Inhibition of Membrane-bound Lytic Transglycosylase A by Inhibitors of Vertebrate Lysozyme P1 and P2

By

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ABSTRACT

Inhibition of Membrane-bound Lytic Transglycosylase A by Inhibitors of Vertebrate Lysozyme P1 and P2

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The proteinaceous inhibitors of vertebrate lysozyme (Ivy) p1 and p2 from Pseudomonas aeruginosa are two putative inhibitors of lytic transglycosylase (LT). The LTs are bacterial enzymes essential for the cleavage of glycosidic linkages in the peptidoglycan (PG) sacculus surrounding the cell. Although initially identified as inhibitors of lysozymes, Ivyp1 and Ivyp2 have previously been shown to inhibit a soluble derivative of membrane-bound LT B from P. aeruginosa. The current study investigated the inhibition of a soluble derivative of membrane-bound LT A (sMltA) from the same bacterium. Inhibition of sMltA by both Ivyp1 and Ivyp2 was confirmed in vitro using a turbidometric assay and the production and purification of all three proteins was improved upon. Thus, useful protocols were established for the continuation of the inhibition studies and more evidence was collected to support our hypothesis that Ivyp1 and Ivyp2, as well as homologs in other Gram-negative bacteria, are LT inhibitors.
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<td>Bicinchoninic acid</td>
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<td>Chloramphenicol</td>
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<td>Glu</td>
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<td>HMW</td>
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<td>Immobilised metal affinity chromatography</td>
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<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
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<td>Ivy</td>
<td>Inhibitor of vertebrate lysozyme</td>
</tr>
<tr>
<td>Kan</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LMW</td>
<td>Low molecular weight</td>
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<tr>
<td>LT</td>
<td>Lytic transglycosylase</td>
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<tr>
<td>Lys</td>
<td>Lysine</td>
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<td>MALDI</td>
<td>Matrix-assisted desorption/ionization</td>
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<td>m-DAP</td>
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<td>MW</td>
<td>Molecular weight</td>
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<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>MWCO</td>
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<td>N-acetyl glucosaminidase</td>
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<td>Nucleotide-binding oligomerization domain</td>
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<tr>
<td>Pat</td>
<td>PG O-acetyl transferase</td>
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<tr>
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<td>Penicillin-binding protein</td>
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<td>Polymerase chain reaction</td>
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<td>Protein data bank</td>
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<td>Peptidoglycan</td>
</tr>
<tr>
<td>Pli</td>
<td>Periplasmic lysozyme inhibitor</td>
</tr>
<tr>
<td>Rcs</td>
<td>Regulator of capsule synthesis</td>
</tr>
<tr>
<td>SB</td>
<td>Super broth</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Slt</td>
<td>Soluble lytic transglycosylase</td>
</tr>
<tr>
<td>spp</td>
<td><em>Species pluralis</em></td>
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<td>SRL</td>
<td>Sucrose, RNase, and lysozyme solution</td>
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<tr>
<td>T4L</td>
<td>T4 phage lysozyme</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris, acetic acid, and EDTA buffer</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
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<tr>
<td>TE</td>
<td>Tris and EDTA buffer</td>
</tr>
<tr>
<td>Tet</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>TOF</td>
<td>Time of flight</td>
</tr>
<tr>
<td>Tse</td>
<td>Type VI secretion effector</td>
</tr>
<tr>
<td>Tsi</td>
<td>Type VI secretion immunity</td>
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<tr>
<td>TTBS</td>
<td>Tween and Tris-buffered saline</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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CHAPTER 1 - INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacillus found ubiquitously in soil. Despite being common in the environment, P. aeruginosa does not usually cause infections in healthy individuals. Rather, only immunocompromised individuals such as AIDS patients, chemotherapy patients, burn victims, and patients with cystic fibrosis are susceptible to infection. Additionally, a recent global study conducted from 2002 to 2011 that monitored intra-abdominal and urinary tract infections caused by Gram-negative bacteria found that worldwide, P. aeruginosa was in the top five most commonly isolated microbes (Morrissey et al., 2013). In fact, 9.4% of the intra-abdominal and 5.4% of the urinary tract infections monitored were caused by P. aeruginosa. Many hospitals appear to harbour multidrug resistant strains of P. aeruginosa and thus, this microorganism is also the cause of many hospital acquired infections. Thus, strains of P. aeruginosa which are resistant to aminoglycosides, β-lactams, or fluoroquinololones have been identified and some strains have been shown resistance to more than one class of antibiotic. Hospital acquired P. aeruginosa is more difficult to treat as compared to community acquired infections (Morrissey et al., 2013). The Centers for Disease Control and Prevention in the United States list multidrug-resistant P. aeruginosa as a serious threat in the US (CDC, 2013). The antibiotic resistance threat report for 2013 published by the CDC estimates that there are 51,000 new healthcare-associated P. aeruginosa infections every year in the US, representing 8% of all healthcare-associated infections. Additionally, 13% of these infections are caused by multidrug-resistant strains of P. aeruginosa and lead to over 400 deaths a year (CDC, 2013).

There exists a need for the development of novel antibiotics that can be effective against the multidrug resistant strains of P. aeruginosa, as well as other highly resistant microbes (such as methicillin-resistant Staphylococcus aureus [MRSA] and vancomycin-resistant Enterococcus [VRE]). The bacterial cell wall layer peptidoglycan (PG) presents as an excellent target for antibiotics because it is a structure both critical to cell integrity and unique to bacteria. In fact, multiple classes of existing antibiotics target the bacterial cell wall or its biosynthetic enzymes, including the β-lactams which target the enzymes of the latter stages of PG biosynthesis, and vancomycin, a glycopeptide which binds PG to inhibit its biosynthesis. An attractive target for a novel class of antibiotics is the lytic
transglycosylases (LTs), enzymes involved in PG degradation and recycling which are therefore critical for cell survival. However, much is unknown regarding the LTs and their regulation within the bacterial cell. Such information may support the postulate that these enzymes are a novel target for the development of one or more new classes of antibiotics.

1.1 Peptidoglycan

Peptidoglycan (PG or murein) is an essential feature of the bacterial cell wall and it is found in all known eubacteria except for members of the Mycoplasmas and Planctomycetes genera. This structure is critical in providing shape and protection against cell lysis due to cytoplasmic turgor pressure, and it is an anchoring site for other cell wall components. PG is composed of cross-linked glycan strands of alternating β-1,4-linked N-acetylglicosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues (Fig. 1.1; reviewed by Vollmer et al., 2008a). Glycan strands vary in length with age and between organisms but all are terminated by a 1,6-anhydroMurNAc residue which contains an intra-molecular linkage between C-1 and C-6. Neighbouring strands are cross-linked to one another by short peptides linked to each MurNAc residue (usually L-Ala-γ-D-Glu-meso-2,6-diaminopimelic acid (m-DAP)-D-Ala-D-Ala in Gram-negative bacteria (including P. aeruginosa and Escherichia coli) or L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala in many Gram-positive species). The terminal D-Ala is lost upon formation of the cross-link between the fourth amino acid of one stem peptide (D-Ala) and the third amino acid of another. Some variation exists in PG between different species, and even within the same species, particularly with Gram-positive bacteria. Variations exist within the glycan strands (chain length), the stem peptides (hydroxylation of amino acids), or the peptide cross-linkages (interpeptide bridge instead of simple cross-link). In general, Gram-positive bacteria have much thicker PG layers compared to Gram-negative bacteria.
Figure 1.1 Peptidoglycan structure. PG is composed of alternating MurNAc and GlcNAc residues linked by β-1,4-glycosidic bonds. The peptide chains on the MurNAc residues are involved in the cross-linking of adjacent disaccharide strands. The red arrow denotes the β-1,4-glycosidic bond.

1.2 Peptidoglycan Biosynthesis and Recycling

PG biosynthesis occurs in three stages involving three ‘compartments’ of the bacterial cell (reviewed by Vollmer and Bertsche, 2008b). The first steps of PG biosynthesis occur in the cytoplasm of the cell. UDP-GlcNAc is converted to UDP-MurNAc, and the amino acids comprising the pentapeptide are then attached sequentially. This ‘muropeptide’ is then added to undecaprenyl phosphate generating Lipid I, followed by the addition of UDP-GlcNAc to form Lipid II, the complete PG precursor which is assembled on the cytoplasmic membrane (Fig. 1.2; reviewed in Barreteau et al., 2008). This precursor is translocated across the cytoplasmic membrane by as yet unknown mechanism. As reviewed in Johnson et al. (2012), the protein that transfers Lipid II across the inner cell membrane is unknown. Ruiz (2008) identified MurJ as the flippase in E. coli, based on bioinformatic methods; however, Fay and Dworkin (2009) showed that B. subtilis can survive without any MurJ homologs. Recently, FtsW has been suggested as the flippase (Mohammadi et al., 2011). Once outside the cell, the murein synthases catalyze the formation of mature PG from the muropeptides (GlcNAc-MurNAc-pentapeptide) via transglycosylation (extension of glycan strands by disaccharide units) and transpeptidation (formation of peptide cross-linkages) reactions. Because the terminal D-Ala-D-Ala dipeptide has a similar structure to penicillin, the murein synthases with transpeptidation activity are also capable of binding penicillin and are thus commonly referred to as penicillin-binding proteins (PBPs; Spratt, 1977). PG synthases may
be bi-functional or mono-functional (reviewed by Vollmer and Bertsche, 2008b). High molecular weight (HMW) PBPs are bi-functional, whereas low molecular weight (LMW) PBPs are mono-functional transpeptidases and have specific functions during PG biosynthesis and cell growth. For example, in *E. coli*, PBP2 is essential for cell elongation and PBP3 is present at the site of septation during cell division (Spratt, 1975).

![Figure 1.2 Peptidoglycan biosynthesis.](image)

**Figure 1.2 Peptidoglycan biosynthesis.** UDP-MurNAc-pentapeptide is added to undecaprenyl phosphate by MraY with the release of UMP to produce the Lipid I molecule. MurG catalyses the addition of GlcNAc to the Lipid I molecule using UDP-GlcNAc as a donor and producing Lipid II. Lipid II is transferred across the inner cell membrane (IM) by an unknown flippase and in the periplasm, the disaccharide pentapeptides are incorporated into growing strands of PG. The PBPs catalyse both the transglycosylation and the transpeptidation (dashed line) reactions, allowing elongation and cross-linking of glycan strands. Adapted from Ruiz (2008).

The PG sacculus is continuously being re-modelled through a process known as peptidoglycan recycling (reviewed in Johnson *et al.*, 2012). Over 50% of the PG sacculus is recycled per generation of cell division although most PG recycling takes place on the sides of the bacterium and not the polar caps. Both PG biosynthesis and hydrolysis are crucial for PG recycling and these two activities are carried out in a concerted way to maintain the integrity of the PG layer. The PG fragments released from the sacculus during PG recycling are important effector molecules for several different processes, including messenger functions in microbial communities, host immune response induction during infection, and monitoring external PG stress such as antibiotic challenge by β-lactam antibiotics.
PG degradation is executed in the periplasm by the PG lytic enzymes which are also referred to as autolysins due to their ability to completely degrade the PG sacculus if left unregulated (reviewed in Vollmer et al., 2008c). There are PG lytic enzymes for each bond in the PG sacculus, shown in Figure 1.3, including the LTs. The muramidases cleave the glycosidic linkages within the glycan strands. These enzymes include the lytic transglycosylases (LTs), which cleave the β-1,4 bond between \( \text{GlcN} \) and \( \text{MurN} \) residues within the glycan backbone, producing a non-reducing 1,6-anhydroMurNAc residue (Höltje et al., 1975). Exogenous lysozymes (from fungi, animals, or bacteriophage) hydrolyze the same bond but do not form the 1,6-anhydro product (Fig. 1.5). \( \text{N-Acetylglicosaminidases} \) cleave the glycosidic bond between adjacent MurNAc and GlcNAc residues, producing the monosaccharide subunits. \( \text{N-Acetylmuramyl-L-alanine amidases} \) cleave the α amide bond between the peptide stem and the MurNAc residue, whereas endo- and carboxypeptidases cleave the peptide bonds between amino acids in the peptide stem.

![Figure 1.3 Peptidoglycan lytic enzymes.](image)

Figure 1.3 Peptidoglycan lytic enzymes. Red arrows point to the bonds cleaved by the enzymes in bold font. LT – lytic transglycosylase, Lys – lysozyme, NAG – \( \text{N-acetylglicosaminidase} \), Ami – amidase, CP – carboxypeptidase, EP – endopeptidase.

Following the release of muropeptides (GlcNAc-anhMurNAc disaccharide units with peptide attached) and GlcNAc-anhMurNAc disaccharides (without peptides) from the PG sacculus, the muropeptides and disaccharides are transported across the cell membrane by AmpG, a permease. \( \text{P. aeruginosa} \) encodes two AmpG permeases, AmpG and AmpGh1, although only AmpG has been shown to have activity (Zhang et al., 2010). Once in the cytoplasm,
the muropeptides undergo bond cleavage between the GlcNAc and MurNAc residues by the N-acetylg glucosaminidase NagZ, releasing the anyhydroMurNAc and GlcNAc fragments. Cytoplasmic amidase AmpD removes the peptides from the anhydridMurNAc-peptides and the released peptides are recycled into the PG synthesis pathway. *P. aeruginosa* encodes three AmpD enzymes, AmpD1, AmpDh2, and AmpDh3 (Juan *et al.*, 2006). Released anhydroMurNAc is recycled into the UDP-GlcNAc synthesis pathway, as is GlcNAc. In this way, the lysis of the PG saccusus, the transport of PG fragments into the cytoplasm, and the further degradation of the PG fragments provide the building blocks for PG synthesis.

Many of the PG recycling enzymes are involved in β-lactamase induction in Gram-negative bacteria (reviewed in Johnson *et al.*, 2012). The β-lactamase in Gram-negative bacteria is encoded by a gene called *ampC*, which responds to changes in PG recycling. An increase in the cytoplasmic levels of the PG degradation product anhydroMurNAc-peptide leads to induction of *ampC* expression, while an increase in the concentration of PG building block UDP-MurNAc-pentapeptide represses *ampC* gene expression. A repressor protein, AmpR, binds to these molecules and undergoes a conformational change to induce or repress gene expression of AmpC (Balcewich *et al.*, 2010). AmpC can degrade broad- and extended-spectrum cephalosporins and is secreted into the periplasm to act on these compounds. Thus, in the absence of *ampD* expression, levels of anhydroMurNAc-peptide increase and lead to derepression (induction) of *ampC* gene expression, conferring increased β-lactam resistance.

It has been shown that the three *ampD* homologs encoded by *P. aeruginosa* allow a stepwise upregulation of *ampC* expression leading to four distinct expression profiles and resistance levels (Juan *et al.*, 2006). It has been suggested that *P. aeruginosa* encodes three homologs of *ampD* not only for the benefit of adjustable β-lactamase expression and β-lactam resistance, but also to allow these processes to occur without disrupting normal PG recycling. AmpG is also essential for β-lactamase induction and β-lactam resistance, since this protein imports into the cytoplasm the PG fragments that allow derepression of the *ampC* gene. During antibiotic threat, PG synthesis is inhibited by the β-lactam antibiotics, and PG fragments produced by the PG recycling pathway accumulate in the cytoplasm since they are not being re-incorporated back into newly synthesised PG. The accumulation of these fragments leads to *ampC* expression. As such, the LTs play a critical role not only in the PG
1.3 Lytic Transglycosylases

Lytic transglycosylases are a class of endogenous bacterial enzymes that cleave the β-1,4-glycosidic bond between alternating sugar residues in the peptidoglycan layer of the bacterial cell wall (reviewed in Vollmer et al., 2008c, Scheurwater et al., 2007). LTs are important for many cellular processes including growth and division, insertion of large protein complexes such as flagella through the PG layer, and PG recycling. There are four families of LTs, with family 1 further divided into five subfamilies, and these families are based on sequence and structural similarity (Blackburn and Clarke, 2001). Bacteria encode multiple LT genes from different LT families; for example, E. coli encodes seven LTs, one from each family and subfamily, and P. aeruginosa encodes one from each family except 1B and 1C, and it also encodes four family 3 LTs. Due to the PG-degrading nature of the LTs and the critical role of the PG layer in maintaining cell wall integrity, the activity of the LTs must be controlled by the cells to prevent lysis. While some mechanisms of LT regulation, such as PG O-acetylation are known, there are still unanswered questions about the full scope of LT regulation.

1.3.1 Classification and structure of lytic transglycosylases

The LTs are classified into four families based on sequence and consensus motifs (Blackburn and Clarke, 2001). Family 1 is the largest family, with five subfamilies and some sequence similarity to goose-type lysozymes. These LTs are associated with the insertion of conjugation and secretion systems into the PG sacculus. Family 2 LTs are involved in septation during cell division, and Family 3 enzymes are implicated in flagella and pili formation and sporulation. Family 4 LTs are found only in bacteriophages and are essential for phage-induced cell lysis.

