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Role of peptidoglycan modifications in predation by Bdellovibrio bacteriovorus 109J

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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ROLE OF PEPTIDOGLYCAN MODIFICATIONS IN PREDATION
BY BDELOVIBRIO BACTERIOVORUS 109J

(Thesis format: Monograph)

by

Diane Cynthia Szmiett

Graduate Program in Microbiology and Immunology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

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

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Abstract

*Bdellovibrio bacteriovorus* is a prey dependent bacterium that traverses the outer membrane and peptidoglycan, and enters the periplasmic space of gram negative bacteria, from where it utilizes the cytoplasmic contents for growth. Soluble lytic transglycosylases could be responsible for creating the entry pore during predation. Bacteria can modify their peptidoglycan by the addition of acetate to the C-6 hydroxyl group of the N-acetylmuramic acid residues, which renders the peptidoglycan insensitive to cleavage by lytic transglycosylases. It was hypothesized that the degree of peptidoglycan O-acetylation of the prey cell affects predation efficiency. In this study it was shown that; (1) *Bdellovibrio bacteriovorus* 109J has minimal peptidoglycan O-acetylation while *Bdellovibrio bacteriovorus* 109JA, *Bdellovibrio exovorus* JSS, and *Bacteriovorax stolpii* UKi2 do not O-acetylate their peptidoglycan, (2) predation efficiency of *Bdellovibrio bacteriovorus* is affected by the degree of peptidoglycan O-acetylation, and (3) the lytic transglycosylase *bd3575* is not essential for predation.

Key Words

*Bdellovibrio bacteriovorus*, *Proteus mirabilis*, lytic transglycosylase, O-acetylation, peptidoglycan
Dedication

I dedicate this thesis to my parents Robert and Krystyna Szmiett whose constant support and sacrifices continue to make it possible for me to achieve my goals. I also dedicate this thesis to Stephanie Wilcocks and Simon Van Aert for their support.
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Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title Page</td>
<td>i</td>
</tr>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iv</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>v</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>viii</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>ix</td>
</tr>
</tbody>
</table>

Chapter 1 – Introduction

1.1 Predatory Prokaryotes                                               1
1.2 *Bdellovibrio* and Like Organisms (BALOs)                           1
1.3 Life Cycles of BALOs                                                2
  1.3.1 Periplasmic life cycle                                           2
    1.3.1.1 Search for a suitable prey cell                            2
    1.3.1.2 Attachment and invasion                                    4
    1.3.1.3 Formation of bdelloplast                                   4
    1.3.1.4 Growth phase and release of attack phase cells             5
  1.3.2 Epibiotic life cycle                                            6
    1.3.2.1 Isolation of epibiotic BALO                                 6
1.4 Peptidoglycan of Gram Negative Bacteria                             7
1.5 *Bdellovibrio bacteriovorus* Peptidoglycan Modifying Enzymes       7
  1.5.1 Background studies                                             7
  1.5.2 Current understandings                                         9
1.6 *Proteus mirabilis*                                                11
1.7 Research Objectives                                                14

Chapter 2 – Materials and Methods

2.1 Bacterial Strains and Culture Conditions                            17
2.2 Analysis of Peptidoglycan                                            17
  2.2.1 Isolation of crude peptidoglycan                               17
  2.2.2 Detection of O-acetylation                                     20
    2.2.2.1 Release and quantification of O-acetyl groups               20
    2.2.2.2 Muramic acid quantification                                20
2.3 Construction of *bd3575* Knockout Mutant of *B. bacteriovorus* 109J 21
  2.3.1 Chromosomal DNA and plasmid isolation                          21
  2.3.2 Polymerase chain reaction                                      21
  2.3.3 Construction of the pΔ3575 suicide vector                      24
  2.3.4 Preparation of competent cells                                 24
  2.3.5 Preparation of electrocompetent cells                          24
  2.3.6 Bacterial transformations                                      26
    2.3.6.1 Heat shock transformations of *E. coli* SY327λ.pir         26
    2.3.6.2 Electroporation of *E. coli* SM10λ.pir                     26
  2.3.7 *B. bacteriovorus* conjugal mating                             26
2.3.8 Counterselection 27
2.4 Agarose Gel Electrophoresis 28
2.5 DNA Sequencing and Genomes 28
2.6 Predation Assays 28
  2.6.1 Predation kinetic assays 28
  2.6.2 Enumeration of prey cells 29
2.7 Biotin Labeling 30
  2.7.1 Biotinylation of *B. bacteriovorus* 109J 30
  2.7.2 Preparation of synchronous co-cultures 30
2.8 Microscopy Techniques 31
  2.8.1 Phase contrast light microscopy 31
  2.8.2 Transmission electron microscopy 31

**Chapter 3 – Results** 32
3.1 *O*-acetylation of Peptidoglycan of Representative BALOs 32
  3.1.1 Isolation and purification of peptidoglycan 32
  3.1.2 Quantification of peptidoglycan *O*-acetylation 35
3.2 Effect of Prey Cell Peptidoglycan *O*-acetylation on predation 35
  3.2.1 Synchronous co-cultures assessed by light microscopy 37
  3.2.2 Comparison of predation kinetics 37
  3.2.3 Comparison of predation aggressiveness 40
3.3 Mutation of gene *bd3575* in *B. bacteriovorus* 109J 40
  3.3.1 Creation of *B. bacteriovorus* 109J Δ3575 42
  3.3.2 Synchronous co-cultures assessed by light microscopy 45
  3.3.3 Electron microscopy of *B. bacteriovorus* 109J Δ3575 50
  3.3.4 Predation kinetics of *B. bacteriovorus* 109J Δ3575 50
  3.3.5 Predation aggressiveness of *B. bacteriovorus* 109J Δ3575 55
3.4 Effects of Biotinylation on Predation by *B. bacteriovorus* 109J 55
  3.4.1 Predation efficiency assessed by light microscopy 59

**Chapter 4 – Discussion** 60
4.1 Summary 66

References 68
Curriculum Vitae 73
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The periplasmic life cycle of <em>B. bacteriovorus</em></td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Activity site of major gram negative peptidoglycan modifying enzymes</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>Two component <em>O</em>-acetylation system in gram negative bacteria</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>Physical map of primers used in this study</td>
<td>23</td>
</tr>
<tr>
<td>5</td>
<td>Physical map of suicide plasmid pSSK10 used in this study</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td><em>B. bacteriovorus</em> 109J peptidoglycan sacculi</td>
<td>34</td>
</tr>
<tr>
<td>7</td>
<td>Predation kinetics of <em>B. bacteriovorus</em> 109J on strains of <em>P. mirabilis</em></td>
<td>38</td>
</tr>
<tr>
<td>8</td>
<td>Amplification of upstream and downstream fragments of gene <em>bd3575</em> from <em>B. bacteriovorus</em> 109J</td>
<td>43</td>
</tr>
<tr>
<td>9</td>
<td>Ligation of upstream and downstream fragments of gene <em>bd3575</em> from <em>B. bacteriovorus</em> 109J</td>
<td>44</td>
</tr>
<tr>
<td>10</td>
<td>DNA sequence of deletion construct from pΔ3575</td>
<td>46</td>
</tr>
<tr>
<td>11</td>
<td>Colony PCR screening of <em>E. coli</em> SM10λpir transformed with plasmid pΔ3575</td>
<td>47</td>
</tr>
<tr>
<td>12</td>
<td>Confirmation of <em>B. bacteriovorus</em> 109J Δ3575</td>
<td>48</td>
</tr>
<tr>
<td>13</td>
<td>DNA sequence of deletion construct in <em>B. bacteriovorus</em> 109J Δ3575</td>
<td>49</td>
</tr>
<tr>
<td>14</td>
<td>Electron micrographs of <em>B. bacteriovorus</em> 109J Δ3575 life cycle</td>
<td>51-53</td>
</tr>
<tr>
<td>15</td>
<td>Predation kinetics of <em>B. bacteriovorus</em> 109J Δ3575</td>
<td>54</td>
</tr>
<tr>
<td>16</td>
<td>Biotin-avidin binding assay</td>
<td>58</td>
</tr>
</tbody>
</table>
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Major peptidoglycan modifying enzymes in <em>Escherichia coli</em> and their homologs in <em>B. bacteriovorus</em> HD100</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>Representative bacteria demonstrated to produce O-acetylated peptidoglycan</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>Degree of peptidoglycan O-acetylation of <em>P. mirabilis</em> strains used in this study</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>Bacterial strains and plasmids used in this study</td>
<td>18</td>
</tr>
<tr>
<td>5</td>
<td>Primers used in this study</td>
<td>22</td>
</tr>
<tr>
<td>6</td>
<td>Yield of peptidoglycan</td>
<td>33</td>
</tr>
<tr>
<td>7</td>
<td>Degree of peptidoglycan O-acetylation of bacteria in this study</td>
<td>36</td>
</tr>
<tr>
<td>8</td>
<td>Standard deviations of predation kinetics of <em>B. bacteriovorus</em> 109J on <em>P. mirabilis</em></td>
<td>39</td>
</tr>
<tr>
<td>9</td>
<td>Predation aggressiveness of <em>B. bacteriovorus</em> 109J on <em>P. mirabilis</em></td>
<td>41</td>
</tr>
<tr>
<td>10</td>
<td>Standard deviations of predation kinetics of <em>B. bacteriovorus</em> 109JΔ3575</td>
<td>56</td>
</tr>
<tr>
<td>11</td>
<td>Predation aggressiveness of <em>B. bacteriovorus</em> 109J Δ3575</td>
<td>57</td>
</tr>
</tbody>
</table>
List of Abbreviations

ala  alanine
BALO  *Bdellovibrio* and like organisms
bp  base pair
CFU  colony forming units
DAP  diaminopimelic acid
DNA  deoxyribonucleic acid
DNase  deoxyribonuclease
DNB  dilute nutrient broth
DNTP(s)  deoxy nucleoside triphosphate(s)
GlcNAc  N-acetylglucosamine
glu  glucose
HEPES  N-[2-hydroxyethyl] piperazine-N’-[2- ethane sulfonic acid]
HM  Hapes buffer
HPLC  high performance liquid chromatography
OD  optical density
kb  kilobase
LB  Luria Bertani
MurNAc  N-acetylmuramic acid
NAcGlu  N-acetylglucosamine
NAcMur  N-acetylmuramic acid
NB  nutrient broth
PCR  polymerase chain reaction
PY  peptone yeast
RNA  ribonucleic acid
RNase  ribonuclease
v/v  volume/volume
w/v  weight/volume
Chapter 1: Introduction

1.1 Predatory Prokaryotes

There are many examples of predation on bacteria by eukaryotic organisms, such as ciliates and amoebae. However predation of bacteria by other prokaryotes is not as common. Currently there are nine known genera of predatory prokaryotes: *Bacteriovorax, Bdellovibrio, Myxococcus, Daptobacter, Ensifer, Micavibrio, Peredibacter, Vampirococcus,* and *Vampirovibrio* (Jurkevitch & Davidov, 2006). Some predatory prokaryotes have limited prey ranges as is the case with *Vampirococcus* (species of *Chromatium*) and *Daptobacter* (genera of *Chromatiaceae*) while *Bdellovibrio* and like organisms (BALOs) are able to prey on several gram negative bacteria (Guerrero *et al.*, 1986). The most well studied predatory prokaryotes typically are *Myxococcus*; studied for its complex developmental life cycle, and the obligate predator *Bdellovibrio*.

