Inhibition of Membrane-Bound Lytic Transglycosylases A, B and F by Membrane-Bound Lysozyme Inhibitor of C-type Lysozyme

by

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A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Molecular and Cellular Biology

Guelph, Ontario, Canada

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ABSTRACT

Inhibition of Membrane-Bound Lytic Transglycosylases A and F by Membrane-Bound Lysozyme Inhibitor of C-type Lysozyme

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Peptidoglycan (PG) is an essential component of bacterial cells that forms a mesh-like sacculus that surrounds the cell. Lytic transglycosylases (LTs) are space-making enzymes that are essential for the cleavage of glycosidic linkages in the PG sacculus. This study proposes that the physiological function of the proteinaceous inhibitor, membrane-bound lysozyme inhibitor of C-type lysozyme (MliC) from P. aeruginosa, is to control LT activity from the same bacterium. This study examined the role of MliC as an inhibitor of the soluble-derivatives of membrane-bound lytic transglycosylases A (sMltA), B (sMltB) and F (sMltF). Inhibition of sMltA and sMltF was demonstrated in vitro at a 1:1 equivalent molar ratio (inhibitor: LT), and inhibition of sMltB was found at a 4:1 equivalent molar ratio using the turbidimetric assay. These results provide the first experimental evidence supporting the hypothesis that the true physiological function of MliC is to control the autolytic activity of LTs.
Acknowledgements

I would like to thank my advisor, Dr. Anthony Clarke for his guidance and encouragement throughout my project. Thank you for supporting me throughout my undergraduate and graduate experiences. You provided me an opportunity to learn and grow not only as a scientist, but as a person and I will be forever grateful. I would also like to thank my advisory committee member Dr. Cezar Khursigara for his words of encouragement and support whenever I needed it. I would also like to thank him for inspiring me to pursue graduate studies and to become a better researcher.

I would also like to extend a thank you to all the past and present members of the Clarke Lab. I would like to especially thank David Sychantha for his mentorship and teaching. Thank you answering my many questions I asked as an undergraduate and for teaching me the essential skills for me to become successful in the future. I would also like to thank Chris Vandennende, for his technical support and counsel. Thanks also go to Carys Jones, Francesca Herlihey, Ashley Brott, and Joshua Chun, along with David Sychantha and Chris Vandennende for their friendships and support thought my studies.

I would like to thank my friends and family. I am forever grateful to my parents who supported me not matter the choices I made. And for making sacrifices so I can pursue my goals. Lastly I could like to thank my wife-to-be Gabriela Zimmermann. Thank you for the unconditional love and support throughout this journey, and for being there in the good times and the bad.
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List of Abbreviations

Ami     Amidase
Amp     Ampicillin
AnhydroMurNAc  1,6-Anhydro-N-acetylmuramic acid
BCA     Bicinchorinic acid
BSA     Bovine serum albumin
Cm      Chloramphenicol
CP      Carboxypeptidase
C-type  Chicken-type
DNA     Deoxynucleotide acid
EP      Endopeptidase
FITC    Fluorescein isothiocyanate isomer I
GEWL    Goose egg-white lysozyme
GlcNAc  N-acetylglucosamine
GST     Glutathione-S-transferase
HEWL    Hen egg-white lysozyme
IMAC    Immobilized metal affinity chromatography
IM      Inner membrane
IPTG    Isopropyl β-D-1-thiogalactopyranoside
Ivy     Inhibitor of vertebrate lysozyme
Kan     Kanamycin
LB      Luria-Bertani
LT      Lytic transglycosylase
Ni²⁺-NTA Ni²⁺-nitrilotriacetic acid
m-DAP   meso-diaminopimelic acid
Mip     MltA interacting protein
MliC    Membrane-bound lysozyme inhibitor of C-type lysozyme
Mlt     Membrane-bound lytic transglycosylase
MurNAc  N-acetylmuramic acid
MWCO    Molecular Weight Cut-off
<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>NBT-BCIP</td>
<td>Nitro-blue tetrazolium 5-bromo-4-chloro-3-indolylphosphate</td>
</tr>
<tr>
<td>Oat</td>
<td>O-acetyltransferase</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OM</td>
<td>Outer membrane</td>
</tr>
<tr>
<td>PBP</td>
<td>Peptidoglycan-binding protein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein data bank</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PG</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PliC</td>
<td>Periplasmic lysozyme inhibitor of C-type lysozyme</td>
</tr>
<tr>
<td>RlpA</td>
<td>Rare lipoprotein A</td>
</tr>
<tr>
<td>SB</td>
<td>SuperBroth</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Slt</td>
<td>Soluble lytic transglycosylase</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
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Chapter 1. Introduction