Many bacterial species encode several LTs, usually with at least one from each family. E. coli encodes seven LTs: Slt70 (Family 1A), MltC (Family 1B), EmtA (Family 1C), MltD (Family 1D), YfhD or MltF (Family 1E), MtlA (Family 2), and MltB (Family 3) (Vollmer et al., 2008c). On the other hand, P. aeruginosa encodes nine LTs (one from each family),
although it lacks Family 1C and 1E enzymes and it produces four different Family 3 isozymes (Blackburn and Clarke, 2002). Structures have been solved for families 1A, 2, and 3 LTs from *E. coli* (Fig. 1.4) as well as a family 4 LT from bacteriophage λ. All structures, except that of family 2 enzymes, are mainly alpha-helical, but MltA also has a beta-barrel (van Straaten et al., 2005). Additionally, MltA has an aspartate residue (Fig. 1.5) in place of glutamate for the catalytic residue, which is unique to the family 2 LTs. Slt70 is also unique in that it is the only soluble LT and is shaped like a ring (Thunnissen et al., 1994). The LTs all have a deep groove (or hole in the case of Slt70) in which the saccharide residues of the PG strand lie as demonstrated by co-crystal studies (Fig. 1.5; reviewed in Vollmer et al., 2008c). For example, van Straaten et al. (2007) conducted co-crystal studies with MltA from *E. coli* and chitoheaxoase, and van Asselt et al. (2000) conducted a similar study with Slt35 from *E. coli* and PG fragments. This substrate-binding groove accommodates 3-6 saccharide units, depending on the LT. Most LTs are exo-acting and digest PG strands from the end of the strand, releasing muropeptides into the periplasm. However, family IC (and possibly 1E/MltF) and the family 4 LTs of bacteriophage origin show endo-activity and digest PG strands from the middle, releasing larger PG fragments (Leung et al., 2001; Kraft et al., 1998). Some LTs have stringent substrate specificity; for example, Slt70 from *E. coli* only cleaves PG with peptides linked to the glycan strands but MltA does not require the presence of peptides (Ursinus and Höltje, 1994). Additionally, the differences in substrate specificity, enzyme shape, and mode of activity may all contribute to the specific roles of each family of LT within the bacterial cell. It has also been suggested that bacteria may encode several LTs as protection against a lethal mutation in any of the LT genes (Scheurwater et al., 2007). Although an LT knockout strain of *E. coli*, called MHD79, is able to grow with only one functional LT (MltF), this strain is observed to have difficulty with septation and the cells grow in chains (Heidrich et al., 2002).
LTs and lysozymes all have deep grooves near the active site which accommodate the sugar residues of the PG backbone. Represented here in cartoon configuration are members from each LT family and both c- and g-type lysozymes. Family 1a (Slt70 from *E. coli*; PDB 1QTE), Family 2 (MltA from *E. coli*; PDB 2AE0), Family 3 (Slt35 [MltB derivative] from *E. coli*; PDB 1QUS), Family 4 (LT from bacteriophage λ; PDB 1D9U), c-type lysozyme (HEWL from *Gallus gallus*; PDB 2VB1), g-type lysozyme (GEWL from *Salmo salar*; PDB 3MGW).

As seen in Figure 1.4, the lysozymes have similar tertiary structures as the LTs, which is not surprising given that these two types of enzymes cleave the same bond in the same substrate. Notably, Slt70 and goose egg white lysozyme (GEWL) have similar structures. Families 1A, 3, and 4 LTs have a characteristic lysozyme fold, which is also seen in hen egg white lysozyme (HEWL). Key differences between the two types of enzymes include the presence of a second catalytic residue in c-type lysozymes, the endo-activity of most lysozymes, and the use of a water molecule to cleave the glycosidic linkage by hydrolysis. The main result of these differences lies in the reaction mechanism that each enzyme uses to cleave the β-1,4 glycosidic linkage.
1.3.2 Lytic transglycosylase activity

Unlike the lysozymes, which cleave the same β-1,4 glycosidic linkage in the PG backbone, the LTs are not hydrolases but cleave the glycosidic linkage in a two-step process involving a reaction intermediate. The different reaction mechanism results in a 1,6-anhydroMurNAc residue via an intramolecular rearrangement (Fig. 1.6). Bond cleavage by lysozymes leads to a terminal reducing MurNAc residue. The anhydroMurNAc product is an important signalling molecule for several bacterial and host processes, such as the regulation of PG recycling (reviewed in Johnson et al., 2012) or in the activation of host immune responses (reviewed in Scheurwater et al., 2008).
Figure 1.6 LT and lysozyme activity. Cleavage of the β1,4-glycosidic linkage by LTs (top) produces a 1,6-anhydroMurNAc product, while cleavage by lysozymes (bottom) involves a water molecule and does not form the rearranged MurNAc residue. The peptide side chains of the MurNAc residues are denoted by R in this figure.

LTs have a single catalytic residue, usually glutamate (MltA uses aspartate), which is proposed to act initially as an acid to donate its proton to the glycosidic oxygen of the linkage between adjacent GlcNAc and MurNAc residue (reviewed in Scheurwater et al., 2007). Bond cleavage occurs and the resulting oxocarbonium ion transition state on the MurNAc residue is thought to be stabilized by its N-acetyl group, through the formation of an oxazoline ring intermediate (Fig. 1.7; Scheurwater et al., 2007). The deprotonated catalytic residue now acts as a base to abstract a proton from the C-6 hydroxyl of MurNAc, allowing for an intramolecular nucleophilic attack on the C-1 and leading to the formation of the 1,6-anhydroMurNAc and resolution of the oxazolinium intermediate. Support for this reaction mechanism has been provided by inhibition studies using N-acetylglucosamine thiazoline (NAG thiazoline), which structurally resembles the oxazolinium intermediate (Reid et al., 2004).
The glutamic acid residue of LTs acts as the catalytic residue for both steps in the reaction. The two transition states between substrate and intermediate product and final product are indicated with brackets.

1.3.3 Control of lytic transglycosylases

Since the LTs cleave bonds in the PG sacculus, a structure that must remain intact for cell survival, the activity of the LTs must be tightly regulated to prevent degradation of the sacculus leading to cell lysis. There are several known mechanisms of LT control in bacteria. One level of control is conferred by their association with the divisomes that form at the sites of cell elongation and cell division, which ensure that lytic enzymes remain associated with synthetic enzymes (Höltje, 1998). Membrane-bound LTs are often associated with the inner leaflet of the outer membrane in Gram-negative bacteria (E. coli
LTs – reviewed in Hölzje (1998); *P. aeruginosa* MltB – Blackburn and Clarke, 2002) and PBPs are bound to the outer leaflet of the inner membrane. Many studies have indicated specific interactions between certain LTs and PBPs, lending evidence to the divisome hypothesis (reviewed in Pfeffer *et al*., 2012). When the divisomes form, it is thought that the two membranes are brought together on either side of the PG sacculus. A second level of control of LT (and lysozyme) activity involves the chemical modification to PG which physically prevents cleavage of the β-1→4 glycosidic bond (Scheurwater *et al*., 2007). The most common form of this modification concerns O-acetylation.

The O-acetylation of PG involves the addition of an O-linked acetate group to the C-6 of muramic acid residues (Blackburn and Clarke, 2002). This precludes the formation of the 1,6-anhydro product by the lytic transglycosylases (Fig. 1.8) (Blackburn and Clarke, 2002). The O-linked acetate inhibits exogenous lysozyme activity, which also requires a free C-6 hydroxyl group for binding in the active site cleft of the enzyme (Brumfitt *et al*., 1958). The presence of O-acetylated PG has been detected in many Gram-negative and Gram-positive species, including many pathogens, and the extent of this modification is generally between 10 and 70% of the cellular PG (reviewed by Vollmer, 2008d). Due to the lack of O-acetylated PG precursors in the cytoplasm, it is inferred that O-acetylation occurs after the incorporation of new muropeptides into the PG sacculus (reviewed by Clarke and Dupont, 1992). Hence, O-acetylation is considered a maturation event after incorporation of newly synthesized PG, following an increase in transpeptidation of the incorporated strands.

Figure 1.8 O-Acetylated PG. The addition of an acetate group to the C6 hydroxyl of MurNAc residues prevents both LTs and lysozymes from cleaving the β1,4-glycosidic bond.

The addition of O-linked acetate molecules to the PG sacculus inhibits most known muramidases and all LTs (Vollmer, 2008d). This inhibition has implications in bacterial
infections, as it is more difficult for the host immune system to both lyse bacterial cells with O-acetylated PG and clear these PG fragments from the body. The PG fragments that persist in the human body lead to some of the symptoms of bacterial infection, including somnogenicity, pyrogenicity, arthritogenicity, and stimulation of the NOD1 and NOD2 receptors (Clarke and Dupont, 1992). In many organisms, there is also a correlation between the degree of lysozyme resistance and the percentage of MurNAc residues which are O-acetylated (Dupont and Clarke, 1991). In a study of *Staphylococcus* spp., all pathogenic species were resistant to lysozyme and those tested contained O-linked acetate in their sacculi, whereas all non-pathogenic species were susceptible to lysozyme and none tested positively for O-acetylated PG (Bera *et al.*, 2006).

Although LTs are inhibited by the O-acetylation of PG, there is no conclusive evidence that supports this modification as a tightly regulated mechanism for controlling LT activity. Nonetheless, it is quite likely that the process of O-acetylation and de-O-acetylation is a mechanism for spatial and temporal regulation of LT activity in bacteria that O-acetylate their PG (Weadge and Clarke, 2006). However, not all bacteria contain O-linked acetate in their PG sacculi, including important pathogens such as *E. coli* and *P. aeruginosa*, thus necessitating the need for an alternate means of regulating LT activity. Indeed, potential mechanisms have been identified very recently in these species.

### 1.4 Proteinaceous Inhibitors of Lytic Transglycosylases

#### 1.4.1 Inhibitor of vertebrate lysozyme

Ivy (inhibitor of vertebrate lysozyme) was first identified as the product of the *ykfE* gene in *E. coli* and as an inhibitor of C-type lysozyme (Monchois *et al.*, 2001). This observation was discovered by coincidence, when it was noticed that the gene product of *ykfE* was co-purifying with HEWL that had been added to the cell culture to aid in lysis. The identities of the two proteins were confirmed by mass spectrometry and co-crystallisation experiments were conducted. The inhibition of C-type lysozymes (both HEWL and human lysozyme) by Ivyc (which is Ivy in *E. coli*) was confirmed *in vivo* and it was hypothesized that the inhibitor binds to lysozyme by a slow tight competitive inhibition model with no conformational change (Monchois *et al.*, 2001).
Early fluorescence and gel filtration studies suggested that Ivy forms homodimers (Monchois et al., 2001) and this was confirmed subsequently by X-ray crystallography. Indeed, the structures of Ivy, both in dimer form and in complex with two HEWL molecules, have been determined (Abergel et al., 2007). Ivy monomers contain a novel protein fold, comprised of a β-sheet of five antiparallel β-strands with two helices on the outside of the sheet and one amphipathic helix on the other side (Fig. 1.9). The residues involved in dimerization are conserved among Ivy homologs in some bacteria but not in others. Species encoding Ivy proteins without these residues are expected to produce Ivy in monomeric form, such as Ivy from P. aeruginosa (Abergel et al., 2007).

The crystal structure of the inhibition complex also led to findings about the mechanism of lysozyme inhibition. An inflexible six-residue loop (CKPHDC) from Ivy is positioned in the active site of HEWL and directly prevents activity (Abergel et al., 2007). A histidine residue in the loop (H60) forms two hydrogen bonds with the catalytic residues in the active site of HEWL (D52 and E35). The loop is stabilized by a disulfide bridge between the flanking cysteine residues (C57, C62), as well as an ionic bond between the lysine (K58) and aspartate (D61) residues (Fig. 1.9). Because the loop sequence is specific to the active site of lysozyme, it is a conserved sequence amongst all Ivy proteins identified in this study (Abergel et al., 2007).
Figure 1.9 Interaction between Ivyc and HEWL. Ivyc from *E. coli* forms dimers (a) and each monomer directly inhibits one HEWL molecule. The mode of inhibition involves a rigid loop on Ivyc (red box, b) stabilised by both a disulfide bond between the C57 and C62 residues and a hydrogen bond between the K58 and D61 residues. (c) The H60 residue of the Ivyc loop forms hydrogen bonds with the active site residues E35 and D52 of HEWL (labelled in red). Loop and active site residues are shown in stick configuration and double bonds are shown by dashed lines. PDB 1GPQ.

Using this consensus sequence in a genome search, 35 putative *Ivy* genes were identified in various *Proteobacteria*, all in the alpha, beta, and gamma subdivisions (Abergel et al., 2007). All genes had predicted signal sequences directing them to the periplasm of the bacteria. As in Fig. 1.10, homologs were found in a wide array of species, including all *Enterbacteriales*, except *Salmonella*, all known *Pseudomonadaceae*, all *Burkholderiaceae*, and one *Neisseriaceae* species (Abergel et al., 2007). Most of the genes identified in this study are Ivy orthologs and maintain the conserved inhibition loop sequence. Additionally, six Ivy paralogs were identified (in species of *Pseudomonas*) and these contain a slightly different loop sequence (CExxDxC). *P. aeruginosa* is the only known species that encodes both a homolog (Ivyp1), and a paralog (Ivyp2) of Ivy. Ivy from *E. coli* is referred to as Ivyc to distinguish from Ivy proteins from other species. Both Ivyp1 and Ivyp2 were purified and inhibition of C-type lysozyme was confirmed for Ivyp1, but quite surprisingly, Ivyp2 showed no inhibition (Abergel et al., 2007). This observation is most likely a consequence of
differences in the residues in the inhibition loop of the Ivy paralog. Additionally, unlike Ivyc, Ivyp1 is not dimeric, predicted by a lack of the conserved sequence involved in dimerization (Abergel et al., 2007).

Figure 1.10 Phylogenetic tree of ivy homologs. Gram-negative bacteria from the alpha proteobacteria (dark blue), beta proteobacteria (Burkholderiaceae - green, Neisseriaceae – orange), and gamma proteobacteria (Enterobacteriaceae - purple, Pseudomonadaceae – light blue) are represented. Ivyp1 are homologs which have the conserved CKPHDC loop, while the Ivy2 homologs are less conserved and appear only in species of Pseudomonas. The Ivy homologs of interest (from E. coli and P. aeruginosa) are shown in black font. Adapted from Clarke et al., 2010.
1.4.2 Other lysozyme inhibitors

In addition to the Ivy studies, a second research group identified a different family of lysozyme inhibitors, first in *Salmonella enteritidis*. This protein was also isolated by coincidence when it was noticed that cell extracts from *S. enteritidis* inhibited HEWL even though no Ivy homolog is present in the genome (Callewaert *et al.*, 2008). This novel protein was named PliC (periplasmic lysozyme inhibitor of C-type lysozyme). Homologs were identified in all *Salmonella* species for which sequences are available, based on the conserved COG3895 domain. Additional species containing sequences with this domain include many of the *Proteobacteria*, except those in the epsilon subdivision, as well as *Acidobacteria*, *Cyanobacteria*, and *Bacteroides*. Surprisingly, and in contrast to Ivy, two MliC (membrane-bound lysozyme inhibitor of C-type lysozyme) homologs were identified in Gram-positive bacteria (*Providencia rettgeri* and *Proteus penneri*). All putative homologs are predicted to be periplasmic (PliC) or lipid-anchored (MliC) to the periplasmic side of the outer membrane. Such membrane-bound inhibitors are present in both *E. coli* and *P. aeruginosa*. The inhibitory effect of these proteins was also confirmed in this study (Callewaert *et al.*, 2008).

The structure of MliC from *E. coli* was solved prior to the functional characterisation of the protein and it does not possess the novel fold present in Ivy proteins (Revington *et al.*, 2006). The structure of PliC from *Salmonella typhimurium* has also been solved (Leysen *et al.*, 2011) and reveals a similar structure to MliC (Fig. 1.11) despite only sharing 24-39% sequence homology (Callewaert *et al.*, 2012). Both the MliC and PliC structures are antiparallel beta-barrels composed of 8 strands. The mechanism of inhibition by MliC from *P. aeruginosa* was described by Yum *et al.* (2009) and, similar to Ivy, it involves two conserved loops that directly inhibit the active site of lysozyme (Yum *et al.*, 2009). Co-crystal studies with HEWL have confirmed the presence of the two loops in the active site and also revealed a shallow pocket on MliC which interacts with a loop from HEWL, forming the double key-lock mechanism.
Figure 1.11 MliC and PliC structures. MliC and PliC are shown here in cartoon configuration. Their structures are remarkably identical despite low sequence identity. The side chains of the residues involved in inhibition of lysozyme are shown in stick configuration. MliC from *P. aeruginosa* (PDB 3F6Z) and PliC from *S. typhimurium* (PDB 3OE3) are shown.

In addition to these 2 families of lysozyme inhibitors, proteins inhibiting both g-type and i-type lysozyme have also been identified in Gram-negative bacteria (Vanderkelen et al., 2010; Van Herreweghe et al., 2010). As such, inhibitors to all major types of lysozyme have now been identified. As with Ivy homologs, homologs of MliC/PliC, PliG, and PliI inhibitors are restricted to Gram-negative bacteria (Fig. 1.12). Both proteins have also been crystallised (PliI from *Aeromonas hydrophila* by Leysen et al., 2011; PliG from *Aeromonas hydrophila*, *E. coli*, and *Salmonella enterica* serotype Typhimurium by Leysen et al., 2012). The tertiary structure of PliI is a beta-sandwich composed of two beta-sheets of 4 strands each with an alpha-helix at the C-terminus, while PliG is structured into a beta-sheet with a curve in it (similar to PliC in Fig. 1.11). While PliG and PliI are considered to be in distinct families of proteins, they share some common characteristics with the MliC/PliC family of inhibitors. For example, each of these three families has the same conserved motif SGxxY on a loop that is exposed on the surface of the protein (MliC/PliC has an additional conserved loop as mentioned above). Similar to Ivy and MliC/PliC proteins, the conserved loop in the PliG and PliI families of inhibitors is thought to protrude into the active site of lysozyme. Although co-crystal studies between inhibitors from these two families and their respective lysozymes have not been conducted, the conserved loop of PliI from *A. hydrophila* has been implicated in inhibition from alanine replacement studies (Leysen et al., 2011).
Figure 1.12 Phylogenetic tree of mliC, plII, and plIG homologs. The homologs in black boxes were used to identify other putative homologs. The red boxes denote the only two Gram-positive bacteria known to encode homologs of these inhibitors. The asterisks denote homologs that have been shown to inhibit lysozymes, and the ‡ symbol denotes a homolog that has no known inhibitory activity to lysozyme. Adapted from Pfeffer et al., 2010.