1.2 *Bdellovibrio* and Like Organisms (BALOs)

BALOs are small, highly motile, gram negative bacteria (Jurkevitch, 2006). *Bdellovibrio* strains were first isolated from soil and characterized by Stolp & Starr (1963). They were discovered during titration experiments for bacteriophage. Bacteriophage-induced lysis occurs within approximately 24 hours. After three to four days of incubation novel plaques appeared which they discovered were created by *Bdellovibrio* (Stolp & Petzold, 1962). There are two ecological groups of *Bdellovibrio* (Baer *et al.*, 2004): the freshwater and terrestrial group and the marine or halophilic group of organisms that can be found in the ocean, seas, and other salt waters.
1.3 Life Cycles of BALOs

Due to their prey dependence BALOs are characterized by their biphasic life cycles. To date there have been two life cycles identified. Both consist of a non-replicative motile attack phase and a growth phase. However, during the epibiotic life cycle the BALO remains attached to the outside of the cell in comparison to the periplasmic life cycle where the BALO invades the periplasmic space of its prey.

1.3.1 Periplasmic life cycle

The periplasmic life cycle is the most common life cycle among BALOs isolated to date (Fig. 1). The type genus and species used to study the periplasmic life cycle is *Bdellovibrio bacteriovorus*. The periplasmic life cycle consists of a motile attack phase and a periplasmic growth phase.

1.3.1.1 Search for a suitable prey cell

The motile attack phase consists of the bacterium searching for a prey cell (Strauch *et al.*, 2007). BALOs typically have a wide prey range, but not inclusive to all gram negative bacteria (Jurkevitch *et al.*., 2000). Having a wide prey range is advantageous, as attack phase cells must find a suitable prey cell in order to replicate. S-layers have been identified as anti-predation structures for *B. bacteriovorus* (Koval & Hynes, 1991) but not capsules (Koval & Bayer, 1997). Earlier research done in the Conti lab presented some evidence that chemotaxis plays an important role in *Bdellovibrio* finding a suitable prey cell. They found that *Bdellovibrio* had chemotactic responses towards cells (Straley & Conti, 1997). However, these findings proved to be inconclusive as chemotaxis occurred even if the prey cell was susceptible to predation.
Figure 1. The periplasmic life cycle of *B. bacteriovorus*

Attack phase cells: A. Motile attack phase cells encounter their prey cell through random collisions; B. Attachment to the prey cell; C. Penetration of the prey cell outer membrane and peptidoglycan, loss of *B. bacteriovorus* flagellum.

Growth phase: D. Bdelloplast formation, growth of filament; E. Intraperiplasmic growth of filament and replication of genome; F. Septation of filament and release of progeny attack phase cells (Modified from Ruby, 1992).
or not and only occurred at high prey cell concentrations, which could be a result of random collisions. One could argue whether or not chemotaxis towards a substrate is even possible. Because *Bdellovibrio* swim at high speeds they may not be able to respond to a substrate gradient. More conclusive findings have suggested that *Bdellovibrio* utilize aerotaxis to find a suitable environment (Jurkevitch, 2006).

1.3.1.2 Attachment and invasion

When *Bdellovibrio* collides with a cell there is a reversible short recognition period, which is non-specific as *Bdellovibrio* are even known to attach to abiotic services (e.g. cover slips). If the cell is suitable for predation the recognition period can lead to an irreversible attachment in which the predator firmly adheres to the prey cell (Stolp & Starr, 1963; Burnham *et al.*, 1968). In order for BALOs to utilize a susceptible prey cell the prey cell must be in stationary phase. Once irreversibly attached *B. bacteriovorus* initiates signalling cascades, which results in upregulation of cell cycle specific proteins (Thomashow & Cotter, 1992). A notable modification is the rounding of the prey cell as the peptidoglycan cross-links begin to be cleaved (Lerner *et al.*, 2012). *Bdellovibrio* then “squeeze” through the prey cell outer membrane and the peptidoglycan layer and takes up residence in the periplasmic space (Burnham *et al.*, 1968). During invasion the flagellum of the BALO is lost.

1.3.1.3 Formation of bdelloplast

During invasion some prey cells rounds up and are converted into an osmotically stable bdelloplast that provides a protected environment for optimal growth of the predator (Lerner *et al.*, 2012). *N*-deacetylation of the prey cell peptidoglycan by *B. bacteriovorus* prevents further degradation of the peptidoglycan and prevents premature lysis of the
bdelloplast (Thomashow & Rittenberg, 1978c). The bdelloplast is also resistant to invasion by another BALO, termed double invasion (Lerner et al., 2012). The formation of the bdelloplast marks the beginning of growth phase. Something to note is that not all prey cells are converted into a bdelloplast during the life cycle. One exception is the prey cell *Delftia acidovorans* that maintains its rod shape during the entire life cycle.

**1.3.1.4 Growth phase and release of attack phase cells**

The transition into the growth phase is marked by the up regulation of hydrolytic enzymes that degrade the contents of the prey cell. The degradation of the prey cell cytoplasmic contents makes available the molecules necessary for growth of the BALO, which elongates into a filament (Hespall et al., 1973; Thomashow & Rittenberg, 1978a; Thomashow & Rittenberg, 1978b; Thomashow & Rittenberg, 1978c). Transcriptional studies conducted by Lambert et al. (2010) identified several genes that are expressed specifically during the growth phase. The most common genes identified are those responsible for peptidoglycan modification, transport, and replication and division proteins.

Upon an unknown signal, septa form simultaneously along the filament, giving rise to an average of 4 to 6 progeny when *E. coli* is used as a prey cell (Eksztejn & Varon, 1977; Fenton et al., 2010). Flagellum formation and flagellar motility, while the BALOs are in the bdelloplast, are required for successful release of progeny (Flannagan et al., 2004, Morehouse, 2011). The progeny attack phase cells exit through discreet pores made in the bdelloplast (Fenton et al., 2010). The periplasmic life cycle normally takes approximately 3 hours from attachment to release of the new progeny attack phase cells. A point of interest is that BALOs exhibit plastic phenotypic resistance, which means
that some prey cells are resistant to predation, which occurs in co-cultures that have reached complete predation (Shemesh & Jurkevitch, 2004). Therefore, when growth curves of BALOs are analysed, it is important to take into consideration that the final optical density also includes residual prey cells. When the residual cells are re-cultured and subsequently used as prey cells they are once again susceptible to predation. Also, BALOs do not scatter light significantly, as the predator lyses prey the optical density of the co-culture decreases even though the yield of BALOs has reached between $10^9$ and $10^{10}$.

1.3.2 Epibiotic life cycle

1.3.2.1 Isolation of epibiotic BALO

A predatory prokaryote was isolated from raw sewage in enrichment cultures with *Caulobacter crescentus* (Koval & Hynes, 1991). The isolate, strain JSS, was placed in the *Bdellovibrio* lineage based on a 93% 16S rRNA gene sequence similarity to *B. bacteriovorus*. It was assigned to a new species based on this fact and on its different life cycle (Koval *et al.*, 2013). The new species, *B. exovorus*, has a motile non-replicative attack phase similar to *B. bacteriovorus*, but differs in its ability to invade a prey cell. *B. exovorus* attaches to its prey cell but remains on the outside of the cell. From there *B. exovorus* utilizes the prey cell cytoplasmic contents and divides by binary fission. After predation the prey cell remains, devoid of its cytoplasmic contents, and is termed a ghost cell. In this life cycle no bdelloplast is formed and the prey cell retains its original shape (Koval & Hynes, 1991). This life cycle is termed epibiotic growth (Shemesh *et al.*, 2003).
1.4 Peptidoglycan of Gram Negative Bacteria

Peptidoglycan is a polymer consisting of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues which are covalently linked by a β–1,4 glycosidic bond. These linear glycan chains are further cross-linked by a peptide chain that creates a mesh-like sacculus. The resulting polymer is an integral part of the cell wall and contributes to shape and provides structural support for the cell. The linkage of peptidoglycan is not static as it undergoes reorganization during cell growth and division (Bramhill, 1997). During these cellular events various types of lytic enzymes are produced that cleave specific linkages of the peptidoglycan sacculus (Fig. 2; Smith et al., 2000). Lytic transglycosylases are essential to bacterial cells for their growth and re-arrangement of their sacculus (Moynihan & Clarke, 2011). Lytic transglycosylases specifically cleave the β–1,4 glycosidic bond between GlcNAc and MurNAc residues.

1.5 *Bdellovibrio bacteriovorus* Peptidoglycan Modifying Enzymes

1.5.1 Background studies

Due to their predatory life cycle, modification of prey peptidoglycan by *Bdellovibrio* is a key process in predation as it allows the predator to enter the periplasmic space. Biochemical analysis by Thomashow and Rittenberg (1978b) suggested that a glycanase is involved in prey entry and a peptidase is involved in rounding of the prey cell into the osmotically stable bdelloplast. The activity of these peptidoglycan modifying enzymes decreases once *B. bacteriovorus* has entered the periplasm, resulting in 20% of the prey peptidoglycan being solubilized. Thomashow & Rittenberg (1978c) also showed that *B. bacteriovorus* N-deacetylates prey cell peptidoglycan amino sugars. They suggested that
Figure 2. Activity sites of major gram negative peptidoglycan modifying enzymes
(Modified from Lambert et al., 2009)
this activity could have a role in rendering the bdelloplast lysozyme and autolysin insensitive.

1.5.2 Current understanding

Since 1978, many homologs to known peptidoglycan remodeling enzymes have been identified in the genome of the type strain of *B. bacteriovorus* HD100T (Rendulic *et al.*, 2004; Table 1; Fig. 2). Access to the genome sequence has led to the confirmation of the earlier observations made by Thomashow & Rittenberg. It was recently shown that two D-ala-D-ala carboxypeptidases (genes *bd0816* and *bd3459*) are upregulated in *B. bacteriovorus* HD100 during prey cell entry (Lambert *et al.*, 2010). Studies showed that the gene products are responsible for remodeling the peptidoglycan of the prey cell, which leads to rounding of the prey cell into a bdelloplast and prevents double invasions of one prey cell (Lerner *et al.*, 2012). Gene *bd3279* has been identified as a potential peptidoglycan deacetylase and is reported in Lerner *et al.* (2012) as being under study in the Sockett lab. The transcriptional studies of Lambert *et al.* (2010) also showed that *bd3575*, encoding a lytic transglycosylase, was highly upregulated during invasion.

