial stages of *Bd. bacteriovorus* attack. One of these genes, *treA*, is involved in maintaining osmotic pressure by breaking down trehalose in the periplasm possibly promoting influx of water. It is known that membrane-derived oligosaccharides, like trehalose, are not used as an energy source but are leaked outside the prey cell early in the predatory life cycle (Ruby and McCabe, 1988), unlike periplasmic proteins, which are retained. It is possible that BALOs ‘trick’ the prey cell into upregulating osmoprotectants, which increases the influx of water into the periplasmic space to allow invasion to occur. It would be interesting to observe whether the modifications to the cytoplasm of the prey cell prior to invasion include a decrease in volume.

The resealing of the entry pore created during BALO invasion is a topic typically glanced over by simply saying “it is resealed”. A small scar on the surface of bdelloplasts (Shilo, 1969) is the only sign that the outer membrane of the prey cell has been breached. However, further analysis of this site was not possible until the advent of more advanced imaging techniques, such as CEM and CET. Repair mechanisms by the prey cell were thought to reseal the entry pore (Abram *et al.*, 1974; Shilo, 1969). However, transcriptomic analysis of prey cell proteins upregulated in response to *Bd. bacteriovorus* identified a single protein that may be involved in membrane repair (Lambert *et al.*, 2010). It is possible that the time point chosen for this study was too early and invasion had not yet occurred. Regardless, the results of this study show that the flagellum was shed after invasion occurred, sealing the large entry pore created. It is known that the flagellum is shed early in the life cycle. The loss of the flagellum may

then signal for the next stages in the predatory life cycle to begin. BALOs are not the only bacteria that shed their flagellum. *C. crescentus* also has a developmental life cycle whereby cell division results in two morphologically distinct cells: a motile swarmer cell and a stalked cell. After about one third of the cell cycle, the swarmer cell sheds its flagellum and a stalk is synthesized at the pole that previously contained the flagellum. In addition, DNA replication is initiated (Sherker and Laub, 2004). This process coincides with a signalling cascade that initiates the growth phase (Jensen and Shapiro, 1999). This process may be similar to what occurs in BALOs. The next step in identifying the plug-like structure is to use computer modelling software to develop a three dimensional map of the plug structure to determine how the plug is inserted into the outer membrane of the bdelloplast.

5.2. Role of TFP in predation by *Bd. bacteriovorus*

The environmental impact of BALOs and their role in shaping microbial communities relies on the ability to prey on a wide range of bacteria. Therefore, to determine what facilitates a successful interaction between the predator and prey is a goal to help understand the cell biology of BALOs. There are two different interactions that occur prior to successful predation on a prey cell. Initially there is a reversible attachment in which the predator will search for potential prey cells, attaching to both Gram-positive and Gram-negative cells and even to abiotic surfaces. After a period of time the predator will either detach to continue searching for prey or it will form an irreversible attachment and begin to modify the prey cell for invasion. The surface characteristics that promote irreversible attachment are not known. Due to the wide prey

range, BALOs must have a general mechanism for recognizing a variety of surface characteristics on the Gram negative prey cell.

To determine surface characteristics of the prey cell which are recognized by *Bd. bacteriovorus*, a transposon mutagenesis library of *E. coli* ML35 was created and a high throughput screening method to identify prey cell mutants that were not susceptible to predation by *Bd. bacteriovorus* 109J was developed (Chanyi and Koval, Unpublished data). However, after screening roughly 2000 mutants, no predation resistant mutants were obtained and thus prey cell characteristics that facilitated predator-prey interactions could not be identified. It is believed that irreversible attachment by a BALO to a prey cell may be either a multifactorial event or the factor involved may be essential for prey cell viability. The latter conclusion is supported by the fact that if a bacterium is susceptible to predation, it does not develop resistance. Shemesh and Jurkevitch (2004) described a plastic phenotypic resistance whereby cells remaining in coculture after predation is complete are resistant to further attack. However, if these cells are re-cultured and exposed to the BALO, they are once again susceptible to predation. This suggests that a receptor may be present that is down-regulated late in stationary phase in a few cells of a batch culture. More studies have to be performed to determine if these cells represent a similar phenotype to that of dormant persister cells found in biofilms (Goneau *et al.*, 2014).