Finally, a fifth group of lysozyme inhibitors has been recently identified in P. aeruginosa (Russell et al., 2011). This protein has named Tsi3 and has been shown to inhibit Tse3, a lysozyme that is part of the type VI secretion system in P. aeruginosa. Only species of Pseudomonas encode Tsi3 homologs. These enzymes are unrelated to all other known families of lysozyme inhibitors.
While these five families of lysozyme inhibitors are mostly restricted to Gram-negative bacteria, their distribution amongst these species differs between families. Most bacteria encode an inhibitor specific to one type of lysozyme, but others encode two types of inhibitors (Callewaert \textit{et al.}, 2012). \textit{Aeromonas} is the only genus known to encode inhibitors for each type of lysozyme (c-type, g-type, and i-type). \textit{E. coli} encodes Ivyc, MliC, and PliG thus being able to inhibit both c- and g-type lysozymes, while \textit{P. aeruginosa} is only capable of inhibiting c-type lysozymes, although this species encodes multiple c-type lysozyme inhibitors including Ivyp1, Ivyp2, and MliC, as well as Tsi3. As such, the lysozyme inhibitors seem to be redundant, as is seen with the LTs themselves as well as other enzymes involved in PG recycling such as the PBPs and AmpG in \textit{P. aeruginosa}. There exists also the potential for other as of yet undiscovered LT inhibitors, supported by the evidence that knockouts of Ivyp1, Ivyp2, MliC, and Tsi3 in \textit{P. aeruginosa} are viable (unpublished data). If these proteinaceous inhibitors are critical for LT regulation in some Gram-negative bacteria, a knockout of all LT inhibitors should not survive.

1.4.3 Inhibitors of lytic transglycosylases or lysozymes?

Until recently, it had been postulated that the function of the Ivy, MliC/PliC, PliG, and PliI proteins is to protect invading bacteria from host lysozymes, especially in cases where the outer membrane may be permeabilized by other host factors (Abergel \textit{et al.}, 2007). Indeed, these proteins have been shown to increase lysozyme tolerance when bacteria with permeabilized outer membranes are grown in media supplemented with lysozyme (Callewaert \textit{et al.}, 2008, Deckers \textit{et al.}, 2004). Various other roles for the lysozyme inhibitors have been suggested, including providing protection from lysozymes produced by bacteriophage or other microorganisms such as fungi (Monchois \textit{et al.}, 2001), protecting Gram-positive bacteria in populations of bacteria (for example, in biofilms or in the intestine) (Abergel \textit{et al.}, 2007), or exacerbating the symptoms of infection by preventing the cleavage of PG by lysozymes and enabling large PG fragments to persist in the host (Callewaert \textit{et al.}, 2008).

Despite these suggestions, it is paradoxical that Ivy, PliC, and MliC are only present in Gram-negative bacteria because the PG of these organisms is protected by the outer membrane and is thus not normally accessible to lysozymes (Monchois \textit{et al.}, 2001). The PG
of Gram-positive bacteria, on the other hand, is exposed to the environment and is thus readily available to exogenous lysozymes. Despite this, only two lysozyme inhibitors have been discovered in Gram-positive bacteria. It has been suggested that perhaps the true function of these enzymes is to control the activity of LTs, as these enzymes have identical substrate specificity to the lysozymes and a similar active site (Callewaert et al., 2005; Callewaert et al., 2008; Clarke et al., 2010). Callewaert et al. (2005) conducted a study to determine the specificity of Ivyc for different lysozymes and discovered that T4 phage lysozyme (T4L) is partially inhibited (Callewaert et al., 2005). These findings led to the suggestion that Ivyc can also inhibit the LTs, as they have a similar structure to T4 lysozyme (Callewaert et al., 2005). In 2008, Callewaert et al. suggested that MliC might have a role in PG turnover due to the close proximity of the mliC gene to an anhydro N-acetylated muramic acid kinase (anmK) gene on the chromosome (Callewaert et al., 2008). AnmK is involved in PG turnover and MliC might have a role in this process by inhibiting the LTs to prevent cell lysis during remodelling of the sacculus (Callewaert et al., 2008). In bacteria that do not contain O-acetylated PG, it has been presumed until now that the activity of LTs was controlled mainly by the association of these enzymes in the cell growth and division enzyme complexes in the periplasm. However, this may not be sufficient control for all LTs, as some are soluble and not membrane-bound (for example, Slt70 from E. coli and the three SltB enzymes in P. aeruginosa). Clarke et al. (2010) observed a strict correlation between the absence of PG O-acetylation in species that produce a proteinaceous “lysozyme” inhibitor, including Ivy. Thus, they proposed that in Gram-negative species that do not produce O-acetylated PG, Ivy (and possibly other inhibitors, such as MliC) are responsible for the control of LT activity during cell wall turnover and division (Clarke et al., 2010).

There is additional evidence for Ivy as an LT inhibitor based on how gene expression of Ivyc in E. coli is controlled (Clarke et al., 2010). The Ivyc gene is under the regulation of the Rcs (regulator of capsule synthesis) phospho-relay system which is activated by β-lactam antibiotics or other PG stress factors (Laubacher and Ades, 2008). When activated, the regulon allows the cell to survive in the presence of β-lactam antibiotics. This would be due to an increase in Ivy production, as cell death by β-lactams is not due to the inhibition of PG synthesis, but rather by the ongoing activity of LTs in the enzyme complexes. Thus, an increase in Ivy would enable the cell to control unbalanced lytic activity (Clarke et al., 2010).
However, it is highly unlikely that these proteinaceous inhibitors are the only control mechanism for LTs in Gram-negative bacteria that lack O-acetylated PG. A double knock-out of Ivyc and MliC in E. coli was able to survive, demonstrating that LT activity must not be completely uncontrolled despite the lack of the known inhibiting proteins (Callewaert et al., 2008). This evidence suggests that either the presence of as-yet undiscovered classes of LT inhibitors in E. coli or an alternative LT regulation mechanism.

In 2010, Clarke et al. tested the hypothesis that Ivy was an LT inhibitor, due to mounting evidence that did not support these proteins as lysozyme inhibitors. Other evidence supporting these proteins as LT inhibitors includes the fact that no known activity could be shown for either Ivyp2 from P. aeruginosa or PliG from Bordatella avium through lysozyme inhibition studies. Clarke et al. (2010) confirmed that Ivyp1 and Ivyp2 from P. aeruginosa can inhibit a soluble derivative of MltB from P. aeruginosa. This experiment represented the first experimental evidence that Ivy proteins may act as inhibitors of LTs, especially because of the inhibitory activity of Ivyp2 which previously had no known activity when tested against HEWL.

To further support the hypothesis that Ivy presents a control mechanism for LTs, a correlation was made between its production only in bacteria that do not O-acetylate their PG. Thus, the genome sequences of all bacterial species known to encode Ivy orthologs or paralogs were analyzed for the presence of poa and oap gene clusters responsible for encoding the proteins involved in PG O-acetylation, and the PG from a selection of these species was analyzed for the presence of O-acetylated PG (Clarke et al., 2010). As expected, none of the bacteria known to produce Ivy homologs were found to carry homologs of OatA (O-acetyl transferase A from S. aureus), Apel3 (O-acetylPG esterase 3 from B. anthracis), PatB (PG O-acetyl transferase B), or Apel (from N. gonorrhoeae) (Clarke et al., 2010). To further test this hypothesis, the PG of select bacteria from the list of those that produce any form of Ivy were tested for PG O-acetylation. None of the PG sacculi isolated from these bacteria contained any more than 1% O-linked acetate, confirming that bacteria that produce Ivy proteins do not have O-acetylated PG (Clarke et al., 2010). Conversely, no organisms known to O-acetylate their PG contain either homologs or paralogs to any of the proteinaceous inhibitors identified thus far. The only exceptions are P. penneri and P.
rettgeri, which are known to both O-acetylate their PG and encode MliC homologs (and are the only Gram-positive bacteria known to encode a lysozyme inhibitor).

1.5 Hypothesis

Both Ivyp1 and Ivyp2 have been shown to inhibit a soluble derivative of MltB from P. aeruginosa and it has been postulated that they act to control these LTs in vivo. However, it remains to be seen whether or not these proteinaceous inhibitors can act upon other LTs, specifically, LTs from other families. I hypothesise that these proteins function as inhibitors of all LTs and serve to provide control of these potentially autolytic enzymes within the periplasm. To test this hypothesis, the ability of Ivyp1 and Ivyp2 to inhibit another LT from P. aeruginosa, MltA, was investigated. MltA is a family 2 LT and it is hypothesised that Ivyp1 and Ivyp2 will exhibit a similar potency (less than 50% residual activity after incubation with a 4-fold molar excess of inhibitor) in inhibition toward MltA as has been seen with MltB, a family 3 LT. Successful inhibition of MltA by Ivyp1 and Ivyp2 would be significant because MltA has important differences compared to MltB, such as its beta-barrel structure, unique active site residue, aspartate, and specialised function in cell septation. Furthermore, I hypothesise that MltA will show an exo-acting pattern of activity, similar to MltB, and as seen with MltA from E. coli (Romeis et al., 1993) as this project is also the first to measure the lytic activity of MltA from P. aeruginosa by turbidometry. MltA activity and inhibition studies were conducted using homologous preparations of recombinant MltA from P. aeruginosa and recombinant Ivyp1 and Ivyp2 also from P. aeruginosa.
CHAPTER 2 – MATERIALS AND METHODS

2.1 Chemicals and Reagents

All chemicals were purchased from Sigma-Aldrich (Oakville, ON), Fisher Scientific (Ottawa, ON), FroggaBio (Toronto, ON), or Bio Basic (Markham, ON), unless otherwise specified. All media was purchased from Difco (Mississauga, ON) or Fisher Scientific. All enzymes were purchased from New England BioLabs (NEB, Ipswich, MA) or Kapa Biosystems (Woburn, MA). Molecular biology kits were purchased from Roche Diagnostics (Laval, QC) or Qiagen (Mississauga, ON). All antibiotics were purchased from Sigma-Aldrich. Mouse anti-His antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA), and goat anti-mouse antibody with an alkaline phosphatase conjugate was purchased from Bio-Rad Laboratories, Inc. (Mississauga, ON).

2.2 Strains and Plasmids

_E. coli_ DH5α was used for plasmid isolation. _E. coli_ BL21 (DE3) and _E. coli_ BL21 (DE3) pLysS (Table 2.1) were used for gene expression. _E. coli_ MHD79 was obtained from J.V. Höltje and was also used for gene expression. All expression plasmids were derivatives of pET-21b(+), pET-28a(+), or pET-30b(+) plasmids from Novagen (Darmstadt, Germany) (Table 2.2). All cultures were grown at 37 °C with shaking. Luria-Bertani broth (LB, Appendix A) was used in cultures grown for plasmid passaging and purification, and cultures for protein production were grown in Super Broth (SB, Appendix A). For pACNB54-1 expression only, Terrific broth (Appendix A) was used. Solid media were prepared by adding 15 g/L agar to LB. Plates were incubated upside-down at 37 °C overnight. Media were supplemented with appropriate antibiotics when necessary: kanamycin (Kan; 50 μg/mL final concentration) was used for growth of _E. coli_ carrying either pET-28a(+) or pET-30b(+) and their derivative plasmids; ampicillin (Amp; 100 μg/mL final concentration) was supplemented for growth of _E. coli_ harbouring pET-21a(+) and its derivatives; and chloramphenicol (Cam; 34 μg/mL final concentration) was used for growth of _E. coli_ BL21 (DE3) pLysS. For some growth analyses of _E. coli_ MHD79 (DE3), tetracycline (Tet; 12.5 μg/mL final concentration), Kan (50 μg/mL), and Cam (20 μg/mL) were supplemented in the media. All strain and plasmid stocks were stored at -80 °C in 25% v/v glycerol.
Table 2.1 List of bacterial strains and genotypes used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> DH5</td>
<td>F–Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(rK-, mK+) phoA supE44 thi-1 gyrA96 relA1 λ-</td>
<td>Novagen</td>
</tr>
<tr>
<td><em>E. coli</em> BL21 (DE3)</td>
<td>F–ompT hsdS B(rB–, mB–) gal dcm (DE3)</td>
<td>Novagen</td>
</tr>
<tr>
<td><em>E. coli</em> BL21 (DE3) pLysS</td>
<td>F–ompT hsdS B(rB–, mB–) gal dcm (DE3) pLysS (CamR)</td>
<td>Novagen</td>
</tr>
<tr>
<td><em>E. coli</em> MC1061*</td>
<td>F–Δ(ara-leu)7696 [araD139]B/r Δ(codB-lacI)3 galK16 galE15 ρ- e14- mcrA0 relA1 rpsL150(strR) spoT1 mcrB1 hsdR2 (r-m+)</td>
<td>Casabdan and Cohen (1980)</td>
</tr>
<tr>
<td><em>E. coli</em> MHD79 (DE3)</td>
<td>F–Δ(ara-leu)7696 [araD139]B/r Δ(codB-lacI)3 galK16 galE15 ρ- e14- mcrA0 relA1 rpsL150(strR) spoT1 mcrB1 hsdR2 (r-m+) Δ(sltY, mltA, mltB, mltC, mltD, emtA) (CamR, KanR, TetR) (DE3)</td>
<td>I. Selander</td>
</tr>
</tbody>
</table>

* Not used in this study; parent strain for *E. coli* MHD79.
Table 2.2 List of plasmids and derivatives used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristics</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET-21b(+)</td>
<td>IPTG-inducible expression vector, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Novagen</td>
</tr>
<tr>
<td>pACCC1-FN</td>
<td>pET-28a(+) derivative with recombinant <em>P. aeruginosa ivyp1</em> encoding full-length Ivyp1, C-terminal His-6-tag; Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>F. Ng</td>
</tr>
<tr>
<td>pACCC2</td>
<td>pET-28a(+) derivative with recombinant <em>P. aeruginosa ivyp1</em> encoding Ivyp1&lt;sub&gt;Δ2-23&lt;/sub&gt;, C-terminal His-6-tag; Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>C. Clarke (2010)</td>
</tr>
<tr>
<td>pACCC3-FN</td>
<td>pET-28a(+) derivative with recombinant <em>P. aeruginosa ivyp2</em> encoding full-length Ivyp2, C-terminal His-6-tag; Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>F. Ng</td>
</tr>
<tr>
<td>pACCC4</td>
<td>pET-28a(+) derivative with recombinant <em>P. aeruginosa ivyp2</em> encoding Ivyp2&lt;sub&gt;Δ2-23&lt;/sub&gt;, C-terminal His-6-tag; Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>C. Clarke (2010)</td>
</tr>
<tr>
<td>pACCC2-IS</td>
<td>pET-28a(+) derivative with recombinant <em>P. aeruginosa ivyp1</em> encoding Ivyp1&lt;sub&gt;Δ2-23&lt;/sub&gt;, C-terminal His-6-tag, t147c replacement; Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>I. Selander</td>
</tr>
<tr>
<td>pACIS1</td>
<td>pET-21a(+) derivative with recombinant <em>P. aeruginosa ivyp1</em> encoding full-length Ivyp1, C-terminal His-6-tag; Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>I. Selander</td>
</tr>
<tr>
<td>pACIS2</td>
<td>pET-21a(+) derivative with recombinant <em>P. aeruginosa ivyp2</em> encoding full-length Ivyp2, C-terminal His-6-tag; Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>I. Selander</td>
</tr>
<tr>
<td>pACIS3</td>
<td>pET-21a(+) derivative with recombinant <em>P. aeruginosa ivyp1</em> encoding Ivyp1&lt;sub&gt;Δ2-23&lt;/sub&gt;, C-terminal His-6-tag; Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>I. Selander</td>
</tr>
<tr>
<td>pACIS4</td>
<td>pET-21a(+) derivative with recombinant <em>P. aeruginosa ivyp2</em> encoding full-length Ivyp2&lt;sub&gt;Δ2-23&lt;/sub&gt;, C-terminal His-6-tag; Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>I. Selander</td>
</tr>
<tr>
<td>pNBAC54-1</td>
<td>pET-28a(+) derivative with recombinant <em>P. aeruginosa mltB</em> encoding sMltB&lt;sub&gt;Δ2-17&lt;/sub&gt;, C-terminal His-6-tag; Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>N. Blackburn (2002)</td>
</tr>
<tr>
<td>pACCV4</td>
<td>pET-28a(+) derivative with recombinant <em>P. aeruginosa mltA</em> encoding sMltA&lt;sub&gt;Δ2-25&lt;/sub&gt;, C-terminal His-6-tag; Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>C. Vandenende</td>
</tr>
</tbody>
</table>

2.3 DNA Techniques

2.3.1 General DNA techniques

Preparation of purified plasmid DNA from 5 mL cell cultures was routinely carried out using plasmid isolation kits from Qiagen (QIAprep Spin Miniprep kit) or Roche Diagnostics (High Pure Plasmid Isolation kit). The only change made to the suggested protocols when using kits from either company was eluting in a lower volume of Elution Buffer than suggested (30-50 μL) to increase DNA concentration. Isolated plasmids were stored at -20 °C and nucleotide sequencing was performed by Laboratory Services Division, University of Guelph
(Guelph, ON). The DNA concentrations were measured using a Beckman DU® Series 350 UV/Vis spectrophotometer (Beckman Coulter Inc., Mississauga, ON). Digestions of 1 µg DNA were performed for 2 h at 37 °C with 1 unit of required restriction endonuclease(s) and 2 µL of appropriate 10× NEB buffer, and made to a final volume of 20 µL with sterile deionised water. DNA was separated by agarose gel electrophoresis using 0.8% w/v agarose gels (Appendix A) in TAE buffer (Appendix A) at 90 V for 1 h. DNA samples were prepared for electrophoresis by mixing 5 µL of DNA (stock or diluted) with 1 µL of 6× DNA loading dye (Thermo Fisher Scientific). Five µL of both sample DNA and DNA marker were routinely loaded on the gel. Marker used was the 1kb DNA ladder (NEB). DNA was visualized by staining with Sybr Safe DNA gel stain (Invitrogen Life Technologies, Burlington, ON) and exposure to UV light.