Bacteria are able to modify their peptidoglycan in many ways, some of which affect the activity of autolysins. Peptidoglycan *O*-acetylation was first discovered in *Enterococcus faecalis* (formerly *Streptococcus faecalis*) (Abrams, 1958) and *Micrococcus luteus* (Brumfitt *et al.*, 1958). In both cases the initial discovery was made due to the lysozyme resistance the modification conferred to the organism. Since then, acetate has been found in the peptidoglycan of 49 other species, both gram negative and gram positive (Moynihan & Clark, 2010). *O*-acetylation is the addition of acetate groups to the C-6
Table 1. Major peptidoglycan modifying enzymes in *Escherichia coli* and their homologs in *Bacteriovorus* HD100

<table>
<thead>
<tr>
<th>Activity</th>
<th>Gene</th>
<th>Annotated <em>Bdellovibrio</em> homolog(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transglycosylases</td>
<td><em>sltY</em></td>
<td><em>bd</em>1285</td>
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<td></td>
<td></td>
<td><em>bd</em>2711</td>
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<td><em>bd</em>3575</td>
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<td><em>mltA</em></td>
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<td><em>mltB</em></td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td><em>phpG</em></td>
<td>N/A</td>
</tr>
<tr>
<td>DD-carboxypeptidase and DD-endopeptidase</td>
<td><em>dacB</em></td>
<td><em>bd</em>3244</td>
</tr>
<tr>
<td>DD-endopeptidase</td>
<td>N/A</td>
<td><em>bd</em>3459</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>bd</em>0816</td>
</tr>
<tr>
<td></td>
<td><em>dacE</em></td>
<td><em>bd</em>0436</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>bd</em>1951</td>
</tr>
<tr>
<td></td>
<td><em>dacA</em></td>
<td><em>bd</em>2044</td>
</tr>
<tr>
<td>DD-carboxypeptidase</td>
<td><em>dacD</em></td>
<td>N/A</td>
</tr>
<tr>
<td>O-acetylation</td>
<td>N/A</td>
<td><em>bd</em>1677</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>bd</em>1668</td>
</tr>
<tr>
<td>N-deacetylation</td>
<td>N/A</td>
<td><em>bd</em>3279</td>
</tr>
</tbody>
</table>

N/A = no annotated gene
hydroxyl groups of N-acetylmuramyl (MurNAc) residues. This modification of the MurNAc residue attenuates the active site of lysozyme and lytic transglycosylases, inhibiting their function (Moynihan & Clark, 2010). The degree of peptidoglycan O-acetylation is determined as a measure of base-labile acetate in relation to the total amount of MurNAc residues in the peptidoglycan. O-acetylation has been found to range from 20-60% (Table 2) and depends upon culture conditions, stage of the growth, species, and strain (Moynihan & Clark, 2010).

In gram negative bacteria O-acetylation is achieved by a two-component system that is encoded within OAP (O-acetylation of peptidoglycan) clusters (Weadge et al., 2005). PatA (peptidoglycan O-acetyltransferase) is an integral membrane protein predicted to translocate acetate from the cytoplasm to the periplasmic space, while PatB is a peripheral membrane protein located in the periplasm that adds acetate to the N-acetylmuramyl residues (Weadge et al., 2005). The OAP cluster also encodes an O-acetylpeptidoglycan esterase (Ape, a potential esterase) that is responsible for removing the acetate groups from the peptidoglycan (Fig. 3).

The gram negative O-acetylation system proteins have been putatively identified in the B. bacteriovorus HD100 genome: bd1667 (PatA) and bd1668 (PatB) (A. Clarke, personal communication). The status of O-acetylation of peptidoglycan of B. bacteriovorus attack phase cells is unknown.

1.6 Proteus mirabilis

Proteus mirabilis is a gram negative pathogenic bacterium that is usually associated with urinary tract infections. Previous studies have demonstrated that B. bacteriovorus
<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Degree of peptidoglycan O-acetylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram negative</td>
<td></td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em></td>
<td>56-63%</td>
</tr>
<tr>
<td><em>Neisseria gonorrhoeae</em></td>
<td>35-52%</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>20-52.8%</td>
</tr>
<tr>
<td>Gram positive</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus anthracis</em></td>
<td>33-58%</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>22-72%</td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em></td>
<td>60-70%</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>35-60%</td>
</tr>
</tbody>
</table>

*Reference (Moynihan & Clarke, 2011)*
Figure 3. **Two component O-acetylation system in gram negative bacteria**

A: PatA translocates acetate from the cytoplasm to the periplasmic space; B: PatB adds acetate to the C-6 hydroxyl groups of N-acetylmuramyl peptidoglycan residues; C: Ape1a removes acetate (Adapted from Moynihan & Clarke, 2011). L.T. stands for lytic transglycosylase.
is able to utilize *P. mirabilis* as a prey cell for growth (Dashiff *et al.*, 2010). Dupont and Clarke (1991) demonstrated that the degree of peptidoglycan O-acetylation in *P. mirabilis* can vary between strains of one species. Table 3 lists four *P. mirabilis* strains used in this study and their degree of peptidoglycan O-acetylation. *P. mirabilis* is utilized in this study to investigate the role peptidoglycan O-acetylation of the prey cell has on predation by *B. bacteriovorus*.

### 1.7 Research Objectives

Our laboratory is interested in the invasion process of the periplasmic life cycle. To this end, my thesis has three main objectives: (1) to determine if the peptidoglycan of selected attack phase BALOs is O-acetylated; (2) to assess the effect the degree of peptidoglycan O-acetylation of the prey cell *P. mirabilis* has on predation by *B. bacteriovorus*. It is hypothesized that peptidoglycan O-acetylation of *P. mirabilis* hinders the ability of *B. bacteriovorus* to create an entry pore in the prey cell peptidoglycan, which would result in delayed entry; and (3) to further investigate the role the annotated lytic transglycosylase *bd3575* has on the life cycle of *B. bacteriovorus*. One sentence in the Lerner *et al.* (2010) paper mentioned that deletion of the gene *bd3575* still allowed invasion to occur, but no data were provided either on construction of the mutant or evaluation of predation. It is hypothesized *bd3575* is involved in creating the entry pore in the prey cell during invasion because the transcriptional studies of Lambert *et al.* (2010) showed that *bd3575* was the only lytic transglycosylase upregulated during the initial stages of predation. To determine if the
Table 3. Degree of peptidoglycan $O$-acetylation of *P. mirabilis* strains used in this study

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Degree of peptidoglycan $O$-acetylation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Proteus mirabilis</em> 25933</td>
<td>25%</td>
<td>Dupont and Clarke (1991)</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> 7002</td>
<td>43%</td>
<td>Dupont and Clarke (1991)</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> P19</td>
<td>52.8%</td>
<td>Dupont and Clarke (1991)</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> MCB330</td>
<td>49.8%</td>
<td>PhD of John Pfeffer, University of Guelph</td>
</tr>
</tbody>
</table>
lytic transglycosylase contributes to efficient prey cell entry the gene in *B. bacteriovorus* was mutated.
Chapter 2: Materials and Methods

2.1 Bacterial Strains and Culture Conditions

*E. coli* strains, *Stenotrophomonas maltophilia* X26332 and *Proteus mirabilis* strains were routinely grown in Luria-Bertani medium (Luria & Burrows, 1957) at 30°C for 24 hours. If required the *E. coli* strains were grown in LB medium containing chloramphenicol (25 µg/mL). *B. bacteriovorus* 109J was maintained on *E. coli* ML35. *B. exovorus* JSS was maintained on *S. maltophilia* X26332. Co-cultures were prepared by adding a 1:4 ratio of predator to prey in 3 mM HEPES buffer (pH 7.6) with 1 mM CaCl₂ and 0.1 mM MgSO₄ (HM buffer). The co-cultures were incubated for 24 to 48 hours at 30°C with shaking. Prey independent strains *B. bacteriovorus* 109JA and *Bacteriovorax stolpii* UKi2 were grown in PY medium (Mahmoud & Koval, 2010) overnight with shaking at 30°C. All bacterial strains and plasmids used in this study are listed in Table 4.

2.2 Analysis of Peptidoglycan

2.2.1 Isolation of crude peptidoglycan

Prey dependent BALO strains were grown in 1 L or 2 L co-cultures and incubated at 30°C for 48 hours. Four litres in total were pooled together for each batch and centrifuged at 2,000 x g for 10 minutes to remove residual prey cells. Prey independent strains of BALOs were also grown in 1 L or 2 L cultures, incubated at 30°C for 48 hours, and each batch consisted of 4 L pooled together. Two litres of *S. maltophilia* and *P. mirabilis* were incubated for 48 hours at 30°C.
Table 4. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> ML35</td>
<td>Rittenberg and Shilo (1970)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> SY327λpir</td>
<td>Miller and Mekalanos (1988)</td>
</tr>
<tr>
<td><em>Escherichia coli</em> SM10λpir</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><em>Stenotrophomonas maltophilia</em> X26332</td>
<td>Joanna Brooke, DePaul University, Chicago, USA</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> 25933</td>
<td>Daniel Kadouri, UMDNJ, New Jersey, USA</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> 7002</td>
<td>Daniel Kadouri, UMDNJ, New Jersey, USA</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> MCB330</td>
<td>Anthony Clarke, University of Guelph, Guelph, Ontario</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> P19</td>
<td>Anthony Clarke, University of Guelph, Guelph, Ontario</td>
</tr>
<tr>
<td><em>Bdellovibrio bacteriovorus</em> 109J</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td><em>Bdellovibrio bacteriovorus</em> 109JA</td>
<td>John Tudor, St. Joseph’s University, Philadelphia, USA</td>
</tr>
<tr>
<td><em>Bdellovibrio exovorus</em> JSS</td>
<td>Koval et al. (2013)</td>
</tr>
<tr>
<td><em>Bacteriovorax stolpii</em> UKi2</td>
<td>John Tudor, St. Joseph’s University, Philadelphia, USA</td>
</tr>
<tr>
<td><em>Bdellovibrio bacteriovorus</em> 109JΔ3575</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Plasmids**

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSSK10</td>
<td>Silvia Piñeiro, University of Maryland, Baltimore, USA</td>
</tr>
<tr>
<td>pΔ3575</td>
<td>This study</td>
</tr>
</tbody>
</table>
Peptidoglycan was isolated using a boiling sodium dodecyl sulfate (SDS) procedure that was adapted from previously described methods (Hoyle & Beveridge, 1984; Dupont & Clarke, 1991). During the procedure it was important to ensure that the pH did not exceed 6.8, as this may lead to premature hydrolysis of any O-linked acetate from the samples (Vollmer, 2008).

To purify peptidoglycan cells were collected by centrifugation at 8,000 x g for 15 minutes at 4°C. The pellet was resuspended in 250 mL of Buffer A (25 mM sodium phosphate, pH 6.5) and added drop-wise to an equal volume of boiling 8% SDS in Buffer A, resulting in a 4% SDS final concentration. The suspension was boiled under reflux for 3 hours then left to cool overnight. The suspension was then centrifuged at 160,000 x g for 1 hour at 25°C to pellet the SDS-insoluble crude peptidoglycan. The pellet was washed and resuspended in 250 mL of Buffer A and pelleted (160,000 x g, 1 hour, 25°C). The pellets were washed a total of 3 times and after the final wash the pellet was resuspended in 30 mL of Buffer B (10 mM Tris-HCl, 10 mM NaCl, pH 6.5).

The crude peptidoglycan preparations were then subjected to enzymatic treatment using DNase I, RNase A and MgSO₄ with final concentrations of 10 µg/mL, 50 µg/mL and 20 mM, respectively, and incubated overnight at 37°C. After incubation heat treated pronase (60°C for 2 hours) was added to a final concentration of 200 µg/mL and the suspension was incubated overnight at 60°C. The enzyme-treated peptidoglycan was then added drop-wise to an equal volume of boiling 8% SDS in Buffer A and left boiling under reflux for a minimum of 3 hours. The SDS insoluble peptidoglycan was then centrifuged (160,000 x g, 1 hour, 25°C) and washed three times as before in Buffer A to remove SDS. The final pellet was resuspended in 10 mL of Buffer A, placed in a
50-mL Falcon tube, and frozen at -80°C. Once frozen the sample was lyophilized and stored with desiccation at room temperature.