Type IV pili have been considered as candidate structures to provide attachment and the mechanical force necessary for the penetration process. The genome sequence of *Bd. bacteriovorus* HD100^T (Rendulic *et al.*, 2004) and strain Tiberius (Hobley *et al.*, 2012) identified a large number of TFP genes, suggestive of their importance in the

predatory life cycle. Evans *et al.* (2007) and Mahmoud and Koval (2010) showed that the PilA protein, and therefore TFP in general, are required for predation to occur. Burnham *et al.* (1968) showed that once a predator is attached to a prey, neither vortexing nor light sonication were able to separate predator from prey. TFP may provide the flexibility and strength necessary for these initial interactions. Predators in coculture with anti-PilA antibodies present were unable to attach to the *E. coli* prey cell (Mahmoud and Koval, 2010). This confirmed the role of TFP in attachment; however, their role in invasion was unknown.

Medina *et al.* (2008) used a transposon mutagenesis approach to identify genes necessary for predation on solid surfaces. They identified multiple candidate genes, and, most interestingly, *bd3852* was identified as essential. This gene encodes the PilT2 protein, which has a high amino acid sequence similarity to the PilT protein found in *P. aeruginosa*. PilT proteins are ATP-dependent motors involved in the depolymerisation of PilA from the TFP fiber. This action causes a retraction of the TFP. Therefore, it was proposed that the retraction of TFP powered by PilT2 was necessary for successful invasion into a prey cell (Medina *et al.*, 2008). However, this function was speculative and no further work has been performed to confirm this theory.

The turgor pressure exerted by the prey cell is a formidable barrier the predator must overcome to gain entry into the periplasmic space. The PilT protein is proposed as one of the strongest biological motors studied. PilT is able to generate forces up to 140 pN per pilus (Maier *et al.*, 2002). Although few BALO predators are observed with more than 1-3 pili, bundling of fibers may be promoted upon initial anchoring to the membrane of the prey cell. This was observed in *N. gonorrhoeae* that produced a higher ordered

bundle of pili, which generated forces up to 8 to 10 times higher than the single pilus alone (Biais *et al.*, 2008).

By creating markerless in-frame deletion mutants of the *pilT* genes in *Bd. bacteriovorus*, it was shown that these genes are not required for predation on an *E. coli* prey cell in liquid coculture. Successful invasion of the periplasmic space occurred in the absence of either one or both of these genes, forming stable bdelloplasts and following the same stages of the predatory life cycle. Similar to what was previously described by Medina *et al.* (2008), the *pilT2* mutant was unable to form plaques on an agar surface or prey on a biofilm. Thus, retraction of *Bd. bacteriovorus* TFP is not the motor that drives the invasion process but is required for successful predation within a biofilm. TFP may be required to penetrate the extracellular polymeric substance secreted by biofilms. This function may have a large impact on the bacterial predators in nature as biofilms may represent a natural reservoir of these bacteria (Kadouri and O'Toole, 2005).

The characterization of a BALO that does not invade the periplasmic space provides the perfect model to distinguish between predation-specific and invasion-specific genes. The genome of the epibiotic predator *Bd. exovorus* JSS is much smaller (2.66 mbp) than that observed for periplasmic predators such as *Bd. bacteriovorus* HD100 (3.78 mbp) (Pasternak *et al.*, 2014). Similar to previously sequenced BALO genomes, *Bd. exovorus* JSS possessed a wide array of genes encoding for components of the TFP apparatus. The genes were present in a very similar genome organization to that of *Bd. bacteriovorus* HD100. Homologues of *pilT1* and *pilT2* were found in *Bd. exovorus* JSS, both having 99.5% gene sequence similarity to their homologues in *Bd. bacteriovorus*. The presence of these genes in the *Bd. exovorus* genome combined with

the results of the current studies strongly suggests that retraction of TFP is not required for the invasion process during the periplasmic life cycle.