2.3.2 Competent cell preparation

_E. coli_ DH5α, BL21 (DE3), BL21 (DE3) pLysS, and MHD79 (DE3) cells were prepared by a variation on the method of Cohen et al. (1972). Overnight cultures were sub-cultured 1/10 in 50 mL LB broth and grown at 37 °C with agitation for 1.5-2 h. Cultures were chilled on ice, cells were collected by centrifugation (4230 × g, 20 min, 4 °C), and resuspended in 50 mL cold solution α (Appendix A). Cells were again collected by centrifugation and resuspended in 10 mL solution β (Appendix A). Cells were prepared into 200 µL aliquots and left on ice for 2-3 h before storage at -80 °C.

2.3.3 Transformation of _E. coli_

Thawed chemically-competent _E. coli_ cells were transformed with 1 µL plasmid DNA (approx. 200 ng) by incubation on ice for 20-30 min, followed by a 3-5 min heat shock at 37 °C and then another 5 min on ice. LB broth (500 µL) was added to cells prior to incubation with agitation at 37 °C for 40-60 min. Cells were collected by centrifugation (21 100 × g, 1 min, 4 °C), resuspended in the retained supernatant after decanting, and plated onto solid LB agar with appropriate antibiotics. Plates were incubated at 37 °C overnight and 5 mL tubes of LB broth supplemented with appropriate antibiotics were inoculated from isolated colonies the following day.
2.3.4 Lysogenisation of *E. coli* MHD79 with λDE3 lysogen

The λDE3 lysogenisation kit was purchased from Novagen. Lysogenisation of *E. coli* MHD79 and verification of lysogenisation was performed as per the manufacturer’s specifications.

2.3.5 Site-directed mutagenesis (SDM) of pACCC2

Purified pACCC2 (200 ng, 20 ng, and 2 ng) was incubated with forward and reverse primers (0.2 ng each), 10 μL 5× GC Buffer with 2 mM Mg²⁺, high-quality deoxynucleoside triphosphates (dNTP) mix (500 μM final concentration), and 1 μL (1 unit) of Kapa HiFi HotStart DNA polymerase, in a final volume of 50 μL. PCR buffer, dNTP mix, and DNA polymerase were from a Kapa HiFi HotStart PCR kit (Kapa Biosystems). Polymerase chain reaction (PCR) was performed as described in Table 2.3. Amplified DNA was purified from PCR products using the High Pure PCR Product Purification kit from Roche Diagnostics to the manufacturer’s specifications, except that DNA was eluted in 50 μL of elution buffer instead of 100 μL. The template DNA in the PCR reaction was digested with 20 units of *DpnI* (NEB) with 5 μL 10× buffer #7 (NEB) in a final volume of 50 μL. The reaction was incubated for 1 h at 37 °C, followed by a heat shock for 20 min at 80 °C to inactivate the restriction endonuclease. The PCR product (both undigested and digested by *DpnI*) was visualised by agarose gel electrophoresis. The PCR product was then transformed into *E. coli* DH5α and plated on solid LB agar with appropriate antibiotics. Amplified plasmid, pACCC2-IS, was verified by Sanger sequencing (Laboratory Services Division).

Forward primer sequence: 5’-ccgtccaccagcctgagcctggagggccagccctacgtc-3’

Reverse primer sequence: 5’-ccaggacgtagggctggccctccagctggtggacg-3’

The underlined codon was changed from agt to agc by site-directed mutagenesis.
Table 2.3 PCR cycle for site-directed mutagenesis of pACCC2

<table>
<thead>
<tr>
<th>Cycle Step*</th>
<th>Temperature (°C)</th>
<th>Time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>30</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>30</td>
</tr>
<tr>
<td>Annealing</td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td>Elongation</td>
<td>68</td>
<td>200</td>
</tr>
<tr>
<td>Final Elongation</td>
<td>68</td>
<td>220</td>
</tr>
<tr>
<td>Hold</td>
<td>10</td>
<td>∞</td>
</tr>
</tbody>
</table>

* Steps 2-4 repeated 16 times.

2.3.6 Sub-cloning of recombinant *ivypl*, *ivypl*, truncated *ivypl* and truncated *ivypl* from pACCC plasmids into pET-21b(+)

Parent plasmids (pACCC1-FN, pACCC2-IS, pACCC3-FN, and pACCC4) and pET-21b(+) were digested with *HindIII* and *XbaI* (NEB). Ten μL aliquots of isolated plasmids (approx. 2 μg) were digested with 1 unit of each *HindIII* and *XbaI*. BSA solution (NEB) was added to the digestion reaction (0.2 μL of 100× BSA) and the final volume was brought to 20 μL with water. Digestion products were separated by agarose gel electrophoresis. Gel extraction of full-length and truncated *ivypl* and *ivypl* inserts and linearised pET-21b(+) was performed using the Agarose Gel DNA Extraction kit (Roche Diagnostics) according to the manufacturer’s specifications. Ligations were performed using 3 μL insert DNA, 1 μL linearised plasmid DNA, 1 unit T4 DNA ligase (NEB), and 2 μL T4 ligase buffer in a final volume of 20 μL. Ligation reactions were incubated at 4 °C for 72 h, transformed into *E. coli* DH5α, and plated on solid LB agar with appropriate antibiotics. Positive transformants were screened by e-lyse (described below) and analytical digestions. New constructs (pACIS1, pACIS2, pACIS3, and pACIS4; Table 2.4) were verified by Sanger sequencing (Laboratory Services Division).
Table 2.4 Old and new *ivy* constructs

<table>
<thead>
<tr>
<th>pET-28a(+) plasmid name</th>
<th>Insert</th>
<th>pET-21b(+) plasmid name</th>
</tr>
</thead>
<tbody>
<tr>
<td>pACCC1-FN</td>
<td><em>ivyp1</em></td>
<td>pACIS1</td>
</tr>
<tr>
<td>pACCC2-IS</td>
<td><em>ivyp1Δ2,23</em></td>
<td>pACIS3</td>
</tr>
<tr>
<td>pACCC3-FN</td>
<td><em>ivyp2</em></td>
<td>pACIS2</td>
</tr>
<tr>
<td>pACCC4</td>
<td><em>ivyp2Δ2,23</em></td>
<td>pACIS4</td>
</tr>
</tbody>
</table>

2.3.7 E-lyse

The E-lyse protocol was adapted from the method of Eckhardt (1978) and was used for the screening of positive transformants to identify those containing the inserted gene of interest. Patch plates were made by inoculating colonies from the original transformation plate and grown overnight at 37°C. Colonies were scraped off the patch plate using sterile toothpicks and resuspended in 10 μL TE buffer (Appendix A) and 10 μL SRL lysis solution (Appendix A). Ten μL of this cell suspension were loaded into a 0.8% w/v agarose gel in TB buffer (Appendix A) containing 0.02% w/v sodium dodecyl sulfate (SDS) and the electric current was not applied until the wells cleared. Gels were run at 20 V for 20 min, then voltage was increased to 90 V and electrophoresis was continued for 35 min.

2.4 Growth Curves

The *E. coli* MHD79 (DE3) isolates harbouring pACIS1, pACIS2, and pET-21b(+); and *E. coli* BL21 (DE3) harbouring pACCC1-FN, pACCC3-FN, and pET-28a[+] were inoculated from colonies on solid media (transformed the day before) and grown overnight in LB broth supplemented with the appropriate antibiotics (Amp for *E. coli* MHD79 (DE3) and Kan for *E. coli* BL21 [DE3]). Cultures were diluted 1/100 the following day in 250 mL of LB broth with the same antibiotics. The OD<sub>600</sub> of the cultures was measured every 20 min for 8 h with 0.5 mL samples, until the OD<sub>600</sub> neared 1.0 when 0.25 mL samples were measured. Samples were topped up to 1.0 mL with sterile LB, and the OD<sub>600</sub> was adjusted appropriately.
2.5 Protein Production

2.5.1 Over-expression of *ivyp1*, *ivyp2*, truncated *ivyp1*, truncated *ivyp2*, truncated *mltB*, and truncated *mltA*

Prepared competent cells were transformed with the appropriate plasmid and plated on LB agar supplemented with appropriate antibiotics. Starter cultures (15 mL LB broth with appropriate antibiotics) were inoculated from isolated colonies and grown overnight. The starter cultures were sub-cultured into fresh SB or Terrific broth, with appropriate antibiotics. One L of SB was inoculated with 10-15 mL of starter culture. Cultures were grown at 37 °C with agitation to mid-exponential phase. Recombinant gene expression was then induced with 0.1 mM -1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Bio Basic). After 3 h of growth at 37 °C with agitation, cells were collected by centrifugation (4230 × g, 20 min, 4 °C) and pellets were stored at -20 °C. For truncated *mltB* over-expression only, cell culture was cooled to approx. 15 °C by swirling the flask on ice for 10 min prior to the addition of IPTG, as recommended by Blackburn and Clarke (2002). Truncated *mltB* over-expression was performed for 4 h at 15 °C with agitation to slow recombinant gene expression and therefore reduce unwanted cell lysis by sMltB (Blackburn and Clarke, 2002), and cells were collected by centrifugation, as described above.

2.6 Protein Purification

2.6.1 Osmotic shock

Osmotic shock was performed on frozen pellets of *E. coli* MHD79 (DE3) carrying pACIS plasmids. The protocol was modified from the QiaExpressionist (Qiagen). Cells from 1 L of culture were routinely collected in three 500 mL bottles, each with approx. 330 mL culture. The pellet in each bottle was resuspended in 20 mL 20% w/v sucrose solution (Appendix A), incubated on ice for 10 min, and collected by centrifugation (4230 × g, 20 min, 4 °C). Pellets were then resuspended in 20 mL of 5 mM MgSO₄ solution, incubated on ice for 10 min, and collected by centrifugation as above. The supernatant, containing the periplasmic contents of the cells, was collected and dialysed against immobilized metal affinity chromatography (IMAC) buffer (Appendix A) in preparation for protein purification.
2.6.2 Cell lysis

For the recovery of proteins with all other over-productions, osmotic shock was not performed. Instead, 1-2 L frozen cell pellets were resuspended in 25 mL cold IMAC buffer. DNase I (Bio Basic), RNase A (Roche Diagnostics), and 1 or 2 ethylenediamine tetraacetic acid (EDTA)-free protease inhibitor tablets (Roche Diagnostics) were added to the cell suspension prior to incubation on ice for 10-30 min. Cells were routinely lysed by SLM Aminco French Pressure Cell Press (American Instruments Exchange, Inc., Haverhill, MA) at 1000 psi. Cells were passed through the pressure cell 3-4 times and kept on ice. For convenience, cell lysis was sometimes performed using a BeadBeater (BioSpec Products Inc., Bartlesville, OK) or by sonication. The BeadBeater was used as per the manufacturer’s specifications, and sonication was performed using a 1/4" sonicator probe and a Vibra-Cell™ VCX130 sonicator (Sonics and Materials Inc., Newtown, CT). Sonication was performed at 45% amplitude for 6 min with 10 sec pulses, 15 sec apart. No significant differences in the extent of cell lysis were observed when these alternative methods were used. Following lysis, insoluble material was collected by centrifugation (26 890 × g, 20 min, 4 °C) and cleared lysate was kept for subsequent protein purification.

2.6.3 Immobilised metal affinity chromatography (IMAC)

For purification of Ivyp1, Ivyp2, and sMltB, cleared cell lysate was incubated with nickel-nitriloacetic acid (NTA) resin (Qiagen or Thermo Fisher Scientific). Co²⁺ TALON® Metal Affinity Resin (Clontech Laboratories, Mountain View, CA) was used for purifications of sMltA. When protein was purified from 1 L of original cell culture, 0.5 mL affinity resin was used. Sometimes protein was purified from 2 L of cell culture and the amount of resin was increased to 0.75 mL. If the osmotic shock protocol was carried out first, then 1 mL of resin was used, since the periplasmic contents from 1 L of cell culture were suspended in 60 mL. Prior to addition to cell lysate, resin was washed three times with 1 mL of appropriate IMAC buffer. For Ivy purifications, lysate was incubated with resin for 1-4 h. For sMltB purifications, lysate was incubated with resin for 1 h, and for sMltA, lysate was incubated for 5-60 min. Incubations with resin were always performed at 4 °C with agitation. After the incubation was complete, the resin and lysate were loaded into gravity columns and washed with 150 mL of each wash buffer. If 2 L of cell culture was used for protein purification,
then the volume of wash buffer was increased to 200 mL. Purified proteins were eluted from the column by washing with 5-10 mL of elution buffer, which was passed through the column three times to ensure efficient elution. When protein was purified from 2 L of cell culture, volumes were increased to 10-15 mL of elution buffer.

Ivyp1 and Ivyp2 were purified by a variation on the method of Clarke et al. (2010). Changes included purifying by imidazole gradient instead of pH gradient. Additionally, the IMAC buffers used contained 25 mM buffer (rather than 50 mM) at pH 7.4 and 500 mM NaCl. Glycerol at 10% v/v or Triton X-100 at 0.1% v/v was sometimes added to enhance solubility of purified Ivy proteins. Generally, His-tagged protein was bound in IMAC buffer with 10 mM imidazole, and the column was subsequently washed with IMAC buffer containing 10 mM, 20 mM, and 50 mM imidazole. Ivy was eluted in IMAC buffer containing 250 mM imidazole. The purification of Ivyp1 and Ivyp2 was optimised during this study.

sMltB was purified following a variation on the method of Blackburn and Clarke (2002). Triton X-100 was eliminated from the IMAC buffer and two of the three elution buffers (pH 6.0 and pH 5.0 buffers) were eliminated. Purified protein was eluted at pH 4.5. sMltA was purified by a variation on the method established by C. Vandenende (unpublished data). The recommended IMAC buffer contained 50 mM NaPO₄ at pH 8.0 with 300 mM NaCl, 10 mM MgCl₂, and 0.1% v/v Triton X-100. The IMAC protocol was similar to that described above. sMltA was purified by pH gradient with binding at pH 8.0, and washing at pH 8.0 and pH 7.0. Protein was eluted at pH 6.5 (recommended) or pH 6.0 in IMAC buffer. sMltA purification conditions were optimised for this study.

### 2.6.4 Dialysis

Protein samples were dialysed into assay buffer directly after purification by IMAC. Initially, protein was dialysed against 4 L of assay buffer with 1-2 h buffer changes (two changes that day, one overnight, and one change the following morning).

### 2.6.5 Protein concentration

Amicon Ultra-15 Centrifugal Filter units (EMD Millipore Corp., Billerica, MA) were used to concentrate protein samples after dialysis. A 10 kDa molecular weight cut-off (MWCO) was
used for concentration of Ivyp1 and Ivyp2, and a 30 kDa MWCO was used for sMltA concentration. sMltB did not require concentration after purification. Dialysed protein (5-15 mL) was added to the upper reservoir of the filter unit and protein was concentrated by centrifugation (1300 × g, 5-10 min, 4 °C). Centrifugation steps were repeated as necessary until volume in the upper reservoir had decreased to 1-3 mL.

2.7 Protein Analyses

2.7.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analyses were performed for every protein purification by the method of Laemmli (1970). For most purifications, 15% separating gels and 4% stacking gels were used; however, for some sMltB and sMltA purifications, 12% separating gels were used. Gel recipes can be found in Table A.1 in Appendix A. Two gels were always run at the same time, and one was stained for protein visualisation and the other was used for Western immuno-blotting. Preparation of protein samples regularly loaded on gels, as well as loading volumes, are listed in Table A.2 in Appendix A. Sample buffer used was Lane Marker Reducing Sample Buffer (5×) (Thermo Fisher Scientific). Marker used was PiNK Plus Prestained Protein Ladder (Genedirex, www.genedirex.com) or Precision Plus Protein Dual Color Standards (Bio-Rad Laboratories, Inc.) and 5 μL of ladder was always loaded. Prepared samples were boiled at 95 °C for 15 min prior to loading. Gels were run at 150 V until the dye front reached the bottom of the plates (approx. 1.5 h). Gels were stained for 20-60 min with GelCode Blue Stain Reagent (Thermo Fisher Scientific) using the microwave protocol specified by the manufacturer. Images of gels and blots were manipulated using Image Lab™ (Bio-Rad Laboratories, Inc.) and this software was also used to calculate the observed molecular weights of analysed proteins. This tool uses a standard curve generated from the protein ladder (log of the molecular weight of the protein standards vs. Rf value) and calculates the molecular weight of unknown proteins by fitting their Rf values to the standard curve.