2.2.2 Detection of O-acetylation

2.2.2.1 Release and quantification of O-acetyl groups

Chemical release of O-linked acetate from the purified peptidoglycan samples was achieved through mild base hydrolysis. The peptidoglycan samples were incubated in the presence of 1 M NaOH for 1 hour at ambient temperature, which hydrolyses any ester linkages. The quantification of acetate was determined by two different methods: (1) the Megazyme Acetic Acid Assay kit (Megazyme International Ireland Ltd., Wicklow, Ireland) with the protocol carried out according to the manufacturer’s instructions with one notable exception: the assay and reagent volumes were halved and; (2) samples loaded into a Rezex ROA-Organic Acid H⁺ column at 45°C using a Beckman System Gold HPLC instrument. The elution was performed at a flow rate of 0.6 mL/minute with 5 mM H₂SO₄, and acetic acid was detected at 205 nm. Blank and standard samples were also analyzed.

2.2.2.2 Muramic acid quantification

The desiccated SDS-insoluble peptidoglycan pellets were resuspended in 4 M HCl and incubated in vacuo for 18 hours at 96°C to hydrolyze the samples into their constituent amino sugar and amino acid components. Excess acid was subsequently removed by desiccation and samples resuspended in an equivalent volume of sterile HPLC-grade distilled water. Samples were then analyzed in triplicate by high performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) as previously described by Clarke (1993a).
using a Dionex system and Chromeleon® version 6.80 software.

2.3 Construction of bd3575 Knockout Mutant of B. bacteriovorus 109J

The in-frame markerless deletion method of Steyert & Piñeiro (2007) was used to construct a B. bacteriovorus bd3575 mutant. A truncated version of the gene containing 9 bp of the wild type bd3575 gene was created and inserted into the pSSK10 suicide plasmid. The truncated version of the gene was incorporated into the genome of B. bacteriovorus via two homologous recombination events between the plasmid and the genome.

2.3.1 Chromosomal DNA and plasmid isolation

Chromosomal DNA was isolated using Illustra™ bacterial genomicPrep Mini Spin Kit or DNeasy® Blood & Tissue kit (Qiagen). An EZ-10 Spin Column Plasmid DNA Kit (Bio Basic Inc) was used for plasmid extractions. All DNA preparations were completed according to manufacturer’s directions.

2.3.2 Polymerase chain reaction

i-pfu polymerase (Frogga) was used for all cloning and sequencing. Taq polymerase (Fermentas) or 2x Taq FroggaMix (FroggaBio) was used for colony screening. All PCR conditions were carried out according to the manufacturer’s instructions. All primers used in this study are listed in Table 5 and the physical map of the primers is illustrated in figure 4. The primers designed for the bd3575 deletion construct were based off the B. bacteriovorus HD100 genome.
Table 5. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>bd3575F&lt;sub&gt;1&lt;/sub&gt;</td>
<td>5’-ATCAGTCATATGAAGGGAATGTAATCCGC-3’</td>
</tr>
<tr>
<td>bd3575R&lt;sub&gt;1&lt;/sub&gt;</td>
<td>5’-GCGACGGATATCCTGATAGTTCTAAAGAGG-3’</td>
</tr>
<tr>
<td>bd3575F&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5’-GACCCGGATATCTAGTTCAACAAGACCTAC-3’</td>
</tr>
<tr>
<td>bd3575R&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5’-CCGGACCTCGAGTCTTTGAACACATCAAC-3’</td>
</tr>
<tr>
<td>bd3575F</td>
<td>5’-ATCAGTCGCCGGGAGACAATGATTCGC-3’</td>
</tr>
<tr>
<td>bd3575R</td>
<td>5’-CAACGAACGGGGATGGATGCGCATC-3’</td>
</tr>
<tr>
<td>bd3575F&lt;sub&gt;i&lt;/sub&gt;</td>
<td>5’-AGCGACCTGGGCCTGTACCGTGGGT-3’</td>
</tr>
<tr>
<td>bd3575R&lt;sub&gt;i&lt;/sub&gt;</td>
<td>5’-GCCACATAAAGCTGGAAGCGGCTCTGC-3’</td>
</tr>
</tbody>
</table>

*Restriction enzyme sites Nde<sub>1</sub> (CATATG), Sph<sub>1</sub> (GCATGC), and Xho<sub>1</sub> (CTCGAG) are underlined.
Figure 4. Physical map of primers used in this study
2.3.3 Construction of the \( p\Delta3575 \) suicide vector

To knockout the \( bd3575 \) gene, allelic exchange using an in-frame deletion cloned on the suicide plasmid pSSK10 was used (Fig. 5). Primers \( bd3575F_1 \) and \( bd3575R_1 \) were designed to include the upstream region of the \( bd3575 \) gene (478 bp) and \( bd3575F_2 \) and \( bd3575R_2 \) were made to include the downstream region of the gene (637 bp) (Table 5). Amplification of the upstream and downstream fragments from \( B. bacteriovorus \) chromosomal DNA was confirmed by agarose gel electrophoresis. The two fragments were ligated together using \( Sph1 \), then digested and ligated into the plasmid pSSK10 using the \( Nde1 \) and \( Xho1 \) restriction sites.

2.3.4 Preparation of competent cells

The \( E. coli \) SY327\( \lambda \)pir strain was used for heat shock transformation with the \( p\Delta3575 \) suicide vector to produce large quantities of the plasmid. Competent \( E. coli \) SY327\( \lambda \)pir were prepared by growing the cells at 37°C to an \( OD_{600} \) of 0.5. The 20-mL culture was chilled on ice for 30 minutes then centrifuged. The pellet was resuspended in 3 mL cold 0.1 M \( CaCl_2 \) and chilled for 30 minutes. The cells were then centrifuged at 4°C for 10 minutes at 3,000 x \( g \) and the pellet was resuspended in 1 mL cold 0.1 M \( CaCl_2 \) with 10% (v/v) glycerol. One hundred \( \mu L \) aliquots of cells were stored at -80°C until required.

2.3.5 Preparation of electrocompetent cells

Electrocompetent cells were prepared fresh by growing \( E. coli \) SM10\( \lambda \)pir to an \( OD_{600} \) of 0.5. The 20-mL culture was chilled on ice for 20 minutes then centrifuged for 10 minutes at 7,000 x \( g \). The pellet was rinsed and resuspended in 20 mL of double distilled water. The cells were centrifuged for 10 minutes at 7,000 x \( g \) rinsed and
Figure 5. Physical map of suicide plasmid pSSK10 used in this study (Adapted from Steyert & Piñeiro 2007).
resuspended in 1 mL 10% (v/v) glycerol. The cells were then centrifuged for 2 minutes at 7,000 x g, rinsed and resuspended in a final volume of 150 µL 10% (v/v) glycerol.

2.3.6 Bacterial transformations

2.3.6.1 Heat shock transformation of E. coli SY327λpir

One 100-µL aliquot of E. coli SY327λpir competent cells was thawed. The isolated pΔ3575 suicide vector (5 µL) was added to the competent cells and chilled for 30 minutes on ice. The suspension was subjected to heat shock for 2 minutes at 42°C then immediately chilled on ice for 5 minutes. One mL of LB medium was then added to the Eppendorf tube and the culture was incubated for 1 hour at 37°C. After incubation, 100 µL of the suspension was plated on LB agar containing chloramphenicol (25 µg/mL) and kanamycin (50 µg/mL) and the culture incubated overnight at 37°C.

2.3.6.2 Electroporation of E. coli SM10λpir

Fifty µL of freshly prepared E. coli SM10λpir electrocompetent cells were placed in a sterile electroporation cuvette (Molecular BioProducts), which was cold (stored at -20°C). Five µL of purified plasmid was added, briefly mixed, and electroporated at 200 Ω resistance, 25 µFD capacitance, and 1.8 kV (BioRad Gene Pulser). The cells were directly added to 1 mL of LB medium and then incubated for 2 hours at 37°C. After incubation, 100 µL of the suspension was plated on LB agar containing chloramphenicol (25 µg/mL) and kanamycin (50 µg/mL) and incubated overnight at 37°C.

2.3.7 B. bacteriovorus conjugal mating

A 20-mL overnight co-culture of B. bacteriovorus 109J was centrifuged at 3,000 x g for 10 minutes to remove residual E. coli prey cells. The supernatant was then filtered using
a 0.45 µm nitrocellulose filter and then centrifuged for 20 minutes at 10,000 x g. The pellet was resuspended in 2 mL of DNB (dilute nutrient broth, 1/10 concentration of nutrient broth). A sterile 0.45 µm nitrocellulose filter was then placed on a DNB agar and was left to dry for 30 minutes before the 100 µL of the prepared *B. bacteriovorus* 109J was added to the top filter. This was left to dry for 30 minutes. A 20 mL overnight culture of the donor *E. coli* SM10λpir containing pΔ3575 was centrifuged at 5,000 x g for 10 minutes. The pellet was resuspended in 2 mL DNB and 100 µL was placed on the nitrocellulose filter. The donor *E. coli* and *B. bacteriovorus* 109J were incubated overnight at 30°C.

After incubation the nitrocellulose filter was placed into 5 mL HM buffer and mixed thoroughly then incubated at 37°C for 30 minutes. The suspension was then used to make a dilution series (10⁰ – 10⁻⁶). A 20 mL overnight culture of *E. coli* ML35 was centrifuged at 5,000 x g for 10 minutes and the pellet was resuspended in 2 mL HM. One hundred µL of the dilution and 200 µL of the *E. coli* ML35 were added to 500 µL of 50°C 0.6% DNB agar containing chloramphenicol (25 µg/mL) then poured onto 1.2% DNB agar containing chloramphenicol (25 µg/mL). The presence of chloramphenicol selects for *B. bacteriovorus* 109J that have been transformed and contain the pΔ3575 suicide vector. The integration of the plasmid into the genome was determined by colony PCR using the primers bd3575F₁ and bd3575R₂ (Table 5).

### 2.3.8 Counterselection

Merodiploid *B. bacteriovorus* were grown in HM buffer co-cultures with *E. coli* ML35 in the absence of antibiotics to allow excision of the plasmid from the *B. bacteriovorus* 109J genome by homologous recombination. After several series of co-cultures in the
absence of any selective pressure \textit{B. bacteriovorus} was then co-cultured with prey cell \textit{E. coli} ML35 in HM buffer containing 5% sucrose. The presence of sucrose selects for \textit{B. bacteriovorus} 109J that have lost the suicide plasmid (absence of \textit{sacB} gene) via a secondary homologous recombination event. Colony PCR was used to identify exconjugates that retained the \textit{bd3575} deletion construct versus the wild type gene.

2.4 Agarose Gel Electrophoresis

DNA samples were loaded into the wells (6 µL/well) of a 0.8 % agarose gel and separated using 70V of current for 40 minutes. All samples were run in comparison to a 1 kb DNA ladder GeneRuler™ (Fermentas).

2.5 DNA Sequencing and Genomes

Sequencing of all PCR products was performed at Robarts Research DNA Sequencing Facility (Western University, London, Ontario). The genome sequence of \textit{B. bacteriovorus} HD100 (NC_005363.1) is available online from the National Centre for Biotechnology Information (NCBI).

2.6 Predation Assays

2.6.1 Predation kinetic assay

To assess predation kinetics of \textit{B. bacteriovorus} 109J and \textit{B. bacteriovorus} \textit{A3575} on their respective prey cells the predators were grown in co-cultures on \textit{E. coli} ML35 overnight at 30°C. The 20 mL cultures were then centrifuged at 3,000 x g for 10 minutes to remove any residual prey cells. A 20-mL overnight culture of the prey cells
was centrifuged at 5,000 x g for 10 minutes and the pellet was resuspended in 10 mL HM buffer. Each of the predator prey co-culture pairings were prepared in 125 mL flasks containing 20 mL HM buffer, predator and prey were added in a 1:4 ratio, respectively. Predation kinetics were measured by adding 200 µL to each well of a 100-well BioScreen plate that was incubated at 30°C for 48 hours with shaking (high). Optical density readings (OD₆₀₀) were taken every 15 minutes. The OD₆₀₀ from HM buffer, predators in HM buffer, and prey cells in HM buffer were used as controls. Predation assays were performed in triplicate. When analysing growth curves of BALOs it is important to note two things. The initial optical density represents stationary phase prey cells and attack phase *Bdellovibrio*. The predators consume and lyse the prey cell, which results in a decrease in optical density interpreted as growth of *Bdellovibrio*. Also, the final optical density includes residual prey cells and the new *Bdellovibrio* progeny.