The role of TFP during predation appears to be simple. TFP are necessary for the initial interaction between the predator and prey. The strong adherence to a cell allows the predator to scan the prey giving time to search for a secondary interaction that provides the signal(s) necessary to continue with the predation process. If the signal(s) are not found, the predator releases from the surface of the cell and continues the hunt.

The majority of wildtype *P. aeruginosa* cells have a pilus. However, when PilT was deleted in *P. aeruginosa* the pilus was extended but unable to retract resulting in cells with a hyperpilated phenotype. It is unknown why in a batch culture so few BALOs have TFP on their surface, knowing they are essential for initiating predation. The current study identified that approximately 27% of wildtype *Bd. bacteriovorus* cells have TFP on the surface. This value is similar to those of previous studies, which identified that anywhere between 25% and 30% of cells have visible TFP (Evans *et al.*, 2007; Mahmoud and Koval, 2010). There was a significant increase in the number of cells with TFP in both the *pilT1* and *pilT2* mutants, as assessed by immunofluorescence microscopy (33.4% and 29.2%, respectively). It is possible that only a minority of the population are able to prey, and therefore, only this minority would express TFP. The *pilT2* gene was not essential for predation in cocultures by *Bd. bacteriovorus*. Therefore, to help understand the role of this gene in BALOs, a *pilT2* mutant in *Bd. exovorus* was sought. Unfortunately, a *pilT2* mutant was unable to form plaques on an agar surface, and therefore a prey-independent strain must be used to create the mutant. After many

attempts by the laboratories of Jurkevitch (Pasternak *et al.*, 2014) and Koval, a prey independent strain was unable to be made.

It is still unknown how BALOs enter the periplasmic space of the prey cell. Flagellar motility (Lambert *et al.*, 2006) and TFP retraction have all been ruled out. It is not only important to understand how BALOs recognize their prey but also to understand the fundamental aspects of their life cycle. It was believed that BALOs were capable of preying only upon other Gram negative cells since invasion into the periplasmic space was a necessary step before DNA replication could occur. The characterization of an epibiotic predator raises many questions. Could it be possible to ‘trick’ *Bd. exovorus* into killing Gram positive pathogens? Initial reversible attachment already occurs; therefore, by manipulating the secondary interactions possibly mediated by surface receptors and providing the intermediary signals to stimulate progression into growth phase, it may be possible.

5.3. Bacterial predators for therapeutic use

The studies regarding the therapeutic use of *Bd. exovorus* against *S. maltophilia* arose out of serendipity when a researcher in the *Stenotrophomonas* field contacted the Koval laboratory regarding whether or not a BALO predator could be used to disrupt biofilms of *S. maltophilia*. With the emergence of new antibiotic resistant pathogens on almost a daily basis, it is important to develop novel weapons that can be used to combat these infectious agents. The use of bacterial predators is currently an under-explored avenue with some promising potential. There have been extensive studies on the ability of bacterial predators to kill Gram negative pathogens and disrupt biofilms (Dashiff and Kadouri, 2011; Dashiff *et al.*, 2011; Fratamico *et al.*, 1996; Markelova, 2010; Van Essche

et al., 2011). BALOs are known to have a wide prey range. For example, *Bd. bacteriovorus* can prey on *Acinetobacter*, *Aeromonas*, *Bordetella*, *Burkholderia*, *Campylobacter*, *Citrobacter*, *Enterobacter*, *Helicobacter*, *Klebsiella*, *Listonella*, *Morganella*, *Proteus*, *Serratia*, *Salmonella*, *Shigella*, *Vibrio* and *Yersinia* (Dashiff *et al.*, 2011; Markelova, 2010).