2.7.2 Western immuno-blotting

Western immuno-blotting was carried out for most protein purifications by a variation on the method of Burnette (1981). After SDS-PAGE was performed as described above, the gel
was laid against a 0.45 μM nitrocellulose membrane (Bio-Rad Laboratories, Inc.), submerged in Western transfer buffer (Appendix A), and a 45 V current was applied for 1 h. The blot was rinsed twice in 1× TBS (Appendix A), left in blocking buffer (Appendix A) for 1 h or overnight, then rinsed once more in 1× TBS. The blot was soaked in primary antibody solution (Appendix A) for 1 h or overnight and then rinsed twice in 1× TTBS (Appendix A) and once in 1× TBS. Then, the blot was soaked in secondary antibody solution (Appendix A) for 1 h, rinsed 4 times with 1× TTBS, once with 1× TBS, and finally, the blot was developed in 1-Step NBT/BCIP Solution (Thermo Fisher Scientific) until bands appeared. Blots were stored in water. All washes were done for 5 min and the entire protocol was performed with gentle agitation.

2.7.3 Protein quantification

Protein concentration was measured using the bicinchoninic acid (BCA) protein assay kit from Thermo Fisher Scientific. The microwell plate protocol was followed to the manufacturer’s specifications. Plates were read at 595 nm in a microplate reader. Bovine serum albumin (BSA) standards of 1 mg/mL or 2 mg/mL (Sigma-Aldrich) were diluted to concentrations between 0 and 1 mg/mL. The BSA standards were used to produce a standard curve of sample absorbance at 595 nm vs. protein concentration. The equation of the line was used to solve unknown protein concentrations, based on the absorbance measured.

2.7.4 Matrix-assisted laser desorption/ionisation – time of flight (MALDI-TOF) mass spectrometry

Protocols for sample preparation for MALDI-TOF were obtained from the Advanced Analysis Centre (University of Guelph, Guelph, ON) and can be found online at http://www.uoguelph.ca/~bmsf/. Samples for MALDI-TOF mass spectrometry were prepared by separating protein samples by SDS-PAGE. Gels were stained as usual and bands of interest were excised from the gel. A region of the gel containing no protein was used as a control. Proteins (in-gel) were subjected to a reduction by 10 mM DTT and an alkylation treatment with 55 mM iodoacetamide. Following these treatments, gel fragments were soaked in a trypsin solution for protein digestion. Digested peptides were extracted from the gel using water and then using 5% v/v formic acid in 50% v/v acetonitrile. Finally,
digested protein samples were desalted using ZipTip® pipette tips with C18 reversed-phase media (EMD Millipore Corp.) following the manufacturer’s specifications. Peptides were eluted from the ZipTip columns with the matrix solution. Matrix used was CHCA (α-cyano-4-hydroxycinnamic acid). Prepared samples were spotted onto a stainless steel chip using the dried droplet method. Peptide mapping was performed using a Bruker Reflex III (Bruker Daltonics, Germany) with a 337 nm nitrogen laser and positive ionisation at the Mass Spectrometry Facility (Advanced Analysis Centre, University of Guelph, Guelph, ON). Obtained masses were compared to masses produced by in silico trypsin digests of proteins of interest using PeptideMasses (ExPASy, Swiss Institute of Bioinformatics, Lausanne, Switzerland). For samples containing unknown protein, experimental masses were used to search the RefSeq database (NCBI, Bethesda, MD) using ProFound (Laboratory of Mass Spectrometry and Gaseous Ion Chemistry, The Rockefeller University, New York, NY).

2.8 Measurement of Lytic Activity

The turbidometric assay of Hash (1967) was used to measure the lytic activity of lysozyme and lytic transglycosylases. Inhibition of LTs by Ivyp1 and Ivyp2 was also measured using this method. Micrococcus luteus (ATCC 4698, Sigma-Aldrich) whole cells were resuspended in assay buffer with 10 μg/mL BSA (Sigma-Aldrich) at a final concentration of 0.8 μg/mL. Cell suspensions were sonicated for 3-5 min at 45% with 10 sec pulses, 10 sec apart. For a positive control, 500 μL of cells were mixed with 0.14 μM hen egg-white lysozyme (HEWL, Sigma-Aldrich) in a 1.5-mL cuvette and made to 1 mL with assay buffer (final cell concentration of 0.4 μg/mL). Cell suspensions were monitored at 600 nm for 30 min to 2 h. Negative controls were performed with no enzyme present. To monitor lytic activity, 1.5-6 μM (final concentration) purified sMltB or sMltA was added to the cell suspension. Negative controls for Ivy were performed using 3-15 μM purified Ivyp1 or Ivyp2. As a positive control for Ivyp1, 1.5-6 μM Ivyp1 was incubated with 0.14 μM HEWL for 15 min at room temperature prior to the addition of the cell suspension. Positive controls for Ivyp1 and Ivyp2 were performed by pre-incubating Ivyp1 or Ivyp2 with sMltB. Finally, Ivyp1 and Ivyp2 were pre-incubated with sMltA to monitor sMltA inhibition by Ivy.

The protocol of Clarke et al., 2010 was used for the inhibition of sMltB, but a few changes were made. Final cuvette volumes were decreased to 1 mL from 1.5 mL and the OD was
monitored at 600 nm instead of 660 nm, as in the publication. Buffer components were kept the same. For sMltA inhibition, the assay buffer was optimised for sMltA activity and Ivy solubility.
CHAPTER 3 – RESULTS

The overarching goal of this research was to purify Ivyp1, Ivyp2, and sMltA to homogeneity in order to test the hypothesis that Ivyp1 and Ivyp2 function as inhibitors of sMltA in vitro. The optimisation of the three protein purifications and that of the inhibition assay comprise the bulk of this work.

3.1 Construction of pACIS1, pACIS2, pACIS3, and pACIS4

Both full-length and truncated ivyp1 and ivyp2 genes were excised from the previously created pACCC plasmids (Clarke et al., 2010), and ligated into pET-21b(+). The purpose of these experiments was to have the ivy genes in a pET vector with an Amp-resistance cassette instead of a Kan-resistance cassette. Amp resistance was important because these genes were to be over-expressed in E. coli MHD7 (DE3), which is already resistant to kanamycin. Thus, to select for positive transformants by the pET vectors, a different antibiotic resistance cassette was required. Before starting the sub-cloning experiment, a point mutation in pACCC2 was identified which required correction. This nucleotide change was identified when all the plasmids were sent for Sanger sequencing to verify the inserted genes. The results of the sequencing were compared to the known sequence for this gene in P. aeruginosa PAO1 (gene PA3902, Pseudomonas.com).

3.1.1 Site-directed mutagenesis of pACCC2

Site-directed mutagenesis was used to correct this point mutation in the ivyp1Δ1-23 gene in the pACCC2 plasmid. The affected nucleotide was c.147C>T. While this mutation is silent (AGC>AGT; Ser>Ser), E. coli does not encode a tRNA gene for the AGT anti-codon (Genomic tRNA Database, University of California, Santa Cruz). Thus, it was thought that the ivyp1Δ1-23 gene never expressed from pACCC2 because E. coli does not encode a tRNA gene with an anti-codon to the AGT codon. Primers covering the region containing the point mutation were designed and used in PCR to amplify the entire plasmid with the corrected mutation. A DpnI digestion was performed on the PCR product to digest the template DNA and digestion products were visualised by agarose gel electrophoresis (Fig. 3.1). The point mutation was confirmed by Sanger sequencing. The plasmid with the point mutation
corrected was named pACCC2-IS. \textit{ivyp}1_{Δ1-23} was sub-cloned into pET-21b(+) following this correction. \textit{ivyp}1_{Δ1-23} and \textit{ivyp}2_{Δ1-23} were originally cloned into pET-28a(+) vectors (Clarke \textit{et al.}, 2010) with the first 23 codons truncated because the first 23 amino acids encode the predicted signal sequence. This signal sequence is predicted to be cleaved upon export of the protein to the periplasm (Clarke \textit{et al.}, 2010).

![Figure 3.1](image)

**Figure 3.1 DpnI-digested and undigested PCR product following SDM of pACCC2.** Purified PCR products were digested with 20 units of DpnI. Undigested (lane 1) and digested (lane 2) PCR products were analysed by agarose gel electrophoresis. Undigested (lane 3) and digested (lane 4) 1:10 dilutions of the PCR product were also analysed. M is 1 kb DNA ladder. Sizes of standards are reported in kb.

3.1.2 Sub-cloning of recombinant \textit{ivyp}1, \textit{ivyp}2, truncated \textit{ivyp}1, and truncated \textit{ivyp}2 from pACCC plasmids into pET-21b(+)

Genes encoding Ivyp1 and Ivyp2, both full-length and truncated, were sub-cloned into pET-21b(+) vectors so that the constructs would have ampicillin resistance instead of kanamycin resistance (as in the original pET-28a(+) vectors). The pACCC plasmids (1-4) and pET-21b(+) were digested with \textit{XbaI} and \textit{HindIII}. Gene inserts from the pACCC digests and linearised pET-21b(+) were then gel excised for ligation (Fig. 3.2). Figure 3.2 shows the digestion products for pET-21b(+), pACCC1-FN, and pACCC3-FN and similar experiments were carried out with pACCC2 and pACCC4. The pET-21b(+) vector is 5.4 kb in size, the \textit{ivyp}1 full-length insert is 507 bp, and the \textit{ivyp}2 full-length insert is 516 bp. In Fig. 3.2, the 5.4 kb parent vector can be seen in each lane, and the inserted \textit{ivy} genes (approx. 0.5 kb) can be seen in the pACCC1-FN and pACCC3-FN digestion lanes.
Following the ligation reactions and transformations in *E. coli* DH5α, colonies were screened by e-lyse. Individual clones were verified by analytical digestion (Fig. 3.3) and Sanger sequencing. Four individual clones were screened by analytical digestion for each construct and all four clones were found to be harbouring pET-21b(+) vectors with the *ivy* genes inserted. In Figure 3.3, the *ivy* inserts can be seen in the digested lanes (lanes 2-5 and 7-10) for pACIS1 and pACIS2, around the 0.5 kb marker. The digested pET-21b(+) vector (5.4 kb in size) can be seen between the 5 and 6 kb markers on the ladder. All four plasmids were constructed successfully with the goal of over-expressing the genes from these plasmids in *E. coli* MHD79 (DE3).

![Figure 3.2 Digestion products of pET-21b(+), pACCC1-FN, and pACCC3-FN.](image)

Parent plasmids (pET-21b(+), pACCC1-FN, pACCC3-FN) were digested with *XbaI* and *HindIII* and the entire digestion reactions were loaded onto an agarose gel for gel extraction. The above image was taken prior to gel extraction of the digested DNA. Lanes: 1, 2, pET-21b(+); 3, 4, pACCC1-FN; 5, 6, pACCC3-FN; M, 1 kb DNA ladder. Sizes of standards are reported in kb.
3.2 Production and Purification of Recombinant Ivyp1 and Ivyp2 Over-produced in E. coli MHD79 (DE3)

*E. coli* MHD79 (DE3) is a strain of *E. coli* lacking six of seven LT genes (Heidrich et al., 2002). It was considered to be a good expression host for *ivy* genes because observations made by others in the laboratory (unpublished) suggested that LT(s) might co-purify with Ivy proteins. However, due to the defective cell wall metabolism experienced by this strain, cell growth and division occurs slowly compared to strains of *E. coli* with the LT genes intact. Additionally, *E. coli* MHD79 (DE3) cells do not septate as easily, producing chains of cells 30-50 cells long (Heidrich et al., 2002). It was uncertain whether chains of cells would scatter light in the same way as a cell suspension of fully-septated cells and thereby complicating the monitoring of growth. Therefore, growth curve analysis was performed to identify the mid-exponential phase of *E. coli* MHD79 (DE3) cell culture, as compared to *E. coli* BL21 (DE3). This growth curve analysis was important in ensuring that cells were induced to express the *ivy* genes at the optimal point in their growth.

3.2.1 Growth curve analysis of *E. coli* MHD79 (DE3)

The growth of *E. coli* MHD79 (DE3) [pACIS1], *E. coli* MHD79 (DE3) [pACIS2], and *E. coli* MHD79 (DE3) [pET-21b(+)] in LB broth was monitored to determine when these cultures reached mid-exponential phase (Fig. 3.4[a]). The growth of *E. coli* BL21 (DE3)
[pACCC1-FN], *E. coli* BL21 (DE3) [pACCC3-FN], and *E. coli* BL21 (DE3) [pET-28a(+)] was also monitored for comparison (Fig. 3.4[b]). The times needed for each strain to reach mid-exponential phase (represented by $OD_{600} = 0.7$ for these growth curves, which is slightly higher than usual) in liquid culture are summarised in Table 3.1. *E. coli* MHD79 (DE3) grew more slowly than *E. coli* BL21 (DE3), and the maximum $OD_{600}$ for the *E. coli* MHD79 (DE3) culture was less than that of the *E. coli* BL21 (DE3) culture. Additionally, the plasmids carrying *ivyp1* or *ivyp2* genes slowed the growth of both cultures as compared to the empty pET vectors, and this effect was more pronounced in *E. coli* MHD79 (DE3). Interestingly, *E. coli* BL21 (DE3) [pACCC3-FN] grew slower than *E. coli* BL21 (DE3) [pACCC1-FN], while a difference between *E. coli* MHD79 (DE3) [pACIS1] and *E. coli* MHD79 (DE3) [pACIS2] was not seen. These growth metrics were seen consistently throughout this study, and as such, *E. coli* BL21 (DE3) [pACCC3-FN] cultures were always started approx. 40 min ahead of the *E. coli* BL21 (DE3) [pACCC1-FN] cultures such that they could reach $OD_{600} = 0.7$ at the same time.

**Table 3.1 Growth times needed for each strain to reach mid-exponential phase**

<table>
<thead>
<tr>
<th>Plasmid (gene)</th>
<th>Strain</th>
<th>Time (min) to mid-exponential phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>E. coli</em> MHD79 (DE3)</td>
</tr>
<tr>
<td>pACIS1/pACCC1-FN (<em>ivyp1</em>)</td>
<td></td>
<td>240</td>
</tr>
<tr>
<td>pACIS2/pACCC3-FN (<em>ivyp2</em>)</td>
<td></td>
<td>240</td>
</tr>
<tr>
<td>pET-21b(+)/pET-28a(+) (empty vector)</td>
<td></td>
<td>210</td>
</tr>
</tbody>
</table>
Figure 3.4 Growth curve analysis of *E. coli* BL21 (DE3) and *E. coli* MHD79 (DE3). Over-night cultures of both strains of *E. coli* transformed with various plasmids were inoculated 1:100 in 250 mL fresh LB broth, supplemented with appropriate antibiotics. The OD$_{600}$ was measured over a period of 8 h (480 min). Curves shown are representative. *E. coli* BL21 (DE3) growth curves (a) were performed for cells transformed with pACCC1-FN (closed squares), pACCC3-FN (closed triangles), and empty vector pET-28a(+) [open circles]. *E. coli* MHD79 (DE3) growth curves (b) were performed for cells transformed with pACIS1 (closed squares), pACIS2 (closed triangles), and empty vector pET-21b(+) [open circles]. Data are representative.

3.2.2 Production and purification of full-length Ivyp1 and Ivyp2 from *E. coli* MHD79 (DE3)

The first protein purifications attempted from *E. coli* MHD79 (DE3) were the purification of full-length Ivyp1 and Ivyp2. Ivyp1 and Ivyp2 were over-produced in 1 L cultures of *E. coli* MHD79 (DE3) transformed with either pACIS1 or pACIS2, respectively. It was presumed
that Ivyp1 and Ivyp2 would be processed within the cell and exported to the periplasm; thus, osmotic shock was performed to release the periplasmic contents. The contents of the periplasm were dialysed against IMAC buffer (25 mM Tris, pH 7.4, with 500 mM NaCl, 10% v/v glycerol, and 10 mM imidazole). The IMAC was carried out using an imidazole gradient with 10 mM, 20 mM, and 50 mM imidazole washes. Elution of purified recombinant Ivy was performed with 15 mL IMAC buffer containing 250 mM imidazole.