1.1 General Introduction

*Pseudomonas aeruginosa* is a Gram-negative, rod-shaped bacterium that is commonly found in soil, and fresh water environments. It is a commensal organism on human skin that possesses the ability to transform into an opportunistic pathogen in immunocompromised individuals, such as those who suffer with AIDS or cancer, and those where a breach of host tissue barriers is present (*i.e.* burn victims) (Van Delden and Iglewski, 1998). The abundance of this organism on human skin and its ability to transform into an opportunistic pathogen makes it an important nosocomial pathogen that affects a wide range of patients in hospitals. *P. aeruginosa* is also well-known to be the major pathogen involved with infections of Cystic Fibrosis patients, due to its ability to cause persistent infections and form biofilms that are difficult to treat with conventional antibiotics (Van Delden and Iglewski, 1998). *P. aeruginosa* has developed resistance to commonly used antibiotics such as β-lactams, aminoglycosides and fluoroquinolones, and this presents a serious threat to infected patients (Lister *et al*., 2009). An antibiotic resistance threat report recently published in 2017 by the World Health Organization (WHO), listed carbapenem-resistant *P. aeruginosa* as a critical threat that requires immediate attention (WHO, 2017). Another report published by the Centers for Disease control and Prevention (CDC) in 2013 listed that multi-drug resistant *P. aeruginosa* to cause over 51,000 new healthcare cases associated infections per year in the USA, which represents 8% of all healthcare-associated infections (CDC, 2013). In addition, they estimated that over 6,700 multi-resistant *P. aeruginosa* infections would lead to over 440 deaths per year (CDC, 2013).

There is an increasing need for novel antibiotic treatments for multi-drug resistant organisms. *P. aeruginosa* belongs to the ESKAPE group of pathogens (*Enterococcus faecium,
Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, P. aeruginosa and Enterobacter spp.) where treatment options are limited (Rice, 2008). The cellular process involved with the synthesis and recycling of peptidoglycan (PG) represents ideal targets for antibiotic treatments and discovery. There are already classes of antibiotics, such as β-lactams, which target PG biosynthesis and glycopeptides which bind PG to inhibit recycling/biosynthesis (Davies and Davies, 2010). However as previously mentioned, P. aeruginosa has developed resistance to these classes of antibiotics and this presents a major problem with regards to treatment for these infections (Davies and Davies, 2010).

PG still represents a viable target for the development of novel antibiotic treatment however; the discovery of new antibiotics targeting the structure of PG and its biosynthesis is dependent on understanding the enzymes responsible for these pathways. The lytic transglycosylases (LTs) have emerged as an attractive class of PG-active enzymes for antibiotic targeting, as they are involved with PG recycling and degradation (Scheurwater et al., 2008). They are critical to bacterial cell function as well as reproduction, and they act on an essential structure that is unique to most bacteria. A challenge to the development of LTs as a new target is the lack of knowledge of how LTs are controlled and regulated in the cell. Developing an understanding of how LTs are controlled will provide useful information, which may reinforce the proposal that this class of unique enzymes may pose as an attractive antibiotic target in the bacterial cell.

1.2 Peptidoglycan Structure and Architecture

PG is a dynamic, essential structural element of the cell wall that is present in all known eubacteria with the exception of members of Mycoplasmas and Planctomycetes genera. This heteropolymer is made up of glycan strands and peptide chains that form a continuous mesh-like structure known as the sacculus (Vollmer et al., 2008a). The function of the sacculus is to
maintain cellular shape and integrity and to protect the cell from lysis due to internal turgor pressure (Vollmer et al., 2008a). The glycan strands are comprised of alternating units of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues that are linked by short “stem” peptides attached to the C-3 lactyl moiety of MurNAc residues. The alternating GlcNAc and MurNAc residues are joined by β-1,4 glycosidic linkages, which comprise the glycan backbone of PG (Fig. 1.1). PG structure can be modified with variations within the stem peptides and peptide bridges and/or modification to the glycan backbone, such as N-deacetylation, N-glycolylation and O-acetylation (Vollmer, 2008).