**2.6.2 Enumeration of prey cells**

Quantification of colony forming units (CFU) of prey cell in the predator prey pairings taken before predation and after predation was completed to determine predation efficiency. *B. bacteriovorus* 109J and *B. bacteriovorus* Δ3575 grown overnight in 20 mL co-cultures were centrifuged at 3,000 x g for 10 minutes to remove any residual prey cells. Each of the prey cells was grown overnight in 20 mL of their respective medium then centrifuged at 5,000g for 10 minutes and resuspended in 10 mL HM buffer. Co-cultures of each of the predator prey pairings were set-up in 20 mL HM buffer in a 1:4 predator to prey ratio in 125 mL flasks. A 10-fold serial dilution of each of the experimental conditions and controls (HM buffer containing prey cell only) was
carried out to $10^9$. On LB agar 10 µL of each of the experimental conditions were plated. The co-cultures and controls were then incubated at 30°C for 24 hours and the final CFU assay was carried out as per the initial CFU assay. Predation assays were performed in triplicate.

2.7 Biotin Labeling

2.7.1 Biotinylation of *B. bacteriovorus* 109J

Five mL of an overnight culture of *E. coli* ML35 was centrifuged at 6,000 x g for 10 minutes and resuspended in 10 mL of HM buffer. To label *B. bacteriovorus* 109J and *B. bacteriovorus* Δ3575, two mL of an overnight co-culture was centrifuged at 3,000 x g for 10 minutes to remove any residual prey cells. The supernatant was then centrifuged at 10,000 x g for 15 minutes and the pellet was resuspended in 500 µL HM buffer. The suspension was then centrifuged and washed 2 additional times. The pellet was resuspended in a final volume of 500 µL of HM buffer. One hundred and fifty mL of a 10mM EZ-Link™ Sulfo-NHS-Biotin (Thermo Scientific) solution was added to the suspension of predators and left to stand at room temperature for 30 minutes. After incubation the excess biotin was quenched with 500 µL PBS containing 100mM glycine.

2.7.2 Preparation of synchronous co-cultures

Five mL of an overnight culture of *E. coli* ML35 was centrifuged at 6,000 x g for 10 minutes and resuspended in 10 mL of HM buffer. Co-cultures were set up by adding 500 µL of both the predator and prey to 500 µL of HM buffer and were incubated at
30°C. It is beneficial to prepare synchronous co-cultures when assessing and comparing predation because predation will occur simultaneously between co-cultures within 12.

2.8 Microscopy Techniques

2.8.1 Phase contrast light microscopy

For phase contrast light microscopy, 10µL of a sample was placed on a glass slide and examined using a Leitz Laborlux K light microscope equipped with phase contrast optics.

2.8.2 Transmission electron microscopy

For negative stains, 1mL of each culture was centrifuged in an Eppendorf tube and cells resuspended in 200 µL of the supernatant. Cells were adsorbed to formvar-carbon-coated copper grids, washed two times in double distilled water, and stained with 0.25% or 0.5% (w/v) uranyl acetate with bacitracin (50 µg/mL). Specimens were examined using a Philips EM410 transmission electron microscope operating at 60 kV or a Philips 420 transmission electron microscope operating at 60 kV equipped with a digital camera.

Thin sections were prepared and photographed at the University of Guelph. Five mL samples of the 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.
Chapter 3: Results

3.1 O-acetylation of Peptidoglycan of Representative BALOs

Peptidoglycan can be modified by the addition of an acetate group on the C-6 hydroxyl of MurNAc. Many gram negative and gram positive bacteria acetylate their peptidoglycan in order to control digestion of their peptidoglycan (Moynihan & Clarke, 2012). The first objective of this study was to determine if BALOs O-acetylate their peptidoglycan.

3.1.1 Isolation and purification of peptidoglycan

The protocol previously described by Hoyle and Beveridge (1984) and Dupont and Clarke (1991) requires at least 10 mg of purified peptidoglycan to analyze a batch in triplicate. Enough peptidoglycan for analysis can usually be obtained from 2 L of gram negative bacteria. In order to get enough BALO peptidoglycan it was required to scale up to 4 L co-cultures because some predators are lost when the residual prey cells are removed by differential centrifugation.

The amount of base-labile ester-linked acetate in peptidoglycan preparations was quantified by the enzymatic protocol previously described (Hoyle & Beveridge, 1984; Dupont & Clarke, 1991). The yield of peptidoglycan from the isolation and purification procedure is summarized in Table 6. Production of murein sacculi was verified by electron microscopy of one batch of *B. bacteriovorus* 109J (Fig. 6). There were many murein sacculi, which appear as empty cells.
Table 6: Yield of peptidoglycan

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Volume of cells in batch (L)</th>
<th>Yield (mg/L)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Proteus mirabilis</em> MCB330</td>
<td>2</td>
<td>82.5</td>
</tr>
<tr>
<td><em>Stenotrophomonas maltophilia</em> X26332</td>
<td>2</td>
<td>24</td>
</tr>
<tr>
<td><em>Stenotrophomonas maltophilia</em> X26332</td>
<td>2</td>
<td>104.4</td>
</tr>
<tr>
<td><em>Bdellovibrio bacteriovorus</em> 109J</td>
<td>4</td>
<td>3.7</td>
</tr>
<tr>
<td><em>Bdellovibrio bacteriovorus</em> 109J</td>
<td>4</td>
<td>4.1</td>
</tr>
<tr>
<td><em>Bdellovibrio bacteriovorus</em> 109JA</td>
<td>4</td>
<td>6.1</td>
</tr>
<tr>
<td><em>Bdellovibrio exovorus</em> JSS</td>
<td>4</td>
<td>4.8</td>
</tr>
<tr>
<td><em>Bacteriovorax stolpii</em> UKi2</td>
<td>4</td>
<td>3.8</td>
</tr>
</tbody>
</table>

\(^a\)Yield of peptidoglycan from cells harvested from attack phase or stationary phase cultures.
Figure 6. *B. bacteriovorus* 109J peptidoglycan

Electron micrograph of *B. bacteriovorus* 109J peptidoglycan after isolation and purification. Sacculi were negatively stained with uranyl acetate with bacitracin. Black arrowheads indicate sacculi. Bar = 500 nm
3.1.2 Quantification of peptidoglycan O-acetylation

*P. mirabilis* strain MCB330 was used as a control for the isolation and purification of peptidoglycan as the O-acetyl content of the peptidoglycan had been previously analyzed (Table 3). *P. mirabilis* strain MCB330 was found to possess O-acetylated peptidoglycan, 34.39% (Table 7). The degree of peptidoglycan O-acetylation of *S. maltophilia* X26332 was determined, as it was a prey cell used for *B. exovorus* JSS in this study. The amount of base-labile ester-linked acetate in the samples was 1.8%. *E. coli* does not O-acetylate its peptidoglycan (Moynihan & Clarke, 2010) and was not analyzed because any acetate detected in the analysis of *B. bacteriovorus* 109J would have originated from the predator. The degree of peptidoglycan O-acetylation of *B. bacteriovorus* 109J detected by two different methods was 7.04% (±1.01) and 3.22% (±1.79). Prey independent BALOs *B. bacteriovorus* 109JA, *B. exovorus* JSS, and *B. stolpii* UKi2 were all found to have a base-labile ester-linked acetate content of 0%.

3.2 Effect of Prey Cell Peptidoglycan O-acetylation on Predation

Some bacteria modify their peptidoglycan by adding O-acetyl groups to the C-6 hydroxyl groups of MurNAc residues, which renders the peptidoglycan insensitive to lysozyme and endogenous lytic transglycosylases (Moynihan & Clarke, 2010). This O-acetylation may also hinder the cleavage of bonds by lytic transglycosylases of BALOs and impede formation of the entry pore. The second objective of this study was to investigate whether the degree of peptidoglycan O-acetylation of the prey cell had an effect on the predation efficiency of *B. bacteriovorus* 109J.
Table 7. Degree of peptidoglycan $O$-acylation of bacteria in this study

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>% $O$-acylation$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bdellovibrio bacteriovorus</em> 109J</td>
<td>3.2 ± 1.8$^c$</td>
</tr>
<tr>
<td><em>Bdellovibrio bacteriovorus</em> 109J</td>
<td>7.0 ± 1.0</td>
</tr>
<tr>
<td><em>Bdellovibrio bacteriovorus</em> 109JA</td>
<td>0</td>
</tr>
<tr>
<td><em>Bdellovibrio exovorus</em> JSS</td>
<td>0</td>
</tr>
<tr>
<td><em>Bacteriovorax stolpii</em> UKi2</td>
<td>0</td>
</tr>
<tr>
<td><em>Stenotrophomonas maltophilia</em> X26332</td>
<td>1.8 ± 0.1$^b$</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> MCB330</td>
<td>34.4 ± 1.5</td>
</tr>
</tbody>
</table>

$^a$ Mol % of base-labile acetate relative to MurNAc content in peptidoglycan detected by HPLC, unless stated otherwise. One batch (n=1, unless stated otherwise) was analyzed in triplicate (±SD).

$^b$ n=2

$^c$ Base-labile acetate detected by Megazyme Acetic Acid Assay kit.
3.2.1 Synchronous co-cultures assessed by light microscopy

The predation of *B. bacteriovorus* 109J on four strains of *P. mirabilis* (P19, 25933, MCB330, and 7002) was initially assessed by phase contrast light microscopy. It was confirmed that *B. bacteriovorus* 109J was able to utilize all strains for growth. Synchronous co-cultures of each strain of *P. mirabilis* and *B. bacteriovorus* 109J were monitored for 48 hours. Some attachment of *B. bacteriovorus* 109J was seen within 2 hours of incubation in all of the co-cultures. Formation of bdelloplasts occurred approximately 4 hours into incubation and complete predation was reached within 18 hours for all co-cultures. No qualitative differences in predation kinetics were observed.

3.2.2 Comparison of predation kinetics

The predation kinetics by *B. bacteriovorus* 109J on *P. mirabilis* strains was assessed by monitoring growth curves. Co-cultures were followed for 48 hours using a BioScreen apparatus. The decrease in OD$_{600}$ represents the clearing of *P. mirabilis* prey cells by *B. bacteriovorus* 109J.