The SDS-PAGE and Western blot analysis showed that gene expression of \textit{ivyp1} and \textit{ivyp2} was low (Fig. 3.5). For both Ivyp1 and Ivyp2, bands appeared in the lanes containing induced whole cells and cell fraction following osmotic shock (lanes 2 and 4, respectively). All of the expressed full-length Ivyp1 and Ivyp2 appeared to remain in the cytoplasm, instead of being processed and transported to the periplasm as expected (lane 4, Fig. 3.5). Recombinant protein was not purified to any levels detectable by SDS-PAGE. Full-length, His-tagged Ivyp1 and Ivyp2 have predicted molecular masses of 18.8 kDa and 19.2 kDa, respectively (Compute pI/Mw tool, ExPASy). The estimated masses for Ivyp1 was 16.7 kDa by SDS-PAGE analysis and 15.9 kDa by Western blot analysis, as seen in Fig. 3.5. These estimated masses are lower than the expected mass and they are actually closer to the mass of Ivyp1\textsubscript{Δ1-23} (16.5 kDa). Thus, Ivyp1 may have been processed and had its signal sequence removed by the host cell during production. For Ivyp2, the estimated masses are 18.1 kDa by SDS-PAGE analysis and 18.3 kDa by Western blot analysis. These values are in between the predicted mass of Ivyp2 full-length (19.2 kDa) and the predicted mass of Ivyp2\textsubscript{Δ1-23} (16.9 kDa). The estimated masses of recombinant Ivyp1 and Ivyp2 determined by SDS-PAGE analysis were always lower than the predicted masses. The bands of interest are less intense on the Western blot as compared to the SDS-PAGE analysis. This observation was seen throughout this study, and furthermore, Ivyp2 bands often appeared less intense than the Ivyp1 bands on the Western blots, as seen in this figure.
Figure 3.5 SDS-PAGE and Western analysis of full-length Ivyp1 and Ivyp2 IMAC purifications from E. coli MHD79 (DE3) transformants. Protein samples were collected at various points in the purification process and analysed by (a) SDS-PAGE with Coomassie Brilliant Blue staining and (b) Western blot using anti-His-6 antibody. UC: uninduced cell pellet; IC: induced cell pellet; PF: periplasmic fraction; CF: cell fraction; FT: IMAC flow-through; W1: IMAC wash 1; W2: IMAC wash 2; W3: IMAC wash 3; EL: IMAC elution; M: PiNK Plus Prestained Protein Ladder. Molecular masses of standards are reported in kDa. The red box denotes the expected location of respective Ivy proteins, and the estimated molecular masses of the bands of interest are in red font.

Since the recombinant full-length Ivy proteins were not truncated and exported to the periplasm, an attempt was made to purify full-length Ivyp2 from the cytoplasm of E. coli MHD79 (DE3) [Fig. 3.6]. The IMAC buffer compositions were kept identical as with the previous purification. Osmotic shock was performed to remove all periplasmic contents from the cell lysate, and the remaining cell pellets were lysed by French Press. Recombinant protein was subsequently purified by IMAC with no further changes to the protocol, and the results of the purification were analysed by SDS-PAGE and Western blot (Fig. 3.6). The total protein concentration was 0.24 g/L, and 0.85 g/L following concentration by Amicon. Many contaminants were carried along with Ivyp2 in this purification and it was suspected that MltF could be one of these contaminants, since it is the only LT expressed by E. coli MHD79 (DE3) (Scheurwater and Clarke, 2004). Ivyc from E. coli is known to complex with lysozyme during IMAC purifications (Monchois et al., 2001), so it is possible that the same co-purification effect could occur with Ivy and an LT. For these reasons, MALDI-TOF was used to investigate the possible presence of MltF in the IMAC elution from this purification.
3.2.3 Expression of \textit{ivyp1}_{Δ1-23} and \textit{ivyp2}_{Δ1-23} from \textit{E. coli} MHD79 (DE3)

Expression trials were conducted with pACIS3 and pACIS4 in 5 mL tubes of LB supplemented with appropriate antibiotics in an attempt to find better expression conditions, since the full-length genes did not express very well. \textit{ivyp2}_{Δ1-23} expressed best at 37 °C with 0.1 mM IPTG, as seen by SDS-PAGE and Western analysis of whole cell lysates (Fig. 3.7). However, the pACIS3 construct did not express \textit{ivyp1}_{Δ1-23} at all, which is consistent with the lack of expression seen with the parent plasmid pACCC2-IS (Fig. 3.7). Neither the pACCC2-IS plasmid nor the pACIS3 plasmid would express the \textit{ivyp1}_{Δ1-23} gene, despite multiple attempts. Sequence information for both plasmids revealed no nucleotide changes within the cloned gene or the promoter region. The cloned genes in these two plasmids were never successfully expressed in this study.

The estimated mass of the protein of interest was 15.1 kDa by SDS-PAGE analysis and 14.0 kDa by Western blot analysis, which is again lower than expected for truncated recombinant Iyvp2 (16.5 kDa) produced by pACIS4. In this figure, the estimated masses of the bands of
interest on the Western blot are lower than the estimated masses on the SDS-PAGE gel by 1 kDa.

<table>
<thead>
<tr>
<th>pACC2-IS</th>
<th>pACIS4</th>
<th>pACIS3</th>
</tr>
</thead>
<tbody>
<tr>
<td>uninduced</td>
<td>37°C</td>
<td>1 mM</td>
</tr>
<tr>
<td>30°C</td>
<td>1 mM</td>
<td>0.1 mM</td>
</tr>
</tbody>
</table>
| M | Precision Plus Protein Dual Color Standards | Molecular masses of standards are reported in kDa. The red box denotes the expected location of respective Ivy proteins, and the estimated molecular masses of the bands of interest are in red font.

Since Ivyp1Δ1-23 could not be produced by either the pACC2-IS or pACIS3 plasmids, attempts to purify these proteins were abandoned and focus was instead placed on the purification of the full-length Ivy proteins. Additionally, E. coli BL21 (DE3) was used for all subsequent purifications, since this strain grows faster and is able to produce more protein than E. coli MHD79 (DE3).

3.3 Matrix-assisted Laser Desorption/Ionisation - Time of Flight (MALDI-TOF) Mass Spectrometry for the Identification of Ivyp2His-6 and MltF

An SDS-polyacrylamide gel of the same purification presented in Fig. 3.6 was run to separate the various proteins in the IMAC elution. Bands corresponding to both Ivyp2 proteins (the two bands visible in the elution lane of Fig. 3.6 [b]) and two higher molecular weight (MW)
bands around the molecular mass of MltF (estimated to be 59.4 kDa, and 54.6 kDa; predicted mass of MltF is 58.3 kDa) were excised. Protein contained within these bands was digested in-gel by trypsin. These samples, in addition to an undigested whole elution sample, were subjected to MALDI-TOF analysis. In addition to the negative control, a sample of digested BSA was used for a positive control.

The negative control and positive control for this experiment produced the expected results. The negative control contained only peaks corresponding to the CHCA matrix, and the positive control (digested BSA) was successfully identified. Recombinant Ivyp2Δ1-23 (lacking a signal sequence) was successfully identified by MALDI-TOF analysis of the lower MW Ivyp2 band (Table 3.2). A peptide with a mass of 2042.1 Da representing the majority of the signal sequence (NLMNALLLGAAASSLAVAADR) was absent from the analysis of this band, confirming the absence of the signal sequence. The sample with the higher MW Ivyp2 band failed during MALDI-TOF although it is likely that this band represents recombinant full-length Ivyp2.

Table 3.2 Experimental peptide masses obtained from MALDI-TOF following trypsin digestion of Ivyp2His-6

<table>
<thead>
<tr>
<th>Average mass from in silico digest (Da)</th>
<th>Sequence</th>
<th>Experimental peptide mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>617.68</td>
<td>HASFR</td>
<td>617.67</td>
</tr>
<tr>
<td>639.64</td>
<td>DGEYR</td>
<td>639.67</td>
</tr>
<tr>
<td>859.01</td>
<td>ILDEQLK</td>
<td>No peak</td>
</tr>
<tr>
<td>982.18</td>
<td>YLVGGLCEK</td>
<td>982.19</td>
</tr>
<tr>
<td>1107.3</td>
<td>LLVAFDWDK</td>
<td>1107.7</td>
</tr>
<tr>
<td>1194.3</td>
<td>ADPNWYWGGK</td>
<td>1194.5</td>
</tr>
<tr>
<td>1253.5</td>
<td>WLGKPEPAVQK</td>
<td>1253.7</td>
</tr>
<tr>
<td>1347.4</td>
<td>AWQNLVEDER</td>
<td>1347.7</td>
</tr>
<tr>
<td>1367.5</td>
<td>LNELQSTDAGYR</td>
<td>1367.8</td>
</tr>
<tr>
<td>1410.5</td>
<td>LAAALEHHHHHH</td>
<td>1410.9</td>
</tr>
<tr>
<td>2002.2</td>
<td>SHAYGLYVQVPEGLPQDK</td>
<td>2002.7</td>
</tr>
<tr>
<td>2599.9</td>
<td>QGDK</td>
<td>No peak</td>
</tr>
</tbody>
</table>

1. Average masses from an in silico digestion (PeptideMass) of Ivyp2His-6 are shown for comparison. Peptides with masses below 500 Da were filtered out.
2. No peak refers to the absence of an experimental peak for this peptide.
The sample with the high MW band that was a suspect for MltF also failed during the MALDI-TOF experiment. MltF was not identified in the sample with the suspect lower MW band. This particular sample likely contained a mix of two proteins, neither of which produced peptides with masses corresponding to MltF or sMltF. From the MALDI-TOF analysis, one of the proteins in the mixture was in a higher concentration than the other, or was more hydrophilic and more easily digested by trypsin, since one protein seemed to ionise much better than the other. Experimental peptide masses were queried in ProFound but no matching proteins were identified. No bands corresponding to either Ivyp2 or MltF were indentified in the whole elution undigested sample.

3.4 Production and Purification of Recombinant Ivyp1 and Ivyp2 Over-expressed in E. coli BL21 (DE3)

The plasmids pACCC1-FN and pACCC3-FN were over-expressed in E. coli BL21 (DE3) for the production of full-length recombinant Ivyp1 and Ivyp2, respectively. The over-expression and purification protocol for these plasmids was adapted from Clarke et al. (2010). Cells were lysed by French Press and Ivy was purified by IMAC with the usual imidazole gradient. The IMAC buffer used contained 25 mM NaPO_4 at pH 7.4 with 10% v/v glycerol, 0.5 M NaCl, and 10 mM imidazole. The results of this purification were analysed by SDS-PAGE and Western blot (Fig. 3.8). For both Ivyp1 and Ivyp2, a larger band appeared in the induced cell pellet and insoluble fraction lanes, as compared to the band of interest in the final IMAC elution lane. As seen with other purifications, the observed masses estimated for Ivyp1 and Ivyp2 on both the SDS-PAGE and Western blot are lower than the expected masses for each protein. The lower molecular weight bands in the IMAC elution lanes in Fig. 3.8 are the processed Ivy proteins, and this form of the protein is the dominant one following purification by IMAC. The large Ivy band seen in the insoluble fraction for both Ivyp1 and Ivyp2 purifications is due to the inefficient lysis of the E. coli MHD79 (DE3) cells, as seen with E. coli BL21 (DE3). Even after as many as six careful passages through the French Press at 1000 psi, a large cell pellet remained after centrifugation of the lysate. This method produced a protein preparation that was more pure than elutions of Ivy that had been purified from E. coli MHD79 (DE3). Initial total protein concentrations were 0.28 g/L (Ivyp1) and 0.08 g/L (Ivyp2). Protein was concentrated to 0.92
g/L (Ivyp1) and 0.35 g/L (Ivyp2). Recombinant full-length Ivyp1 and Ivyp2 was routinely purified this way for use in all subsequent experiments. Ivyp1 and Ivyp2 were purified by this method for all subsequent purifications, and preparations such as in Fig. 3.9 were used in the turbidometric inhibition assays. Average protein concentrations were around 0.2 g/L of culture, and following concentration by Amicon, between 1.0 g/L and 2.0 g/L.

Figure 3.8 SDS-PAGE and Western analysis of Ivyp1 and Ivyp2 purified in E. coli BL21 (DE3). Samples were taken at various points during protein purification for analysis by (a) SDS-PAGE with Coomassie Brilliant Blue staining and (b) Western blot using anti-His-6 antibody. UC: uninduced cell pellet; IC: induced cell pellet; CL: cleared cell lysate; IS: insoluble fraction following cell lysis; FT: IMAC flow-through; W1: IMAC wash 1; EL: IMAC elution; W2: IMAC wash 2; W3: IMAC wash 3; M, Precision Plus Protein Dual Color Standards. Molecular masses of standards are reported in kDa. The red box denotes the expected location of respective Ivy proteins, and the estimated molecular masses of the bands of interest are in red font.

3.5 Production and Purification of sMltB Over-produced in E. coli BL21 (DE3) pLysS

The sMltB protein was over-produced and purified so that it could be used as a positive control for Ivyp1 and Ivyp2 inhibition. A variation on the purification protocol from Blackburn and Clarke (2002) was used to purify sMltB. sMltB was over-expressed in E. coli BL21 (DE3) pLysS cells and cells were lysed by French Press. Unlike E. coli cells that had over-expressed ivy genes, cells that over-produced sMltB lysed easily. Following cell lysis, recombinant His-tagged protein was purified by IMAC, using a pH gradient. Imidazole gradients are not recommended for the purification of LTs, as imidazole inhibits their lytic activity. Although the interaction between imidazole and the LTs is reversible and imidazole can be removed from the protein preparation via buffer exchange, a pH gradient was used to
avoid potential downstream complications. The IMAC buffer used contained 50 mM NaPO₄ at pH 8.0 with 300 mM NaCl. This method produced a high yield of sMltB with few contaminants (Fig. 3.9). Although sMltB_His₆ is predicted to have a mass of 41 kDa, the masses calculated from the SDS-PAGE analysis and Western blot analysis were 38.2 and 36.9 kDa, respectively. The final protein concentration of purified recombinant sMltB was 0.36 g/L of culture. This preparation was used in turbidometric assays as a positive control for Ivyp1 and Ivyp2 inhibition.

Figure 3.9 SDS-PAGE and Western blot analysis of sMltB purified from E. coli BL21 (DE3) pLysS. Samples were taken at various points during protein purification for analysis by (a) SDS-PAGE with Coomassie Brilliant Blue staining and (b) Western blot using anti-His-6 antibody. UC: uninduced cell pellet; IC: induced cell pellet; CL: cleared cell lysate; IS: insoluble fraction following cell lysis; FT: IMAC flow-through; W1: IMAC wash 1; W2: IMAC wash 2; EL: IMAC elution; M, Precision Plus Protein Dual Color Standards. Molecular masses of standards are reported in kDa. The red box denotes the expected location of sMltB, and the estimated molecular masses of the bands of interest are in red font.

3.6 Production and Purification of sMltA Over-produced in E. coli BL21 (DE3) pLysS

The protocol for the over-expression of pACCV4 in E. coli BL21(DE3) pLysS and subsequent purification of sMltA by IMAC was adapted from C. Vandenende. Initially, the recommended protocol was attempted. As with sMltB, E. coli BL21 (DE3) cells that had over-produced sMltA lysed efficiently. The IMAC buffer used contained 50 mM NaPO₄ at pH 8.0 with 300 mM NaCl and 0.1% v/v Triton X-100 (10 mM MgCl₂ was omitted at the
recommendation of C. Vandenende). Washes were performed with 100 mL of IMAC buffer at pH 8.0 and 100 mL of IMAC buffer at pH 7.0, and bound sMltA was recovered in 5 mL of IMAC buffer at pH 6.5, as recommended. The estimated mass of the recombinant protein by SDS-PAGE and Western blot analysis was 42.2 kDa and 40-42 kDa, respectively (Fig. 3.10). The expected mass of recombinant sMltA is 40 kDa. The Western blot lanes did not run in a straight line, which skewed measurements such that sMltA appears to have migrated further in some lanes compared to other. This method did not yield enough protein for planned experiments; the sample in the elution lane in Fig. 3.10 has been concentrated approx. 5 times after purification. Total protein concentration was 0.0641 g/L, and 0.357 g/L following concentration. It was expected that approx. 0.1 g/L sMltA in 5 μL of IMAC buffer would be purified by this method, but the actual results were lower. Therefore, alternative purification methods were investigated.

Despite the efficient cell lysis of \textit{E. coli} that had over-produced sMtlA, a small amount often remained in the insoluble fraction lane and was evident in Western immuno-blots (Fig. 3.10, insoluble fraction lane). sMltA usually appeared in most fractions during sMltA purifications, including the IMAC flow-through. While the yield and purity of the sMltA
purifications was improved during this study, conditions were not found which promoted complete binding of His-tagged sMltA to the Co$^{2+}$-NTA resin.

The sMltA purification was repeated with changes to help increase the yield and purity of recombinant sMltA. These changes included using IMAC buffers that did not contain Triton X-100 and increasing IMAC wash volumes to 150 mL. A comparison was also made between elution of the recombinant sMltA from the gravity column with IMAC buffer at pH 6.5 and pH 6.0. For this experiment, the cell lysate that had been incubated with Co$^{2+}$ resin was split in half (~ 12.5 mL of cell lysate per column). Each half was poured through a separate gravity column and IMAC was carried out as usual until the last step. During the elution, one column was washed with IMAC buffer at pH 6.0, and the other was washed with IMAC buffer at pH 6.5. The elution at pH 6.0 was found to have a high protein yield compared to elution at pH 6.5 (Fig. 3.11). Unfortunately, BCA assays were not performed on these samples, so data is purely empirical. However, in contrast to Fig. 3.10, the samples loaded in the IMAC elution lanes on Fig. 3.11 were not concentrated by Amicon. Also, the same volume of IMAC elution sample was loaded on both gels, but because the cell lysate was split in half for the second purification, both elution lanes (pH 6.5 and pH 6.0) contain protein purified from the equivalent of 0.5 L of culture. Thus, eluting recombinant sMltA from the Co$^{2+}$ resin at pH 6.0 was more effective than at pH 6.5. Additionally, the purity of these samples was much greater than the initial purification attempt (Fig. 3.10) due to the lack of Triton X-100 and the increased wash buffer volumes. While the sMltA preparation by this method was both pure and concentrated enough to use in subsequent experiments, sMltA had to be purified frequently for testing in various assay conditions, and therefore, attempts were made to increase the yield.
Figure 3.11 SDS-PAGE analysis of sMltA purified from *E. coli* BL21 (DE3) in the absence of Triton X-100 with elutions at pH 6.5 and pH 6.0. Samples were taken at various points during protein purification for analysis by SDS-PAGE with Coomassie Brilliant Blue staining. UC: uninduced cell pellet; IC: induced cell pellet; CL: cleared cell lysate; IS: insoluble fraction following cell lysis; FT: IMAC flow-through; W1: IMAC wash 1; W2: IMAC wash 2; EL: IMAC elution; M, Precision Plus Protein Dual Color Standards. Molecular masses of standards are reported in kDa. The red box denotes the expected location of sMltA, and the estimated molecular masses of the bands of interest are in red font.