In a poultry model of *Salmonella* infection and intestinal/cecal colonization, orally administered *Bd. bacteriovorus* significantly reduced the colonization of *S. enterica* serovar Enteritidis, an important zoonotic pathogen (Atterbury *et al.*, 2011). There was also significant reduction in cecal inflammation. These effects were a direct result of *Bd. bacteriovorus* predation as the same results were not observed when a non-predacious *pilA* mutant was used. This was the first report of using a BALO in an animal model. Another study focussed on the use of *Bd. bacteriovorus* to aid in ocular infections in cows caused by *Moraxella bovis* (Boileau *et al.*, 2011). The authors found that *Bd. bacteriovorus* reduced the number of *M. bovis* cells attached to bovine epithelial cells and the predator remained active in tears for up to 24 h, an important aspect for topical use.

Two major hurdles against using a live antimicrobial agent are (1) the potential to cause tissue damage in the host and (2) the host immune system recognizing and killing the predator before it is able to help combat infection. The first hurdle is a simple one for BALOs: they are unable to invade and lyse eukaryotic cells and therefore their predatory life cycle does not cause tissue damage. The second hurdle could pose a problem. *Bd. bacteriovorus* has a modified lipid A structure (Schwudke *et al.*, 2003). Rather than the typical phosphate head group, *Bd. bacteriovorus* has an α -D-mannose residue. This modification replaces the negative charges in the lipid A and therefore, as a neutral lipid

A, is less immunogenic. There is a decrease in the binding affinity to the LPS receptor on human macrophages, as measured by a decrease in stimulation of TNF- α and IL-6 as compared to an *E. coli* control (Schwudke *et al.*, 2003).

S. maltophilia is an emerging multi-drug resistant bacterium that has been associated with ocular and respiratory infections, pneumonia and bacteremia (Brooke, 2012). It has been also cultured from the mucus of patients with cystic fibrosis (Amin and Waters, 2014; Davies and Rubin, 2007; Samonis *et al.*, 2012; Steinkamp *et al.*, 2005), a respiratory disease that causes fluid buildup in the lungs, which is easily colonized by pathogens such as *Pseudomonas aeruginosa*, *Burkholderia cenocepacia*, *Staphylococcus aureus* and *S. maltophilia*. Unlike other pathogens, the genome of *S. maltophilia* does not encode for typical virulence factors and instead relies on the ability to successfully colonize and form biofilms in harsh environments (Brooke, 2012). It has even been found to be a contaminant in the antiseptic used in hospitals, which is typically used to kill most microorganisms (Wishart and Riley, 1976). There is a large degree of strain variation in the susceptibility to specific antibiotics. To find a more broad acting strategy against *S. maltophilia*, phage therapy was tried. However, this approach was ineffective. The data presented in the current study showed that *Bd. exovorus* was able to prey on 19 *S. maltophilia* isolates, killing on average 99% of the prey cells present. Furthermore, *Bd. exovorus* disrupted biofilms of almost all *S. maltophilia* isolates even in the presence of ciprofloxacin or kanamycin. The concentration of ciprofloxacin used was 2 to 5 times the minimum inhibitory concentration required to eliminate 50% of *S. maltophilia* present (Crossman *et al.*, 2008; Pankuch *et al.*, 1994).

It is important to note that, in a clinical setting, a patient does not have the luxury of knowing an infection is occurring until it has already been established. Therefore, biofilms were pre-formed in the absence of any antibiotic. This study is a first step in the potential use of *Bd. exovorus* to combat infections of *S. maltophilia*. The next step would be to use an animal model to determine if the same results are obtained *in vivo*.

It is also important to understand that many infections are polymicrobial, such as bacterial infections associated with cystic fibrosis. Because BALOs have a wide prey range, but not inclusive to all Gram negative pathogens, a predatory cocktail may be of use; however, this approach has not yet been studied.

5.4. Final statement

With each puzzle piece that is uncovered about BALOs, it seems two or more questions arise. The studies discussed in this thesis have revealed many insights into the cell biology of BALOs. If nothing else, it should be apparent that microorganisms are smarter than we are. With every new antibiotic we develop, resistance is gained. Like probiotics, I believe we should start using predatory bacteria for our own good. BALOs have evolved to kill bacteria, and they do it very well. As a true predator, BALOs will never kill all of their prey. However, in a severe infection this may be enough to allow our own immune system to gain some ground, killing those the BALOs could not. A lot of experimental work needs to be done before this is possible but “if we don’t change our direction we’re likely to end up where we’re headed”.