All co-cultures had a 6 hour lag phase and reached complete predation within 16 hours (Fig. 7). The predation efficiency of *B. bacteriovorus* 109J, in this case the slope of the growth curves, on the four strains of *P. mirabilis* was almost identical. All co-cultures started with an OD$_{600}$ reading of 0.7 and decreased in OD to approximately 0.2. The OD$_{600}$ did not reach zero because this represent the residual *P. mirabilis* prey cells as well as *B. bacteriovorus* 109J progeny. The standard deviation of three replicates was calculated and never exceeded an OD$_{600}$ reading of 0.0085 (Table 8).
Figure 7. Predation kinetics of *B. bacteriovorus* 109J on strains of *P. mirabilis*

Co-cultures containing *B. bacteriovorus* 109J and one of the four strains of *P. mirabilis* were incubated for 24 hours and growth assessed using a BioScreen apparatus. The symbols represent individual co-cultures and are defined as follows: (■) *P. mirabilis* MCB330, (♦) *P. mirabilis* 25933, (▲) *P. mirabilis* 7002, and (●) *P. mirabilis* P19. n=3
Table 8. Standard deviations of predation kinetics of *B. bacteriovorus* 109J on *P. mirabilis*

The standard deviations (OD$_{600}$±SD) of predation kinetics of *B. bacteriovorus* 109J on *P. mirabilis*. n=3

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Proteus mirabilis 25933</th>
<th>Proteus mirabilis 7002</th>
<th>Proteus mirabilis MCB330</th>
<th>Proteus mirabilis P19</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0024</td>
<td>0.0061</td>
<td>0.0077</td>
<td>0.0034</td>
</tr>
<tr>
<td>2</td>
<td>0.0022</td>
<td>0.0055</td>
<td>0.0071</td>
<td>0.0032</td>
</tr>
<tr>
<td>4</td>
<td>0.0016</td>
<td>0.0050</td>
<td>0.0076</td>
<td>0.0033</td>
</tr>
<tr>
<td>6</td>
<td>0.0015</td>
<td>0.0050</td>
<td>0.0077</td>
<td>0.0039</td>
</tr>
<tr>
<td>8</td>
<td>0.0017</td>
<td>0.0053</td>
<td>0.0075</td>
<td>0.0036</td>
</tr>
<tr>
<td>10</td>
<td>0.0018</td>
<td>0.0065</td>
<td>0.0077</td>
<td>0.0041</td>
</tr>
<tr>
<td>12</td>
<td>0.0018</td>
<td>0.0072</td>
<td>0.0077</td>
<td>0.0036</td>
</tr>
<tr>
<td>14</td>
<td>0.0020</td>
<td>0.0069</td>
<td>0.0079</td>
<td>0.0036</td>
</tr>
<tr>
<td>16</td>
<td>0.0014</td>
<td>0.0057</td>
<td>0.0075</td>
<td>0.0038</td>
</tr>
<tr>
<td>18</td>
<td>0.0019</td>
<td>0.0062</td>
<td>0.0080</td>
<td>0.0035</td>
</tr>
<tr>
<td>20</td>
<td>0.0020</td>
<td>0.0063</td>
<td>0.0075</td>
<td>0.0032</td>
</tr>
<tr>
<td>22</td>
<td>0.0023</td>
<td>0.0065</td>
<td>0.0080</td>
<td>0.0023</td>
</tr>
<tr>
<td>24</td>
<td>0.0017</td>
<td>0.0065</td>
<td>0.0085</td>
<td>0.0038</td>
</tr>
</tbody>
</table>
3.2.3 Comparison of predation aggressiveness

The predation aggressiveness is a measure of how well a BALO is able to utilize a prey cell for growth. To assess whether the predation aggressiveness of *B. bacteriovorus* 109J is affected by the degree of peptidoglycan O-acetylation of the prey cell we quantified the residual number of prey cells. The four strains of *P. mirabilis* were incubated for 24 hours in the presence or absence of *B. bacteriovorus* 109J and the fold change in CFU/mL with and without the predator was calculated. *P. mirabilis* strains MCB330 and P19 had an identical fold change of CFU/mL between cultures that did not contain *B. bacteriovorus* 109J and those that did (Table 9). Their peptidoglycan O-acetylation is 49.8% and 52.8% respectively (Table 9). The *P. mirabilis* strain that had the least decrease in CFU/mL was 25933; it had a log$_{10}$ fold change of 2.1 and a percent O-acetylation of 25. There was a fold change difference of 0.5 between the *P. mirabilis* strain that had the smallest degree of peptidoglycan O-acetylation and the one with the greatest, 25% and 52.8% respectively. These results indicated that an increase in O-acetylation of peptidoglycan resulted in an increase in predation efficiency.

3.3 Mutation of gene *bd3575* in *Bdellovibrio bacteriovorus* 109J

The predatory life cycle of *B. bacteriovorus* requires the bacterium to traverse the prey cell outer membrane and peptidoglycan, and enter the periplasmic space of its prey cell. In bacteria, lytic transglycosylases are periplasmic enzymes responsible for the cleavage of β–1,4 glycosidic bonds of the peptidoglycan backbone. Therefore, these enzymes in BALOs are thought to be involved in creating a pore in the prey cell peptidoglycan during invasion. The transcriptional study of Lambert *et al.* (2010) showed that *bd3575,*
Table 9. Predation aggressiveness of *B. bacteriovorus* 109J on *P. mirabilis*

The ability of *B. bacteriovorus* 109J to utilize the four different *P. mirabilis* strains was measured by comparing the final CFU/mL of the *P. mirabilis* strains incubated in the absence or presence of *B. bacteriovorus* 109J for 24 hours. n=3

<table>
<thead>
<tr>
<th>Strain of <em>Proteus mirabilis</em></th>
<th>Published % peptidoglycan O-acetylation</th>
<th>Final prey cell only (CFU/mL)</th>
<th>Final prey cell with <em>B. bacteriovorus</em> (CFU/mL)</th>
<th>Log$_{10}$ Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>25933</td>
<td>25</td>
<td>2.14 x$10^{9}$</td>
<td>1.89 x$10^{7}$</td>
<td>2.1</td>
</tr>
<tr>
<td>7002</td>
<td>43</td>
<td>4.60 x$10^{8}$</td>
<td>1.46 x$10^{6}$</td>
<td>2.5</td>
</tr>
<tr>
<td>MCB330</td>
<td>49.8</td>
<td>1.60 x$10^{9}$</td>
<td>3.51 x$10^{6}$</td>
<td>2.6</td>
</tr>
<tr>
<td>P19</td>
<td>52.8</td>
<td>7.00 x$10^{8}$</td>
<td>1.85 x$10^{6}$</td>
<td>2.6</td>
</tr>
</tbody>
</table>
annotated as encoding a lytic transglycosylase (Rendulic et al., 2004), was highly upregulated during invasion. To determine if the lytic transglycosylase *bd3575* contributes to efficient prey cell entry we mutated the gene in *B. bacteriovorus* strain 109J. In this study we used prey dependent *B. bacteriovorus* 109J versus prey independent strain *B. bacteriovorus* 109JA because a previous study stated deletion of gene *bd3575* is not lethal (Lerner et al., 2010). Therefore, a more accurate analysis of the role the *bd3575* gene product has in the periplasmic life cycle could be analyzed.

Before mutational analysis, the *bd3575* gene of *B. bacteriovorus* was analyzed using InterProScan (Version 4.8). The sequence had a protein signature of a lytic transglycosylase-like catalytic domain. When the protein sequence was scanned with homology and structure prediction software (HHpred) the top sequence alignment was to a soluble lytic transglycosylase.

**3.3.1 Creation of *Bdellovibrio bacteriovorus* 109J Δ3575**

Chromosomal DNA of *B. bacteriovorus* 109J was isolated and the upstream and downstream fragments of gene *bd3575* were amplified using the primers *bd3575*F₁, *bd3575*R₁, *bd3575*F₂, and *bd3575*R₂ (Table 5). The upstream region of *bd3575* was amplified by PCR and run on an agarose gel; the fragment was approximately 500 bp (Fig. 8, Lane 3; Fig. 9, Lane 4). The downstream fragment was amplified by PCR and migrated on the gel to 650 bp (Fig. 8, Lane 5; Fig. 9, Lane 5). After both fragments were digested with *Xho*1 and subsequently ligated together which results in the deletion construct, a band approximately 1100 bp in length (Fig. 9, Lane 3). The deletion construct eliminated the majority of the *bd3575* gene of *B. bacteriovorus* 109J, leaving only 9 bp of the original gene. The deletion construct and suicide vector pSSK10 were
Figure 8. Amplification of upstream and downstream fragments of gene *bd3575* from *B. bacteriovorus 109J*

Agarose gel electrophoresis of PCR products. The primers used to amplify the fragments are indicated in parenthesis. Lanes: 1) 1kb DNA ladder 2) Empty 3) Upstream fragment, 500 bp (*bd3575*F₁ and *bd3575*R₁) 4) Empty and 5) Downstream fragment, 650 bp (*bd3575*F₂ and *bd3575*R₂)
Figure 9. Ligation of upstream and downstream fragments of gene \textit{bd3575} from \textit{B. bacteriovorus} 109J

Agarose gel electrophoresis of PCR products. Lanes: 1) 1kb DNA ladder 2) Empty 3) Ligation product, 1100 bp 4) Upstream fragment, 500 bp and 5) Downstream fragment, 650 bp
then digested with NdeI and SphI and ligated together resulting in plasmid pΔ3575. The deletion construct was confirmed by sequencing the PCR product from pΔ3575 using bd3575F1 and bd3575R2 primers (Fig. 10). Colony PCR using primers bd3575F1 and bd3575R2 was used to screen E. coli SM10λpir that had been transformed with plasmid pΔ3575, which can faintly be seen in Lane 2 around 1100bp (Fig. 11, Lane 2). The transformed E. coli SM10λpir containing the plasmid was then used as a donor for conjugation to the recipient B. bacteriovorus 109J. Positive selection of merodiploid exconjugates was done using a plaque assay in the presence of chloramphenicol and counterselection for allelic exchange was completed in the presence of sucrose. Any B. bacteriovorus 109J that did not undergo a second homologous recombination event, to excise the plasmid pSSK10 containing the counterselection gene sacB, were susceptible to the toxin levansucrase. Using the primer pairs bd3575F1/bd3575R2 and bd3575F/bd3575R a B. bacteriovorus 109J exconjugate was screened for the Δ3575 deletion construct (Fig. 12, Lanes 4 and 5). Using primers designed to amplify the deleted fragment of the gene bd3575, bd3575F1 and bd3575R1, it was confirmed that the wild type gene was not incorporated elsewhere in the genome of B. bacteriovorus 109J (no product) (Fig. 12, Lane 6). The validity of the internal primers was confirmed by PCR with wild type B. bacteriovorus 109J (data not shown). Construction of B. bacteriovorus 109J Δ3575 was also confirmed by DNA sequencing (Fig. 13).