Due to the success of the IMAC elution at pH 6.0, an attempt was made to elute recombinant protein from the Co$^{2+}$ resin at an even lower pH, pH 4.5. For this experiment, two different incubation times for cell lysate with Co$^{2+}$ resin were tested (5 min and 1 h). Due to the high expression levels of *sMltA*, a 5 min incubation time was tested to investigate if this length of time was enough to promote binding of His-tagged sMltA to the resin but not long enough for contaminating proteins to bind. sMltA was purified from 2 L of culture and following lysis, the cell lysate was split in half. The first half was incubated with 0.5 mL Co$^{2+}$ resin for 5 min and the second half was incubated with 0.5 mL Co$^{2+}$ resin for 1 h. IMAC was performed on both columns in the same way. Washes were performed with 150 mL of IMAC buffer (with no Triton X-100) at pH 8.0, pH 7.0, and pH 6.0, and recombinant sMltA was eluted with 10 mL of IMAC buffer at pH 4.5. Due to the recombinant sMltA being eluted at pH 4.5, a pH 6.0 wash was added to this purification. The sample that had been incubated for 1 h produced a higher yield of purified sMltA than the sample that had been incubated for 5 min (Fig. 3.12). Both samples had a similar level of contamination and a similar empirical concentration; therefore samples were pooled for future experimentation. The initial protein concentration was 0.4574 g/L and the sample was concentrated to 1.4266 g/L. Compared to the previous purification (Fig. 3.11), protein yield was high. However, more contaminants eluted at pH 4.5 and purified sMltA was not as pure. The estimated
masses of the sMltA bands by SDS-PAGE and Western blot analysis were comparable to the predicted mass of sMltA (40.6 and 41.2 kDa, respectively, vs. 40 kDa).

Figure 3.12 SDS-PAGE and Western analysis of sMltA purified from *E. coli* BL21 (DE3) with various incubation times with Co<sup>2+</sup> resin and elution at pH 4.5. Samples were taken at various points during protein purification for analysis by (a) SDS-PAGE with Coomassie Brilliant Blue staining and (b) Western blot using anti-His-6 antibody. Two tandem purifications were performed, with incubation times of 5 min and 1 h, respectively. UC: uninduced cell pellet; IC: induced cell pellet; CL: cleared cell lysate; IS: insoluble fraction following cell lysis; FT: IMAC flow-through; W1: IMAC wash 1; W2: IMAC wash 2; W3: IMAC wash 3; EL: IMAC elution; M, Precision Plus Protein Dual Color Standards. Molecular masses of standards are reported in kDa. The red box denotes the expected location of sMltA, and the estimated molecular masses of the bands of interest are in red font.

For all subsequent sMltA purifications, incubation times between 5 min and 1 h were used because the length of incubation of the cell lysate with Co<sup>2+</sup> resin did not seem to improve binding to the resin or the purity of sMltA after elution. Recombinant sMltA was eluted at pH 6.0, following 150 mL washes at pH 8.0 and pH 7.0, as this modification produced a purer sample than elution at pH 4.5. To further increase the yield, protein was purified from 4-6 L of started culture. No more than 2 L cell pellets were lysed together in 25 mL of IMAC buffer. Cell lysate from 2 L of culture (in 25 mL) was always incubated with 0.75 mL Co<sup>2+</sup> resin, and no more than this volume was ever passed through one column. Figure 3.13 is representative of such a purification, where sMltA was purified from 4 L of starter culture and two columns were used. All elutions were pooled at the end of the experiment and protein concentration was approx. 0.2 g/L. Purified recombinant sMltA was concentrated by Amicon to approx. 2.0 g/L. This method was used for all subsequent sMltA preparations and
enzyme purified this way was used in the turbidometric assays to test for inhibition by Ivyp1 and Ivyp2. In Fig. 3.13, the estimated masses of the bands are higher than the predicted mass of recombinant sMltA. In particular, the lanes on the Western blot (b), did not run evenly, and thus masses estimated varied.

**Figure 3.13 SDS-PAGE and Western analysis of sMltA purified by IMAC after a 1 h incubation with CO\textsuperscript{2+} resin and elution at pH 6.0.** Samples were taken at various points during protein purification for analysis by (a) SDS-PAGE with Coomassie Brilliant Blue staining and (b) Western blot using anti-His-6 antibody. Samples 1 and 2 were purified identically. UC: uninduced cell pellet; IC: induced cell pellet; CL: cleared cell lysate; IS: insoluble fraction following cell lysis; FT: IMAC flow-through; W1: IMAC wash 1; EL: IMAC elution; W2: IMAC wash 2; M, Precision Plus Protein Dual Color Standards. Molecular masses of standards are reported in kDa. The red box denotes the expected location of sMltA, and the estimated molecular masses of the bands of interest are in red font.

### 3.7 Inhibition of Lytic Enzymes by Ivyp1 and Ivyp2 using Turbidometry

Earlier, Ivyp1 and Ivyp2 were investigated as potential inhibitors of sMltB to test the hypothesis that the physiological function of the Ivy proteins is to provide substrate-level control of LTs (Clarke et al., 2010). The question remained as to whether the Ivy proteins are general inhibitors of LTs, or that the observed inhibition was specific to sMltB. In the current study, the inhibition of sMltA was investigated, and sMltB was used as a positive control for the Ivy proteins (Clarke et al., 2010). Purified Ivyp1 and Ivyp2 were tested in the turbidometric assay, originally described by Hash (1967), against purified LTs. Inhibition of HEWL by Ivyp1 was also performed for comparison between lysozyme and LT inhibition, since Ivyp1 is known to inhibit HEWL (Abergel et al., 2007).
3.7.1 sMltB inhibition by Ivyp1 and Ivyp2

sMltB was inhibited by Ivyp1 and Ivyp2 as a positive control for both the assay and the inhibitory capability of purified Ivy proteins (Fig. 3.14). Results were comparable to previously published data (Clarke et al., 2010). The experiment was performed as in Clarke et al., 2010, although enzyme and inhibitor concentrations were different. Due to the biphasic nature of sMltB activity, rates of activity were calculated from the first 7 minutes of the assay. A 4 × molar equivalent of Ivyp1 and Ivyp2 was tested against sMltB and it was found that 43.4% of the initial activity of sMltB remained after pre-incubation with Ivyp1 and 54.7% of the initial activity remained after pre-incubation with Ivyp2 (Table 3.3).

Figure 3.14 Inhibition of sMltB by Ivyp1 and Ivyp2. Controls include cells only (open squares), 6 μM Ivyp1 (open triangles), 6 μM Ivyp2 (open circles), and 1.5 μM sMltB (closed squares). Inhibition of 1.5 μM sMltB was performed with 6 μM Ivyp1 (closed triangles) and 6 μM Ivyp2 (closed circles). The OD_{600} was monitored for 45 min. Data are representative.
Table 3.3 Inhibition of sMltB by Ivyp1 and Ivyp2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Activity</th>
<th>% Residual</th>
</tr>
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<tbody>
<tr>
<td>sMltB</td>
<td>0.454</td>
<td>100</td>
</tr>
<tr>
<td>sMltB + 6.0 µM Ivyp1</td>
<td>0.197</td>
<td>43.4</td>
</tr>
<tr>
<td>sMltB + 6.0 µM Ivyp2</td>
<td>0.248</td>
<td>54.7</td>
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1. Each reaction contained 1.5 µM sMltB in 25 mM NaPO₄, pH 5.8, with 100 mM NaCl and 0.1% v/v Triton X-100, and the decrease in OD₆₀₀ of 0.4 µg/mL M. luteus whole cells was monitored over the first 7 minutes of reaction. Rates were normalised by subtracting the rate of settling cells in the absence of enzyme.

3.7.2 sMltA activity and inhibition

The activity of purified sMltA was measured using the turbidometric assay, with M. luteus whole cells as the substrate for the LT. sMltA had not been tested in the turbidometric assays and thus, the assay had to be optimised for this LT. sMltA was active in 25 mM MES or 25 mM NaPO₄ buffer at pH 5.5 with 100 mM NaCl. sMltA was also active in the presence of 10% v/v glycerol, but activity was lost in the presence of 0.1% v/v Triton X-100. Salt concentrations above 100 mM were also inhibitory to sMltA. Activity was lost in the presence of imidazole (concentrations as low as 20 mM) and MgCl₂ (concentrations as low as 10 mM). After optimisation of the assay conditions, three different concentrations of sMltA were tested (1.5 µM, 3.0 µM, and 6.0 µM) by the turbidometric assay (Fig. 3.15). HEWL was used as a positive control. Activity rates were reported as a percent change in the OD₆₀₀ over time and are tabulated in Table 3.4. It was found that sMltA is active on M. luteus whole cell PG but this enzyme has a lower rate of activity than sMltB.
Figure 3.15 Activity of sMltA and HEWL acting on *M. luteus* whole cells. Solubilisation of *M. luteus* whole cells was measured by monitoring the OD$_{600}$ of the cell suspension over 20 min. Purified sMltA was added to the cell suspension at concentrations of: 0 μM (open circles), 1.5 μM (closed squares), 3.0 μM (closed triangles), and 6.0 μM (closed circles). HEWL (0.14 μM – closed diamonds) was used as a positive control. Data are representative.

Figure 3.16 shows the sMltA activity assays from Fig. 3.15 in closer detail as well as the linear relationship between enzyme activity and enzyme concentration. The activity curve of sMltA has a different shape than that of sMltB, which is biphasic and exhibits a change in rate (Blackburn and Clarke, 2001). The activity of sMltA more closely resembles that of sMltF, in that the rate of activity is constant throughout the reaction (Scheurwater *et al*., 2008). Assays were carried out for as long as 2 h and the rate was constant. Enzymatic rate increased with increasing enzyme concentration, and the relationship between enzyme rate and concentration was linear. sMltA at 3.0 μM was measured in triplicate using this assay to produce a mean rate of 0.0509 Δ% OD/min ± 0.0022.
Figure 3.16 Activity of sMltA and relationship between sMltA activity and concentration. Solubilisation of *M. luteus* whole cells was measured by monitoring the $\text{OD}_{600}$ of the cell suspension over 20 min. Purified sMltA was added to the cell suspension at concentrations of: 0 μM (open circles), 1.5 μM (closed squares), 3.0 μM (closed triangles), and 6.0 μM (closed circles). Inset: the activities of different concentrations of sMltA were plotted against enzyme concentrations. $R^2 = 0.924$. Data are representative.

Controls for Ivyp1 and Ivyp2 were conducted using the turbidometric assay to ensure that the optimal conditions for sMltA were also suitable for Ivyp1 and Ivyp2 (Fig. 3.17). Under some buffering conditions, Ivyp1 would precipitate out of solution, causing the $\text{OD}_{600}$ to increase above the initial reading. Conversely, under different conditions, a decrease in the $\text{OD}_{600}$ readings greater than that seen in the negative cell control was observed in the Ivyp2 control. It was found that omitting either glycerol or Triton X-100 in the assay buffer helped maintain Ivyp1 and Ivyp2 in solution without significant changes to the $\text{OD}_{600}$ reading over time. Under optimal conditions for both Ivy inhibitors, the rate of cell settling was similar compared to the negative cell control (Fig. 3.17).
Figure 3.17 Negative control for Ivyp1 and Ivyp2. Turbidity of *M. luteus* whole cells was measured by monitoring the OD<sub>600</sub> of the cell suspension over 20 min. Three µM purified Ivyp1 (squares) and 3 µM purified Ivyp2 (triangles) was added to the cell suspension. Open symbols represent unsuccessful controls, and closed symbols represent ideal Ivy controls. The negative cell control is represented by the open circles. Data are representative.

With optimal assay conditions found for both sMltA and the Ivy proteins, inhibition of sMltA by Ivyp1 and Ivyp2 was tested. sMltA was pre-incubated with a two-fold molar excess of Ivyp1 or Ivyp2 and added to a cell suspension of *M. luteus* whole cells and the decrease in OD<sub>600</sub> over time was monitored (Fig. 3.18). As seen from the data presented in Table 3.4, the initial activity of control sMltA was low compared to that of sMltB despite the use of twice as much enzyme in the reaction (3.0 µM sMltA vs. 1.5 µM sMltB). Nonetheless, inhibition of lytic activity was observed in the presence of both Ivyp1 and Ivyp2. The two-fold molar excess of Ivyp1 and Ivyp2 caused a 77% and 88% inhibition of sMltA, respectively, under the conditions employed. These data suggest that the Ivy proteins are more potent inhibitors of sMltA compared to sMltB, even after accounting for the low activity of sMltA.
Figure 3.18 Inhibition of sMltA by Ivyp1 and Ivyp2. Turbidity of *M. luteus* whole cells was measured by monitoring the OD\textsubscript{600} of the cell suspension over 20 min. The activity of sMltA (3 µM) was monitored in the absence (closed circles) and presence of 6 µM of the proteinaceous inhibitors Ivyp1 (closed squares) and Ivyp2 (closed triangles). Open circles represent the negative cell control. Data are representative.

Table 3.4 Inhibition of sMltA by Ivyp1 and Ivyp2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Activity</th>
<th>Δ% OD/min</th>
<th>% Residual</th>
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</thead>
<tbody>
<tr>
<td>sMltA</td>
<td>0.0509 ± 0.0022(^2)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>sMltA + 6.0 µM Ivyp1</td>
<td>0.0118</td>
<td>23.2</td>
<td></td>
</tr>
<tr>
<td>sMltA + 6.0 µM Ivyp2</td>
<td>0.0061</td>
<td>12.0</td>
<td></td>
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</table>

1. Each reaction contained 3.0 µM sMltA in 25 mM NaPO\(_4\), pH 5.5, with 100 mM NaCl, and the decrease in OD\textsubscript{600} of 0.4 µg/mL *M. luteus* whole cells was monitored over the first 20 minutes of reaction. Rates were normalised by subtracting the rate of settling cells in the absence of enzyme.
2. Standard error was calculated for 3.0 µM sMltA.
CHAPTER 4 – DISCUSSION

4.1 MltA Inhibition and Turbidometric Assays

This study provides the first experimental evidence that Ivyp1 and Ivyp2 from *P. aeruginosa* are inhibitors of sMltA. Thus, MltA is the second LT to be inhibited by the Ivy proteins and this evidence confirms that it is not by chance that Ivyp1 and Ivyp2 can inhibit MltB. This finding is especially significant because MltA and MltB are representatives of Family 2 and 3 LTs, respectively, and are therefore quite different in their structure and possibly enzyme specificity. Therefore, the experiments conducted in this study lend support to the hypothesis that Ivy (and the other inhibitor proteins such as MliC) represents a mechanism of LT regulation.

Both Ivyp1 and Ivyp2 proved to inhibit sMltA more strongly than sMltB and this effect may be due to differences in the active site structure between these two LTs and thus their interactions with Ivy could be different. MltA from *E. coli* is known to accommodate sugar residues with no peptides attached (Romeis et al., 1993), while the peptide chain is required for the activity of MltB from *P. aeruginosa* (Blackburn and Clarke, 2000). Although the overall active site structures of these two LTs are similar (van Straaten et al., 2005; van Asselt et al., 1999), family 3 LTs have residues which protrude into the active site and are presumed to interact with the peptide chain of PG (Blackburn and Clarke, 2002). Another possible reason for the difference in inhibition of lytic activity by Ivyp1 and Ivyp1 in sMltA and sMltB could be the differences in rates of activity and patterns of activity over time between the two enzymes. Since sMltA was shown to have a lower rate of activity, it is possible trace lytic activity that remained in the assays and was not detectable from the error. A third possibility is that Ivy inhibitors are specific to certain families of LTs and inhibit the other LTs more weakly. For example, it is possible that the Ivy family are the primary inhibitors of family 2 LTs whereas another inhibitor family, such as MliC or PliG, are the primary inhibitors of family 3 LTs. In contrast, the fact that *P. aeruginosa* encodes two Ivy homologs and four family 3 LTs suggest that Ivy may be specific to family 3 LTs.

Additionally, the turbidometric assays conducted with MltA as the lytic enzyme have produced new findings. sMltA activity was very low compared to other LTs, such as MltB,
and it is hypothesised that this observation could be due to both the mode of action of MltA and the *in vitro* conditions of the assay. Alternatively, sMltA could have lost activity throughout the purification process and the observed activity was residual. MltA exhibited a pattern of lytic activity seen previously only with MltF from *E. coli* (Scheurwater and Clarke, 2008). In contrast to other characterised LTs, such as MltB but like MltF, MltA exhibits a lower overall rate of activity. Also like MltF, this rate of activity appears to be constant over long periods of time and, presumably, until all the PG in solution has been digested as has been shown with MltF. In comparison, MltB has been shown to have an initially rapid rate which slows down over time and plateaus as originally observed by Blackburn and Clarke (2001) and demonstrated in this study.