5.5. References

- Abram, D., Melo, J.C. and Chou, D. (1974) Penetration of *Bdellovibrio bacteriovorus* into host cells. *J Bacteriol* **118**: 663-680.
- Amin, R. and Waters, V. (2014) Antibiotic treatment for *Stenotrophomonas maltophilia* in people with cystic fibrosis. *Cochrane Database Syst Rev* **4**: CD009249.
- Atterbury, R. J., Hopley, L., Till, R., Lambert, C., Capeness, M. J., Lerner, T. R., Fenton, A. K., Barrow, P. and Sockett, R. E. (2011) Effects of orally administered *Bdellovibrio bacteriovorus* on the well-being and *Salmonella* colonization of young chicks. *Appl Environ Microb* **77**: 5794-5803.
- Biais, N., Ladoux, B., Higashi, D., So, M. and Sheetz, M. (2008) Cooperative retraction of bundled type IV pili enables nanonewton force generation. *PLoS Biol* **6**: 907-913.
- Boileau, M. J. and Clinkenbeard, K. D. (2011) Assessment of *Bdellovibrio bacteriovorus* 109J viability in bovine tears. *J Vet Intern Med* **25**: 759-760.
- Borgnia, M.J., Subramanian, S. and Milne, J.L. (2008) Three-dimensional imaging of the highly bent architecture of *Bdellovibrio bacteriovorus* by using cryo-electron tomography. *J Bacteriol* **190**: 2588-2596.
- Brooke, J.S. (2012) *Stenotrophomonas maltophilia*: an emerging global opportunistic pathogen. *Clin Microbiol Rev* **25**: 2-41.
- Burnham, J.C., Hashimoto, T. and Conti, S.F. (1968) Electron microscope observations on the penetration of *Bdellovibrio bacteriovorus* into gram-negative bacterial hosts. *J Bacteriol* **96**: 1366-1381.
- Butan, C., Hartnell, L.M., Fenton, A.K., Bliss, D. and Sockett, R.E., Subramaniam, S. and Milne, J.L. (2011) Spiral architecture of the nucleoid in *Bdellovibrio bacteriovorus*. *J Bacteriol* **193**: 1341-1350.
- Crossman, L.C., Gould, V.C., Dow, J.M., Vernikos, G.S., Okazaki, A., Sebahia, M., Saunders, D., Arrowsmith, C., Carver, T., Peters, N., Adlem, E., Kerhornou, A., Lord, A., Murphy, L., Seeger, K., Squares, R., Rutter, S., Quail, M.A., Rajandream, M.A., Harris, D., Churcher, C., Bentley, S.D., Parkhill, J., Thomson, N.R. and Avison, M.B. (2008) The complete genome, comparative and functional analysis of *Stenotrophomonas maltophilia* reveals an organism heavily shielded by drug resistant determinants. *Genome Biol* **9**: R74.
- Dashiff, A. and Kadouri, D.E. (2011) Predation of oral pathogens by *Bdellovibrio bacteriovorus* 109J. *Mol Oral Microbiol* **26**: 19-34.

Dashiff, A., Junka, R.A., Libera, M. and Kadouri, D.E. (2011) Predation of human pathogens by the predatory bacteria *Micavibrio aeruginosavorus* and *Bdellovibrio bacteriovorus*. *J Appl Microbiol* **110**: 431-444.

Davies, J.C. and Rubin, B.K. (2007) Emerging and unusual Gram-negative infections in cystic fibrosis. *Semin Respir Crit Care Med* **28**: 312-321.

Evans, K.J., Lambert, C. and Sockett, R.E. (2007) Predation by *Bdellovibrio bacteriovorus* HD100 requires type IV pili. *J Bacteriol* **189**: 4850-4859.

Fratamico, P. M. and Cooke, P. H. (1996) Isolation of *Bdellovibrios* that prey on *Escherichia coli* O157:H7 and *Salmonella* species and application for removal of prey from stainless steel surfaces. *J Food Safety* **16**: 161-173.