3.3.2 Synchronous co-cultures assessed by light microscopy

The predation of B. bacteriovorus 109J Δ3575 was initially assessed by phase contrast light microscopy. Synchronous co-cultures of wild type or mutant B. bacteriovorus 109J containing prey cell E. coli ML35 were monitored for 48 hours. There were no
Figure 10. DNA sequence of deletion construct from pΔ3575

The sequenced PCR product from amplification of DNA from pΔ3575 using primers bd3575F₁ and bd3575R₂. The 9 bp of the original gene are in bold. The cut site from XhoI is underlined.
Figure 11. Colony PCR screening *E. coli* SM10λpir transformed with plasmid pΔ3575

Agarose gel electrophoresis of PCR products (primers *bd3575F*₁ and *bd3575R*₂) from *E. coli* SM10λpir that were electroporated with pΔ3575. Lanes: 1) 1kb DNA ladder 2) Transformed *E. coli* SM10λpir and 3) No product.
Figure 12. Confirmation of *B. bacteriovorus* 109J Δ3575

Agarose gel electrophoresis of PCR products. The template DNA is listed and primers are indicated in parenthesis. Lanes: 1) 1kb DNA ladder 2) *B. bacteriovorus* 109J *(bd3575F/bd3575R)* 3) pΔ3575 *(bd3575F1/bd3575R2)* 4) *B. bacteriovorus* 109J Δ3575 *(bd3575F1/bd3575R2)* 5) *B. bacteriovorus* 109J Δ3575 *(bd3575F/bd3575R)* and 6) *B. bacteriovorus* 109J Δ3575, no product *(bd3575F1/bd3575R1).*
CGGCNTGCCNCCAGAGTTTGCGAAAAATACATCGGCTGATAATTATNNGNA
CCTACAATAAACTGCCTTGAGCAGATGTCTTGGCAAAAGCCGCTCAAC
AGCAAATCCCATCAAAATGCCTGTCAAAATCTGCATACATT
TAATTTACAGATCTCGTCTCAGTCTGACACGTTCAAAACAAACATTT
ACGGCCCCACCCCCAGCTTGCTTAGCCCTCAAAAGGTACGATTTATGCA
GCTATACCTTCTTAAGAACATATGCAATAGTAGATCTTAGTTCACACAAAGA
CTTTTGATCTTTACCAAAAGGTTCTAGACGCTTCAAAAAGCATTTTAA
ACGGCCCCACAACCGCGAGCCAGCTTCAAAAGGTACGATTTATGCA
CAGCTTGTCCAGATGATACCGCTCAACCAATGCCCCATGATATGCTAT
AAACCAGTCTTCAGACCCGTCTCCACACCCATGCTGGAACATGT
GATTCCACCTGCCCCACATCCGCCACCCGAGGTGTTGCGCCGATGGAAGG
TTCTGTTACACGGCAAGCAGGCATGTGATCGGCTGACGGTACAG
CTTTGCATGGCAAGTTTTTCAACCCGGAAACCTTTGTTTGCTCTGAAACT
CGNGTCTTGGCGATTAGTGCA

Figure 13. DNA sequence of deletion construct in *B. bacteriovorus* 109J Δ3575

The sequenced PCR product from amplification of DNA from *B. bacteriovorus* 109J Δ3575 using primers *bd3575F* and *bd3575R*. The deletion construct (15 bp) is in bold.
visible morphological differences between wild type and \textit{B. bacteriovorus} 109J Δ3575. When compared to wild type, \textit{B. bacteriovorus} 109J Δ3575 showed no difference in its ability to prey on \textit{E. coli} ML35. Both co-cultures had evidence of the predator attached to \textit{E. coli} ML35 after 2 hours of incubation. Formation of bdelloplasts occurred approximately 6 hours into incubation for both co-cultures and complete predation was reached after 30 hours.

3.3.3 \textbf{Electron microscopy of \textit{B. bacteriovorus} 109J Δ3575}

The life cycle \textit{B. bacteriovorus} 109J Δ3575 was assessed using electron microscopy. Thin sections were prepared from co-cultures containing \textit{B. bacteriovorus} 109J Δ3575 and prey cell \textit{E. coli} ML35 after 10 hours of incubation. \textit{B. bacteriovorus} 109J Δ3575 is still able to attach to prey cells (Fig. 14A), traverse the outer membrane and peptidoglycan (Fig. 14B), and form a bdelloplast (Fig. 14C). These images confirm the results of the synchronous co-cultures assessed by phase contrast light microscopy.

3.3.4 \textbf{Predation kinetics of \textit{B. bacteriovorus} 109J Δ3575}

The predation kinetics of \textit{B. bacteriovorus} 109J Δ3575 in comparison to \textit{B. bacteriovorus} 109J were assessed using growth curves. To quantify if there were any changes in predation, co-cultures containing a predator and \textit{E. coli} ML35 were monitored for 48 hours using a BioScreen apparatus. The decrease in optical density represents the consumption of \textit{E. coli} ML35, which can be interpreted as \textit{B. bacteriovorus} growth.

Both co-cultures had a 6-hour lag phase and reached complete predation within 16 hours (Fig. 15). The rate at which predation occurred (the slope of the growth curve)
Figure 14A. Initial recognition period of *B. bacteriovorus* 109J Δ3575
Thin section electron micrographs of *B. bacteriovorus* 109J Δ3575 with prey cell *E. coli* ML35 after 10 hours of incubation; i) initial recognition stage. *B. bacteriovorus* 109J Δ3575 still possesses its flagellum and ii) attachment stage. Arrows indicate *B. bacteriovorus* 109J Δ3575. Arrowheads indicate *E. coli* ML35. Bar = 1 μm
Figure 14B. Invasion of *E. coli* ML35 by *B. bacteriovorus* 109J Δ3575

Thin section electron micrograph of *B. bacteriovorus* 109J Δ3575 with prey cell *E. coli* ML35 after 10 hours of incubation. Arrow indicates *B. bacteriovorus* 109J Δ3575. Arrowhead indicates *E. coli* ML35. Bar = 200 nm
**Figure 14C. Formation of bdelloplasts by *B. bacteriovorus* 109J Δ3575**

Thin section electron micrograph of *B. bacteriovorus* 109J Δ3575 with prey cell *E. coli* ML35 after 10 hours of incubation. Arrow indicates *B. bacteriovorus* 109J Δ3575. Arrowhead indicates bdelloplast. Bar = 2 µm
Figure 15. Predation kinetics of *B. bacteriovorus* 109J Δ3575

The optical density of *B. bacteriovorus* 109J Δ3575 and *B. bacteriovorus* 109J co-cultures containing prey cell *E. coli* ML35 was assessed using a Bioscreen apparatus. (●) *B. bacteriovorus* 109J and (▲) *B. bacteriovorus* 109J Δ3575.
was also similar between the two co-cultures. Both co-cultures had a final optical density reading of 0.2; this represents the residual *E. coli* ML35 and the newly synthesized predators. The standard deviation of three replicates was calculated and never exceeded an OD<sub>600</sub> reading of 0.028 (Table 10).

### 3.3.5 Predation aggressiveness of *B. bacteriovorus* 109J Δ3575

The predation aggressiveness is a measure of how well *B. bacteriovorus* is able to utilize a prey cell. To assess whether *B. bacteriovorus* 109J Δ3575 is attenuated in its ability to use *E. coli* ML35 as its prey cell we quantified the fold change in CFU/mL before and after predation, and compared that to growth of *B. bacteriovorus* 109J. Both co-cultures had an average starting 3.13 x10<sup>9</sup> CFU/mL of *E. coli* ML35 (Table 11). After 24 hours of incubation the co-culture containing *B. bacteriovorus* 109J Δ3575 had 2.10 x10<sup>6</sup> CFU/mL of *E. coli* ML35 remaining, a 3.2 Log<sub>10</sub> fold change. Wild type *B. bacteriovorus* 109J had 2.66 x10<sup>6</sup> CFU/mL of residual *E. coli* ML35, a 3.1 Log<sub>10</sub> fold change. The deletion of gene *bd3575* did not affect predation aggressiveness by *B. bacteriovorus* 109J.

### 3.4 Effects of Biotinylation on Predation by *B. bacteriovorus* 109J

In order to better assess early predation kinetics of *B. bacteriovorus* 109J our lab sought out a technique that was more sensitive to quantifying formation of bdelloplasts within liquid co-cultures. In order to achieve this objective we decided to use a biotin-avidin labeling assay. Using co-cultures of biotinylated *Bdellovibrio* and *E. coli* that expresses a fluorescent protein, quantification of attack phase *Bdellovibrio* and bdelloplasts is possible (Fig. 16). Biotin was chosen to label *B. bacteriovorus* because its small size
Table 10. Standard deviations of predation kinetics of *B. bacteriovorus* 109J Δ3575

The standard deviations (OD\textsubscript{600}±SD) of predation kinetics of *B. bacteriovorus* 109J Δ3575 and *B. bacteriovorus* 109J on *E. coli* ML35. n=3

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th><em>Bdellovibrio bacteriovorus</em> 109J Δ3575</th>
<th><em>Bdellovibrio bacteriovorus</em> 109J</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.016</td>
<td>0.012</td>
</tr>
<tr>
<td>2</td>
<td>0.017</td>
<td>0.012</td>
</tr>
<tr>
<td>4</td>
<td>0.021</td>
<td>0.015</td>
</tr>
<tr>
<td>6</td>
<td>0.013</td>
<td>0.0085</td>
</tr>
<tr>
<td>8</td>
<td>0.013</td>
<td>0.0078</td>
</tr>
<tr>
<td>10</td>
<td>0.011</td>
<td>0.0071</td>
</tr>
<tr>
<td>12</td>
<td>0.022</td>
<td>0.0093</td>
</tr>
<tr>
<td>14</td>
<td>0.028</td>
<td>0.0067</td>
</tr>
<tr>
<td>16</td>
<td>0.020</td>
<td>0.012</td>
</tr>
<tr>
<td>18</td>
<td>0.015</td>
<td>0.012</td>
</tr>
<tr>
<td>20</td>
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</tr>
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<td>22</td>
<td>0.011</td>
<td>0.011</td>
</tr>
<tr>
<td>24</td>
<td>0.011</td>
<td>0.013</td>
</tr>
</tbody>
</table>
**Table 11. Predation aggressiveness of *B. bacteriovorus 109J Δ3575***

The ability of *B. bacteriovorus 109J Δ3575* to utilize *E. coli* ML35 was measured by comparing the initial and final CFU/mL of *E. coli* ML35 in comparison to wild type *B. bacteriovorus 109J*. n=3

<table>
<thead>
<tr>
<th>Predator</th>
<th>Initial amount of <em>E. coli ML35</em> (CFU/mL)</th>
<th>Final amount of <em>E. coli ML35</em> (CFU/mL)</th>
<th>Log$_{10}$ Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. bacteriovorus 109J Δ3575</em></td>
<td>3.13 x$10^9$</td>
<td>2.10 x$10^6$</td>
<td>3.2</td>
</tr>
<tr>
<td><em>B. bacteriovorus 109J</em></td>
<td>3.13 x$10^9$</td>
<td>2.66 x$10^6$</td>
<td>3.1</td>
</tr>
</tbody>
</table>
Figure 16. Biotin-avidin binding assay

Beige-coloured flagellated cells represent biotin labeled *B. bacteriovorus*. Red non-flagellated cells represent *E. coli* expressing red fluorescent protein (RFP). Green flagellated cells represent attack phase *B. bacteriovorus* fluorescently labeled with avidin. Rounded cells represent a bdelloplast with *B. bacteriovorus* and the shrinking cytoplasmic contents of the prey cell inside.
does not typically interfere with function of proteins. Also, biotin-avidin binding has a high affinity and is very specific. An N-hydroxysuccinimide (NHS) ester of biotin was used because it is water-soluble and can biotinylate the cell surface.

3.4.1 Predation efficiency assessed by light microscopy

*B. bacteriovorus* 109J labeled with an NHS ester of biotin showed attachment and invasion of prey cells after 24 hours of incubation as compared to unlabeled *B. bacteriovorus* 109J in which predation occurred after 1 hour of incubation. Co-cultures of unlabeled *B. bacteriovorus* 109J reached complete predation in less than 24 hours, which is typical for *Bdellovibrio*. However, biotinylated *B. bacteriovorus* 109J took 48 hours to reach complete predation, a notable delay in its predation efficiency. Therefore, this assay was not suitable for an analysis of the kinetics of invasion.
Chapter 4: Discussion

Bacterial peptidoglycan envelops the whole cell and contributes to the overall shape and provides structural support. Given the importance of peptidoglycan to the cell it is not surprising enzymes exist that cleave the linkages between the residues. The β-1,4 glycosidic bond of peptidoglycan can be cleaved by lysozyme, produced by the innate immune system. This action can ultimately lead to cell lysis (Moynihan & Clarke, 2010). The β-1,4 glycosidic bond can also be cleaved by endogenous lytic transglycosylases that are essential to bacterial cells so they can remodel their peptidoglycan, which is required for the growth and maintenance of the structure. One way bacteria can control the lytic ability of both enzymes is by modifying peptidoglycan by O-acetylation. The addition of acetate to MurNAc residues prevents the activity of lysozyme and lytic transglycosylases through steric hindrance of their active site for the substrate. This modification provides resistance to cleavage of the peptidoglycan by exogenous lysozyme as well as lysis by endogenous lytic transglycosylases. Currently there are only two research groups that study O-acetylation of peptidoglycan, those of Clarke (University of Guelph) and Vollmer (University of Newcastle upon Tyne, UK). At the onset of this study the peptidoglycan O-acetylation status of any BALO was unknown.