Scheurwater and Clarke (2008) hypothesised that the different pattern of activity shown by MltF could be explained by MltF being an endo-acting enzyme, cleaving in the middle of glycan strands instead of from the reducing ends. This suggestion is based on the fact that MltF is most similar to MltE of the family 1 LTs and MltE from *E. coli* is known to be an endo-acting LT (Kraft *et al.*, 1998). However, it was also suggested that the lower rate of activity for MltF could be due to the imperfect conditions of the assay. *In vivo*, the LTs are associated in biosynthetic complexes with other PG-acting enzymes, including the PBPs (reviewed in Pfeffer *et al.*, 2012). Thus, it could be that these associations provide a microenvironment more optimal for LT activity than the *in vitro* conditions provided by the turbidimetric assay. Thus, it is possible that MltA and MltF require additional factors to provide favourable enzymatic conditions, such as the presence of interacting proteins. Finally, the lower activity of sMltA could be caused by the enzyme being engineered for production in the lab. The missing residues involved in membrane anchorage could play an important role in overall enzyme structure or activity. Alternatively, the His-6 tag could interfere with proper enzyme function. In the case of MltB, these structural changes to the enzyme may not be as important, since a soluble derivative of MltB called Slt35 is known to naturally occur in *E. coli* (Engel *et al.*, 1992). It is, therefore, possible that the soluble derivative of MltB from *P. aeruginosa* used in this study can also act more readily on PG than the soluble derivative of MltA.
If MltA is an endo-acting enzyme, a new assay should be developed to measure the rate of activity that is more sensitive to the larger PG fragments produced by endo-acting LTs. The current assay, which is useful for exo-acting enzymes, monitors the lysing of *M. luteus* cells and is, therefore, an inappropriate assay for endo-acting LTs because the majority of the PG fragments released from the sacculus by endo-acting LTs remain insoluble in solution and contribute to the OD readings. Unfortunately, a PG substrate is not commercially available and LTs cannot act upon chitooligosaccharides (Blackburn and Clarke, 2000). Homogeneous PG fragments of a defined length with identical peptide sidechains would be the preferred substrate since other LTs require the peptide chain for proper substrate binding. Even if MltA was not an endo-acting LT, its lower activity requires a more sensitive assay to detect lytic activity from background errors more easily, especially in the case of inhibition trials. Ongoing work in our lab involves the development of a homogeneous PG substrate with peptide chains for such assays. An alternative to a turbidometry-based assay is a high-performance liquid chromatography (HPLC)-based assay which separates the reaction products allowing detection of anhydroMurNAc residues (Blackburn and Clarke, 2000). The anhydroMurNAc residues can be quantified and the initial rate of reaction can be detected. This assay also allows for more exact calculations of rates and kinetic analysis. This method was not investigated because it is tedious and time-consuming, and reliable HPLC equipment was not available.

The most complicated step in the inhibition of sMltA was the establishment of ideal conditions for Ivyp1 and Ivyp2. While Ivy proteins tended to precipitate at the low salt concentrations required for LT activity, the addition of stabilising agents such as glycerol or Triton X-100 caused interfering effects on the Ivy control inhibitions (against HEWL). Ivyp1 was often seen to increase the OD600 and it is hypothesised that this was due to ongoing precipitation of the protein during the course of inhibition reactions. In contrast, Ivyp2 alone was observed to decrease the OD600. This effect had been observed with both Ivyp1 and Ivyp2 in previous research in the laboratory (unpublished data), and it was postulated then that perhaps the detectable PG-degradation was due to the carry-over of LTs from the *E. coli* host used for the production of Ivy. Attempts to overcome this issue by purifying the inhibitor proteins in an LT-knockout strain (*E. coli* MHD79) were not met with any success. However, during the initial MltB inhibition assays (Clarke et al. 2010), Ivyp1 and Ivyp2 were
dialysed into assay buffer containing 0.1% v/v Triton X-100 and the proteins did not exhibit these anomalous patterns. The observed behaviours of Ivyp1 and Ivyp2 in different buffers were inconsistent despite repeated attempts to reproduce conditions that had been successful in one or two earlier trials. Eventually, it was found that buffers with minimal components were best although results were still not always consistent, making the reproducibility of results difficult. While it is unclear why soluble, periplasmic proteins such as Ivy should be so unstable under typical *in vitro* buffering conditions, it can be speculated that the periplasm provides a very specific microenvironment for the inhibitor proteins. It is possible that in the periplasm, Ivy interacts closely with other PG-acting enzymes, such as the PBPs for example, during the PG biosynthesis steps of PG recycling. Also, specific factors in the periplasm, such as anhydromuropeptides released during excessive PG lysis or β-lactam antibiotic threat, may promote the inhibition of LTs by Ivy. Indeed, it is known that Ivy production is induced by β-lactam presence or other PG-related stress (Laubacher and Ades, 2008) and so it is possible that Ivy activity in the periplasm is also being stimulated by other signalling factors. Both Ivyp1 and Ivyp2 were observed to be stable in buffers with higher salt concentrations for up to a month, so it is also possible that the periplasm provides the ions needed to promote Ivy solubility.

4.2 Production and Purification of Ivyp1, Ivyp2, and sMtlA

Recombinant Ivyp1 and Ivyp2 production and purification from *E. coli* was achieved in levels suitable for turbidometric assays. One interesting observation in regard to the production of Ivy was that *E. coli* BL21 (DE3) cells over-producing Ivyp2 always grew more slowly than those over-producing Ivyp1. *E. coli* does not encode an Ivy paralog and so it is possible that Ivyp2 is interfering with normal LT activity in this bacterium, leading to slower growth. Another ongoing problem that was not solved by any methods tried was the difficulty in lysing these cells. Three different lysis methods were attempted as well as a pretreatment of osmotic shock, but nothing improved cell lysis. Lysozyme cannot be added to cells over-producing Ivy as it will co-purify with Ivy (as previously seen by Monchois *et al.*, 2001) and interfere with downstream experiments. It is not surprising that *E. coli* MHD79 (DE3) cells over-producing Ivy proteins did not easily lyse, due to the combination of the under-production of LTs and the over-production of Ivyp1 or Ivyp2, but even *E. coli* BL21
(DE3) cells (with all LT genes intact) were difficult to lyse. Although leftover cell pellets were never weighed for actual calculation of unlysed cells, it is estimated that between 10-20% of the cells over-producing Ivy did not lyse even after rigorous attempts.

Both Ivyp1 and Ivyp2 were purified successfully from *E. coli* BL21 (DE3) by methods similar to those published in Clarke *et al.*, 2010. Differences between the protocols include the use of an imidazole gradient in this study instead of a pH gradient for IMAC elutions, and the inclusion of 10% v/v glycerol in this study. Both a higher overall salt concentration and the presence of a stabilising agent, such as glycerol, helped to keep Ivy soluble following purification. *E. coli* MHD79 (DE3) proved not to be an acceptable host strain for Ivyp1 and Ivyp2 overproduction and purification. Some of the problems seen were associated with the strain itself, which lacks six of seven LTs and consequently has some growth defects. The slow growth associated with this strain can likely be attributed to the difficulty this strain has in septation, but the cells may also have difficulty expanding their sacculi to grow larger before division events (Heidrich *et al.*, 2002). However, other problems identified with Ivy over-production in this strain included the inability of the cell to export full-length Ivy and the overall low expression of both *ivy* genes. It is likely that all of the problems can be attributed to the LT-deficient nature of *E. coli* MHD79 (DE3) which would make this strain more sensitive to an LT inhibitor such as Ivy. Therefore, the bacterium may be degrading excess Ivy to remain viable, leading to the appearance of low expression. Low expression could be ruled out by examining levels of the mRNA encoding recombinant Ivy. Alternatively, expression could be low due to the cell not having enough resources to produce large amounts of Ivy. The *E. coli* MHD79 (DE3) cells are not only struggling to grow and divide due to the lack of LTs, but they are also putting energy into replicating the pET vectors and producing the β-lactamase required for ampicillin resistance while under considerable PG stress. Thus, it is not surprising that this strain is likely not suitable to the over-expression of any protein, especially an LT inhibitor that will further interfere with PG recycling and cell growth.

The MALDI-TOF experiment conducted on samples of purified Ivyp2 from *E. coli* MHD79 (DE3) did not yield any helpful results in terms of identifying the contaminant suspected to be MltF. The method chosen was appropriate as shown by the success in positively
identifying Ivyp2. However, the suspected MltF contaminants were not separated well enough by SDS-PAGE from other contaminants of similar molecular weights and since these proteins were present in low quantities to begin with, the ionisation of the peptides was not successful. With higher resolution on SDS-PAGE and a more concentrated protein sample, it is possible that this technique could have been successful for identifying a contaminant protein. However, MS/MS would have been the best way to confirm the identity of any protein, as it can be used to decipher the exact amino acid composition of a peptide, leading to positive identification of an unknown protein.

The protocol for the production and purification of sMltA from \textit{E. coli} BL21 (DE3) pLysS was improved upon from that developed by C. Vandenende (unpublished data). Important for this study was the production of high yields of enzyme since the assays conducted were carried out in 1-mL volumes. As such, the omission of Triton X-100 and elution at a lower pH during IMAC proved to increase the yields of recombinant protein. However, production of sMltA was never improved to the point where a sufficient amount of protein could be purified from 1 L of bacterial cell culture. This low yield was because a significant amount of protein was lost during each step of the purification process. This loss was increased when cell pellets from more than 1 L of culture were pooled into 25 mL of lysis buffer and run together on one disposable column packed with Ni$^{2+}$-NTA resin. In hindsight, it would have been best to process the cell lysate from each litre of culture individually to minimise the loss of recombinant protein.

\textbf{4.3 Conclusions and Future Directions}

Although this work is promising since it lends further evidence to the hypothesis that Ivyp1 and Ivyp2 are LT inhibitors, there are still many unanswered questions. For example, it would be useful to have kinetic parameters of the interactions between Ivyp1 and Ivyp2, and MltA and MltB. Testing various molar ratios of inhibitor:LT would also be useful in determining the potency of these inhibitors towards the LTs. Additionally, inhibition studies have only been conducted on two of the nine LTs encoded by \textit{P. aeruginosa} and it is likely that the other LTs can be inhibited by Ivyp1 and Ivyp2. A next logical step would be to test a Family 1 LT; however, there are five subfamilies in Family 1 so it would be interesting to see if Ivyp1 and Ivyp2 can inhibit all Family 1 LTs, as well as the three other SltB isozymes in \textit{P. aeruginosa}. 69
aeruginosa. It also remains to be confirmed by crystallography or biochemical studies how Ivyp1 and Ivyp2 interact with their LT targets, although it can be hypothesised with good confidence that this mode of inhibition would be similar to that of the lysozymes (Abergel et al., 2007) given the similarity of their respective structures. Furthermore, an investigation into the LT inhibitory activity of the other putative LT inhibitors is necessary to confirm the role of these proteins as an LT regulation mechanism in some Gram-negative bacteria. To date, none of the MliC/PliC, PliG, or PliI inhibitors have been tested for LT inhibition. Finally, it remains to be seen whether we have found all the potential LT inhibitor families. Their redundancy is not surprising and it is possible that other families of inhibitors exist. Ongoing work in our lab is attempting to answer this question by “fishing” with LTs for potential binding partners. Knock-out studies are also being conducted to determine if viable knock-outs of all known inhibitors in P. aeruginosa can be created.

The results of the experiments conducted in this study provide more support to the hypothesis that Ivyp1, Ivyp2, and likely all “lysozyme” inhibitors are inhibitors of LTs and are involved in LT regulation. Additionally, better protocols were established through this project for the purification of Ivyp1, Ivyp2, and sMltA, as well as a modified protocol for LT inhibition studies using the turbidometric assay. Indeed, these protocols have already been used in the lab by other students with success. While unanswered questions remain, it is clear that the LTs are a good candidate for antibiotic development due the necessity of both their activity and the regulation of this activity. Thus, LT regulation mechanisms such as Ivy and the other inhibitors pose as equally attractive antibiotic targets. Antibiotics targeted to this regulation mechanism could be useful in the treatment of infection by many important pathogenic species such as E. coli, P. aeruginosa, and Klebsiella pneumoniae, among others.
APPENDIX A – MEDIA, SOLUTIONS, AND REAGENTS

A.1 Bacterial media and solutions

- LB medium: 10 g/L tryptone peptone, 5 g/L yeast extract, 10 g/L NaCl
- LB agar (1.5%): LB medium supplemented with 15 g/L agar
- SB medium: 32 g/L tryptone peptone, 20 g/L yeast extract, 5 g/L NaCl
- Terrific broth medium: 12 g tryptone peptone, 24 g yeast extract, 4 mL glycerol in 900 mL water; sterilize and add 100 mL sterile KPO₄ solution
- KPO₄ solution: 2.31 g KH₂PO₄, 12.54 g K₂HPO₄ in 100 mL water

A.2 DNA solutions

- 0.5 M EDTA: 14.61 g EDTA in 100 mL water; pH 8.0 using NaOH
- TAE buffer (50×): 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA solution, add water to final volume of 1 L
- 0.8% w/v agarose gel: 0.24 g agarose in 30 mL TAE buffer
- Solution α: 3 mL 1 M KCH₃CO₂, 10 mL 1M KCl, 1 mL 1 M CaCl₂, 5 mL 1 M MnCl₂, 30 mL 50% v/v glycerol, add water to final volume of 100 mL; pH 5.8 using 0.2 M acetic acid and filter sterilize
- Solution β: 100 µL 1 M MOPS, 750 µL 1 M CaCl₂, 100 µL 1 M KCl, 3 mL 50% v/v glycerol, add water to final volume of 10 mL; pH 6.5 with 1 M KOH and filter sterilize
- TE buffer: 0.06 g Tris, 0.02 g Na₂EDTA•2H₂O in 50 mL water; pH 8.0 using HCl
- SRL lysis solution: 1 mL 25% w/v sucrose solution (in water) with a pinch of HEWL and RNase A
TB buffer (5×): 5.45 g Tris, 2.78 g boric acid, 0.47 g Na₂EDTA•2H₂O in 100 mL water

A.3 Protein solutions and reagents

20% w/v sucrose solution: 1.82 g Tris, 100 g sucrose, 1 mL 0.5 M EDTA, add water to final volume of 500 mL

Table A.1 SDS-PAGE gel preparations

<table>
<thead>
<tr>
<th>Reagents</th>
<th>12% resolving gel</th>
<th>15% resolving gel</th>
<th>4% stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide/Bis (30%)</td>
<td>4 mL</td>
<td>5 mL</td>
<td>1.35 mL</td>
</tr>
<tr>
<td>Water</td>
<td>3.35 mL</td>
<td>2.35 mL</td>
<td>6.05 mL</td>
</tr>
<tr>
<td>1.5 M Tris-HCl pH 8.8</td>
<td>2.5 mL</td>
<td>2.5 mL</td>
<td>-</td>
</tr>
<tr>
<td>0.5 M Tris-HCl pH 6.8</td>
<td>-</td>
<td>-</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>10% w/v SDS</td>
<td>100 μL</td>
<td>100 μL</td>
<td>100 μL</td>
</tr>
<tr>
<td>30% w/v APS</td>
<td>100 μL</td>
<td>100 μL</td>
<td>100 μL</td>
</tr>
<tr>
<td>Tetramethylethylenediamine (TEMED)</td>
<td>5 μL</td>
<td>5 μL</td>
<td>10 μL</td>
</tr>
</tbody>
</table>

Western transfer buffer: 0.84 g NaHCO₃, 0.32 g Na₂CO₃, 0.2 g SDS, 200 mL methanol, add water to final volume of 1 L

5× TBS: 3.03 g Tris, 21.92 g NaCl, add water to final volume of 500 mL; pH 7.5 using HCl

5× TTBS: 6.06 g Tris, 73 g NaCl, 5 mL Triton X-100, add water to final volume of 500 mL; pH 7.5 using HCl

Blocking buffer: 0.9 g BSA in 30 mL 1× TBS

Primary antibody solution: 30 μL primary antibody in 30 mL blocking buffer

Secondary antibody solution: 15 μL secondary antibody in 30 mL blocking buffer

Assay buffer (sMltB): 25 mM NaPO₄, 100 mM NaCl, 0.1% v/v Triton X-100; pH 5.8 using phosphoric acid

Assay buffer (sMltA): 25 mM NaPO₄, 100 mM NaCl; pH 5.8 using phosphoric acid
### A.4 SDS-PAGE sample preparation

#### Table A.2 Sample preparation for SDS-PAGE

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Sample preparation</th>
<th>Volume 5× sample buffer added (μL)</th>
<th>Volume loaded on gel (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell fractions (uninduced and induced)</td>
<td>Resuspend 1mL cell pellets in 100 μL H₂O</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>Cell lysate</td>
<td>Mix 5 μL of lysate with 100 μL H₂O</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>Insoluble material after lysis</td>
<td>Resuspend pellet in 100 μL H₂O, aliquot 5 μL into a new tube and dilute with 100 μL H₂O</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>IMAC flow-through</td>
<td>Mix 5 μL of flow-through with 100 μL H₂O</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>IMAC washes</td>
<td>28 μL, not diluted</td>
<td>7</td>
<td>First wash: 5; other washes: 12-15</td>
</tr>
<tr>
<td>IMAC elution</td>
<td>28 μL, not diluted</td>
<td>7</td>
<td>12-15</td>
</tr>
</tbody>
</table>
REFERENCES