Goneau, L.W., Yeoh, N.S., MacDonald, K.W., Cadieux, P.A., Burton, J.P., Razvi, H. and Reid, G. (2014) Selective target inactivation rather than global metabolic dormancy causes antibiotic tolerance in uropathogens. *Antimicrob Agents Chemother* **58**: 2089-2097.

Gray, K.M. and Ruby, E.G. (1991) Intercellular signalling in the *Bdellovibrio* developmental growth cycle. In: Microbial cell-cell interactions. Dworkin, M., ed. American Society for Microbiology pp. 333-366.

Hobley, L., Lerner, T.R., Williams, L.E., Lambert, C., Till, R., Milner, D.S., Basford, S.M., Capeness, M.J., Fenton, A.K., Atterbury, R.J., Harris, M.A. and Sockett, R.E. (2012) 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. *BMC Genomics* **13**: 670.

Jensen, R.B. and Shapiro, L. (1999) The *Caulobacter crescentus smc* gene is required for cell cycle progression and chromosome segregation. *Proc Natl Acad Sci USA* **96**: 10661-10666.

Kadouri, D. and O'Toole, G.A. (2005) Susceptibility of biofilms to *Bdellovibrio bacteriovorus* attack. *Appl Environ Microbiol* **71**: 4044-4051.

Lambert, C., Evans, K.J., Till, R., Hobley, L., Capeness, M., Rendulic, S., Schuster, S.C., Aizawa, S. and Sockett, R.E. (2006) Characterizing the flagellar filament and the role of motility in bacterial prey-penetration by *Bdellovibrio bacteriovorus*. *Mol Microbiol* **60**: 274-286.

Lambert, C., Ivanov, P. and Sockett, R.E. (2010) A transcriptional "scream" early response of *E. coli* prey to predatory invasion by *Bdellovibrio*. *Curr Microbiol* **60**: 419-427.

Mahmoud, K.K. and Koval, S.F. (2010) Characterization of type IV pili in the life cycle of the predator bacterium *Bdellovibrio*. *Microbiology* **156**: 1040-1051.

Maier, B., Potter, L., So, M., Long, C.D., Seifert, H.S. and Sheetz, M.P. (2002) Single pilus motor forces exceed 100 pN. *Proc Natl Acad Sci USA* **99**:16012-16017.

Markelova, N. Y. (2010) Predacious bacteria, *Bdellovibrio* with potential for biocontrol. *Int J Hyg Environ Health* **213**: 428-431.

Medina, A.A., Shanks, R.M. and Kadouri, D.E. (2008) Development of a novel system for isolating genes involved in predator-prey interactions using host independent derivatives of *Bdellovibrio bacteriovorus* 109J. *BMC Microbiol* **8**: 33.

Pankuch, G.A., Jacobs, M.R., Rittenhouse, S.F. and Appelbaum, P.C. (1994) Susceptibility of 123 strains of *Xanthomonas maltophilia* to eight β -lactams (including β -lactam- β -lactamase inhibitor combinations) and ciprofloxacin tested by five methods. *Antimicrob Agents Chemother* **38**: 2317-2322.

Pasternak, Z., Njagi, M., Shani, Y., Chanyi, R., Rotem, O., Lurie-Weinberger, M.N., Koval, S., Pietrokovski, S., Gophna, U. and Jurkevitch, E. (2014) In and out: an analysis of epibiotic vs periplasmic bacterial predators. *ISME J* **8**: 625-635.

Rendulic, S., Jagtap, P., Rosinus, A., Eppinger, M., Baar, C., Lanz, C., Keller, H., Lambert, C., Evans, K.J., Goesmann, A., Meyer, F., Sockett, R.E. and Schuster, S.C. (2004) A predator unmasked: Life cycle of *Bdellovibrio bacteriovorus* from a genomic perspective. *Science* **303**: 689-692.

Ruby, E.G. and McCabe, J.B. (1988) Metabolism of periplasmic membrane-derived oligosaccharides by the predatory bacterium *Bdellovibrio bacteriovorus* 109J. *J Bacteriol* **170**: 646-652.