The degree of peptidoglycan O-acetylation, the amount of acetate present relative to the amount of MurNAc, has been found to depend upon stage of growth, species, and strain (Moynihan & Clark, 2010). Pfeffer et al. (2006) found that, upon entering stationary phase, cultures of *E. faecalis* had an increase of 10-40% of peptidoglycan O-acetylation. This was taken into account when culturing cells for analysis in this study as
peptidoglycan was purified from BALO attack phase cells or prey cell cultures that had reached stationary phase.

The degree of peptidoglycan O-acetylation was characterized for *P. mirabilis* MCB330 as a control for the purification procedure. During isolation of peptidoglycan, acetate can be lost by premature hydrolysis if the pH of the sample exceeds 6.8. In this study the value 34.39% (±1.45) is less than that previously reported (49.8%) for strain MCB330 (Pfeffer, 2013). This result confirms the technique in that not all of the acetate was lost during isolation and purification of peptidoglycan. The difference in O-acetylation values (15.41%) for the peptidoglycan modification could be due to premature hydrolysis of some O-linked acetate, differences in culture conditions, or the method used to determine the amount of acetate (A. Clarke, personal communication).

In this study cultures were incubated at 30°C and O-linked acetate was determined using an anion exchange column. In comparison the previous study used the Megazyme Acetic Acid Assay kit method to measure the amount of acetate in the sample from cultures incubated at 37°C (Pfeffer, 2013).

The prey dependant strains of BALOs characterized in this study were *B. bacteriovorus* 109J and *B. exovorus* JSS. When analysing prey dependent strains it is important to consider the contribution of acetate from peptidoglycan of residual prey cells, if not all were removed from the samples before isolation. The prey cell for *B. bacteriovorus* 109J is *E. coli*, a bacterium that does not O-acetylate its peptidoglycan (Moynihan & Clarke, 2010). Any acetate detected during analysis of the peptidoglycan preparation will come from the predator peptidoglycan. In this study the degree of peptidoglycan O-acetylation of *B. bacteriovorus* 109J was determined to be 7.04% (±1.01) using HPLC
analysis and 3.22% (±1.79) using the Megazyme Acetic Acid Assay kit analysis. Both the analyses have advantages and disadvantages. HPLC is a more sensitive method but there is a chance that something may co-elute with acetate, resulting in a false positive. In comparison the Megazyme Acetic Acid Assay kit lacks sensitivity at low levels. Given these findings we further investigated the genes previously annotated in the genome of *B. bacteriovorus*. Using BLAST the PatA homolog *bd1667* was confirmed and contained an essential neighbouring hydrolase. However, the consensus sequences of PatB are lacking in the neighboring hydrolase. Furthermore, there was no Ape (potential esterase) found in the genome. The degree of peptidoglycan O-acetylation of *S. maltophilia* and *B. bacteriovorus* 109J is at best minimal considering most bacteria that acetylate their peptidoglycan do so at levels 20% or greater (Moynihan & Clarke, 2010).

The prey cell used in this study for *B. exovorus* JSS was *S. maltophilia*. This was because the alternative prey cell *Caulobacter crescentus* possesses a biphasic life cycle of its own and would have complicated the isolation and purification procedure of peptidoglycan. The degree of peptidoglycan O-acetylation of *S. maltophilia* in this study was 1.8 (±0.12). The amount of acetate detected for the BALO *B. exovorus* JSS was found to be 0%. No BLAST hits for PatA, PatB, or Ape gene homologs were identified in the genome of *B. exovorus* JSS or *S. maltophilia*.

Other BALOs analysed in this study were *B. bacteriovorus* 109JA and *B. stolpii* UKi2. Because they are prey independent strains they do not require analysis of a prey cell and any acetate detected would be a contribution from the BALO. In this study both prey
independent strains had no acetate in their peptidoglycan samples. Genomic analysis of B. stolpii UKi2 is not presented because the genome has not been published.

The amount of O-acetylation detected taken together with the genomic analysis, it is suggested that B. exovorus JSS, B. bacteriovorus 109JA, and B. stolpii UKi2 do not acetylate their peptidoglycan under the conditions used in this study. Therefore, O-acetylation of peptidoglycan is not used as a way to control the lytic activity of enzymes that cleave the β–1,4 glycosidic bond.

During the periplasmic life cycle B. bacteriovorus must traverse the prey cell outer membrane and peptidoglycan. In order to do this B. bacteriovorus would require a lytic transglycosylase to create the entry pore in the prey cell peptidoglycan. Thus it was hypothesized that O-acetylation of the prey cell peptidoglycan may hinder the cleavage of the glycan chains by the lytic transglycosylase of the predator.

The four P. mirabilis strains used in this study were previously characterized for their degree of peptidoglycan O-acetylation: P. mirabilis 25933 (25%), P. mirabilis 7002 (43%), P. mirabilis MCB330 (49.8%), and P. mirabilis P19 (52.8%) (Dupont & Clarke, 1991; Pfeffer, 2013). By assessing predation by B. bacteriovorus on P. mirabilis we controlled for variation in predation efficiency by B. bacteriovorus on different species, which is not well understood. P. mirabilis strains MCB330 (49.8% O-acetylation) and P19 (52.8% O-acetylation) had an identical log10 fold change of CFU/mL that acts as an internal control for this predation assay as they have similar degrees of peptidoglycan O-acetylation.

It was expected that B. bacteriovorus would have a better predation efficiency on strains of P. mirabilis that had a lower degree of peptidoglycan O-acetylation. There was a
log_{10} fold change difference of 0.5 between P. mirabilis strain 25933 (25% O-acetylation) and P19 (52.8% O-acetylation). With twice the amount of O-acetylation the predation of B. bacteriovorus 109J on P. mirabilis was not hindered but was more efficient.

To better assess the efficiency of attachment and invasion of BALOs, which occurs within the first 15-30 minutes of the life cycle, a biotin-avidin binding assay was carried out (Fig. 16). Once labeled, Bdellovibrio attack phase cells were to be added to E. coli prey cells that express a red fluorescent protein. The addition of a green fluorescent avidin, which binds strongly to biotin, would have allowed us to discern the number of Bdellovibrio inside a bdelloplast (no green biotin-avidin fluorescence, red bdelloplasts) or Bdellovibrio that have not started predation (green biotin-avidin fluorescence). Biotinylated B. bacteriovorus 109J took 24 hours to show signs of attachment, much longer than untreated predators. Complete predation was achieved by 48 hours.

Therefore, surface biotin labeling of B. bacteriovorus at the concentrations used in this study affected the attachment and entry of the predator. Due to this attenuation of predation the biotin-avidin binding assay was not a viable method to closely assess initial stages of predation. To my knowledge biotin has not previously been shown to obstruct the function of cells previous to this study. One modification to this protocol would be to use a lower concentration of biotin when labeling B. bacteriovorus. Biotin attaches to amine groups on the cell surface; using less biotin may allow predation to occur and if enough fluorescent streptavidin binds to produce a strong signal the assay may be used for its intended purpose.
Due to the unique predacious life cycle of BALOs, modification of the prey peptidoglycan by the predator is a key process in predation. The enzymes responsible for creation of the pore in the prey cell peptidoglycan have yet to be identified. The transcriptional studies of Lambert at al. (2010) showed that bd3575, encoding a lytic transglycosylase, was highly upregulated during invasion. One sentence in the Lerner at al. (2010) paper mentioned that deletion of this gene still allowed invasion to occur, but no data were provided either on construction of the mutant or evaluation of predation.

In this study an in-frame markerless deletion of the gene bd3575 in B. bacteriovorus 109J was constructed. Thin section electron micrographs of B. bacteriovorus 109J Δ3575 revealed that there were no alterations in the life cycle. During this study B. bacteriovorus 109J Δ3575 traversing the prey cell outer membrane and peptidoglycan was captured (Fig. 13B). Entry occurs quickly and is rarely illustrated. Predation assays also revealed that there were no detectable differences in predation kinetics of B. bacteriovorus 109J Δ3575 on E. coli ML35. Because the transcriptional studies of Lambert at al. (2010) showed that bd3575 was the only lytic transglycosylase upregulated during the initial stages of predation it was thought that it would have a significant role in predation. However, due to genetic redundancy it is most likely that the mutation of the gene was rescued by one of the other 7 lytic transglycosylases.

In this study predation kinetics were assessed using a BioScreen apparatus. One limitation of this method is aeration of cultures is not optimal in the small wells. Future studies could use an alternative method to assess growth curves, such as predation in 125 mL side-arm flasks assessed using a Klett-Summerson photoelectric colorimeter. This method would allow for more variation in experimental design. Future studies
could also investigate the role lytic transglycosylases have in the predation of \textit{B. bacteriovorus} by constructing mutations in the other 7 annotated lytic transglycosylases. One caveat to this study would be to try to distinguish between lytic transglycosylases involved in creating the entry pore for predation and those essential for remodelling of its own peptidoglycan during growth.

4.1 Summary

Bacteria are able to modify their peptidoglycan by the addition of acetate groups to the MurNAc residues. Addition of acetate to the C-6 hydroxyl group of MurNAc residues renders the peptidoglycan insensitive to cleavage by lysozyme and lytic transglycosylases. Prior to this study the peptidoglycan \textit{O}-acetylation status of any BALOs or \textit{S. maltophilia} were unknown. In this study it was determined that \textit{B. bacteriovorus} 109JA, \textit{B. exovorus} JSS, and \textit{B. stolpii} UKi2 do not \textit{O}-acetylate their peptidoglycan and therefore do not use this peptidoglycan modification method to control cleavage of the β–1,4 bonds. The peptidoglycan \textit{O}-acetylation of \textit{B. bacteriovorus} 109J (7.04\% and 3.22\%) and \textit{S. maltophilia} X26332 (1.8\%) is at best minimal.

It is thought that \textit{B. bacteriovorus} requires lytic transglycosylases to create the entry pore during invasion. Therefore, it was hypothesized that the degree of peptidoglycan \textit{O}-acetylation of the prey cell affects predation efficiency. In this study it was suggested that the predation efficiency of \textit{B. bacteriovorus} is affected by the degree of peptidoglycan \textit{O}-acylation of \textit{P. mirabilis}, as the degree of peptidoglycan \textit{O}-acylation increases the predation efficiency also increases.
To further study the role of lytic transglycosylases in the life cycle, the mutant *B. bacteriovorus* Δ3575 was constructed. The gene *bd3575* of *B. bacteriovorus* has been annotated as being a soluble lytic transglycosylases and was shown to be upregulated during attachment and invasion. The loss of gene *bd3575* function did not affect predation of *B. bacteriovorus* on *E. coli* and the phenotype was most likely rescued by one of the other 7 lytic transglycosylases.
References


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