Samonis, G., Karageorgopolous, D.E., Maraki, S., Levis, P., Dimopoulou, D., Spervovasilis, N.A., Kofteridis, D.P. and Falagas, M.E. (2012) *Stenotrophomonas maltophilia* infections in a general hospital: patient characteristics, antimicrobial susceptibility and treatment outcome. *PLoS One* **7**: e37375.

Sanchez-Amat, A. and Torrella, F. (1990) Formation of stable bdelloplasts as a starvation-survival strategy of marine bdellovibrios. *Appl Environ Microbiol* **56**: 2717-2725.

Schwudke, D., Linscheid, M., Strauch, E., Appel, B., Zahringer, U., Moll, H., Muller, M., Brecker, L., Gronow, S. and Lindner, B. (2003) The obligate predatory *Bdellovibrio bacteriovorus* possesses a neutral lipid A containing alpha-D-Mannoses that replace phosphate residues: similarities and differences between the lipid As and the lipopolysaccharides of the wild type strain *B. bacteriovorus* HD100 and its host-independent derivative HI100. *The J Biol Chem* **278**: 27502-27512.

- Shemesh, Y.S. and Jurkevitch, E. (2004) Plastic phenotypic resistance to predation by *Bdellovibrio* and like organisms in bacterial prey. *Environ Microbiol* **6**: 12-18.
- Sherker, J.M. and Laub, M.T. (2004) Cell-cycle progression and the generation of asymmetry in *Caulobacter crescentus*. *Nat Rev Microbiol* **2**: 325-337.
- Shilo, M. (1969) Morphological and physiological aspects of the interaction of *Bdellovibrio* with host bacteria. *Curr Top Microbiol Immunol* **50**: 174-204.
- Steinkamp, G., Wiedemann, B., Rietschel, E., Krahl, A., Gielen, J., Bartheimer, H. and Ratjen, F. (2005) Prospective evaluation of emerging bacteria in cystic fibrosis. *J Cyst Fibros* **4**: 41-48.
- Van Essche, M., Quirynen, M., Sliopen, I., Loozen, G., Boon, N., Van Eldere, J. and Teughels, W. (2011) Killing of anaerobic pathogens by predatory bacteria. *Mol Oral Microbiol* **26**: 52-61.
- Wishart, M.M. and Riley, T.V. (1976) Infection with *Pseudomonas maltophilia*: hospital outbreak due to contaminated disinfectant. *Med J Aust* **2**: 710-712.

Curriculum Vitae

Ryan Chanyi

The University of Western Ontario
Department of Microbiology and Immunology

Education

2009 BMSc The University of Western Ontario
Schulich School of Medicine and Dentistry
Department of Microbiology and Immunology
London, Ontario, Canada

Honors Specialization in Microbiology and Immunology;
Graduated 'with Distinction'

Thesis: Predator-Prey Interactions of *Bdellovibrio* and like Organisms

Training

2009-2014 PhD Candidate

Primary Foci: Microbial interactions; novel therapeutic development; biofilm disruption; molecular biology; light and electron microscopy (transmission, scanning and cryoelectron); comparative genomics

2008-2009 Undergraduate Honors Thesis Student

Primary Foci: Isolation of environmental predators; identification (16S rRNA sequencing, ARDRA, ribotyping); predator-prey interactions of *Bdellovibrio* and like organisms

Publications

- **Chanyi, R.M.** and Koval, S.F. (2014) Role of type IV pili in predation by *Bdellovibrio bacteriovorus*. *PLoS One* **Under Review**.
 - Pasternak, Z., Njagi, M., Shani, Y., **Chanyi, R.**, Rotem, O., Lurie-Weinberger, M.N., Koval, S., Pietrokovski, S., Gophna, U. and Jurkevitch, E. (2014) In and out: an analysis of epibiotic vs periplasmic bacterial predators. *ISME J* **8**: 625-635.
 - **Chanyi, R.M.**, Ward, C., Pechey, A. and Koval, S.F. (2013) To invade or not to invade: two approaches to a prokaryotic predatory life cycle. *Can J Microbiol* **59**: 273–279.
-

Textbook contributions

- **Contributed two electron micrographs to:**
Madigan, M.T., Martinko, J.M., Stahl, D.A. and Clark, D.P. (2012) Brock Biology of Microorganisms. 13th Ed. San Francisco, USA. Pearson Prentice Hall. p500.

Publications in Preparation

- **Chanyi, R.M.**, Koval, S.F. and Brooke, J. Disruption of *Stenotrophomonas maltophilia* biofilms using the bacterial predator *Bdellovibrio exovorus* in the presence of antibiotics.
- **Chanyi, R.M.**, Khursigara, C. and Koval, S.F. Visualization of two predatory life cycles by cryoelectron microscopy and tomography.
- **Chanyi, R.M.**, Szmielt, D., Dharamshi, J., Dresser, A. and Koval, S.F. Identification of *Bdellovibrio* and like organisms using amplified ribosomal DNA restriction analysis and ribotyping.

*Oral Presentations (*presenting author)*

- Koval, S.F*., **Chanyi, R.M.**, Moyles, D. and Khursigara, C. Structural analysis of a bacterial predator-prey interaction. Annual Conference Microscopical Society of Canada. Halifax, NS, Canada, June 2012.

*Poster Presentations (*presenting author)*

- **Chanyi, R.M.*** and Koval, S.F. Twitching motility is essential for predation on a biofilm by *Bdellovibrio bacteriovorus*. Annual Conference. Canadian Society of Microbiologists, Carleton University, Ottawa, ON, Canada, June 2013.
 - **Received Terry Beveridge Poster Award**
- **Chanyi, R.M.***, Dresser, A., Dharamshi, J. and Koval, S.F. Identification of new *Bdellovibrio* and like organisms. ASM Conference. Montreal, QC, Canada, May 2012.
 - **Received Travel Grant from ASM**
- **Chanyi, R.M.*** and Koval, S.F. Role of *pilT1* and *pilT2* in predation by *Bdellovibrio bacteriovorus*. Infection and Immunity Research Forum. University of Western Ontario, London, ON, Canada, November 2011.

- **Chanyi, R.M.*** and Koval, S.F. Characterization of *pilT1* and *pilT2* in the life cycle of *Bdellovibrio bacteriovorus*. Annual Conference. Canadian Society of Microbiologists, Memorial University, St. John's, NL, Canada, June 2011.
 - **Received Canadian College of Microbiologists Poster Award**
- **Chanyi, R.M.***, Mahmoud, K.K., and Koval, S.F. Invasion strategies: role of type IV pili in attachment and invasion of *E. coli* by *Bdellovibrio bacteriovorus*. Annual Conference. Canadian Society of Microbiologists, McMaster University, Hamilton, ON, Canada, June 2010.

Teaching Experience

- **Graduate Teaching Assistant**
The University of Western Ontario. Microbiology & Immunology 2100A (Biology of Prokaryotes) lab component. September to December 2010, 2011, 2012 and 2013.
- **Supervision of Fourth Year Honor Thesis Students**
Responsible for the supervision and training of four fourth year honor thesis project students: Amy Dresser (2010), Jennah Dharamshi (2011), Diane Szmielt (2012) and Zach Findlay (2013).
- **Supervision of Student Volunteers**
Supervised four student volunteers: Ian Lobb, Sarah Walker, Jodie Baer and Fulbert Fu and a USRA student, Emma Farago (2011).

Honors and Awards

- Terry Beveridge Poster Award at the Canadian Society of Microbiologists Annual Conference: June 2013, Ottawa, ON, Canada
 - Microbiology and Immunology Travel Award: 2011, 2012 and 2013
 - Graduate Thesis Research Award Fund: 2013
 - American Society for Microbiology (ASM) Travel Award, 2012
 - Canadian College of Microbiologists Poster Award at the Canadian Society of Microbiologists Annual Conference: June 2011, St. John's, NL, Canada
 - PhD candidacy exam- Passed with distinction: March 2011
 - Graduated BMSc with distinction: June 2009
-