![Figure 1.1 PG structure.](image)

In Gram-negative bacteria, glycan strands terminate with 1,6-anhydroMurNAc residues, the product of lytic transglycosylase (LT) activity, and the length of the glycan chain varies
based on external and internal stresses and growth conditions (Vollmer et al., 2015). In Gram-positive bacteria, the glycan strands terminate with a MurNAc residue (Vollmer et al., 2008a). There is variation amongst the composition of stem peptides. For example, in most Gram-negative bacteria, such as *P. aeruginosa*, the stem peptide usually consists of L-Ala, D-Glu, *meso*-diaminopimelic acid (*m*-DAP), D-Ala and a terminal D-Ala upon synthesis (Fig. 1.1) (Vollmer, 2008). During PG maturation, adjacent strands are cross-linked between the fourth amino acid (D-Ala) of one stem, and the third amino acid (*m*-DAP) of the other stem and the necessary bond energy for this process is provided by the removal of the terminal D-Ala (Vollmer, 2008; Vollmer et al., 2008a).

In Gram-negative bacteria, there is a thin layer of PG usually 2-3 nm wide that is located in the periplasm between the inner membrane (IM) and the outer membrane (OM) (Fig. 1.2 A) (Dmitriev et al., 1999). The outer membrane of the Gram-negative bacteria acts as a physical barrier to preclude external hazards that would normally disrupt or degrade PG. In addition, *P. aeruginosa* contains lipopolysaccharides (LPS), located on the extracellular face of the OM, which presents another line of defense against external hazards (Fig. 1.2A) (Kocínková and Lam, 2011). In contrast, the PG of Gram-positive bacteria consists of a thick layer (30-40 nm) that has a high degree of cross-linking (Fig. 1.2B) (Labischinski et al., 1991). However, these latter cells lack an outer membrane and so their PG is exposed to external threats (eg. lysozymes or antibiotics) that can be detrimental to the integrity of the cell. As PG is an essential and dynamic structure in bacteria, there is a requirement for constant remodeling and recycling to permit cellular growth, cellular division and insertion of membrane-spanning cellular machinery.
Figure 1.2. Cartoon representation of the Gram-negative and Gram-positive cell walls. (A) Gram-negative PG is thin and is located in between the inner (IM) and outer cell membranes (OM). (B) The Gram-positive cell wall has a thicker PG layer. It is located externally to the cell membrane and embedded with teichoic acid, lipoteichoic acids and surface proteins.

1.2.1 Peptidoglycan Biosynthesis and Recycling

Biosynthesis of PG is a dynamic process that occurs in three phases within the bacterial cell (Vollmer et al., 2008b). The first stage/phase of the PG biosynthetic pathway occurs in the cytoplasm with the conversion of UDP-GlcNAc to UDP-MurNAc (Brown et al., 1995). This step requires two essential enzymes MurA, a UDP-GlcNAc enoylpyruvyl transferase and MurB, a UDP-N-acetylenolpyruvylglucamine reductase (Marquardt et al., 1992; Pucci et al., 1992). The assembly of the stem peptide is catalyzed sequentially by four substrate specific amino acid transferases referred to as the Mur ligases (Mur C, D, E and F) which use ATP as an energy
source (Fig 1.3) (Barreteau et al., 2008). Mur C, D and E are responsible for the addition of the first three amino acids of the stem peptide, while Mur F is responsible for attaching the terminal D-Ala-D-Ala dipeptide to the third amino acid in the stem peptide (Fig. 1.3) (Anderson et al., 1996; Sobral et al., 2006). The second stage of PG biosynthesis involves the assembly of Lipid I and Lipid II. The integral membrane protein MraY catalyzes the transfer of UDP MurNAc-pentapeptide to the undecaprenyl phosphate (C55-P) which is located inner surface of the inner membrane yielding Lipid I (undecaprenyl-phosphoryl-MurNAc pentapeptide) (Fig. 1.3) (Bouhss et al., 2004). Then, MurG catalyzes the addition of a GlcNAc moiety from UDP-GlcNAc to Lipid I, which forms Lipid II (undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc) (Mohammadi et al., 2007). The last stage of PG biosynthesis requires the translocation of Lipid II to the outer face of the cytoplasmic membrane. Lipid II is unable to move across the cytoplasmic membrane unassisted (van Dam et al., 2007). The mechanism as to how Lipid II is translocated is currently under debate and there are two flippase candidates proposed to perform this role. The first candidate FtsW, belongs to the SEDS (shape, elongation, division and sporulation) family, which includes FtsW, RodA, and SpoVE (Mohammadi et al., 2011). The other flippase candidate is MurJ, a member of the MOP (multidrug/oligo-saccharidyl-lipid/polysaccharide) exporter superfamily (Hvorup et al., 2003; Ruiz, 2008). Recently, the crystal structure of MurJ from Thermosiphia africanus was determined and shown to possess an inward confirmation facing the cytoplasmic space, thus providing indirect evidence that interaction between the Lipid II intermediate and MurJ could occur (Kuk et al., 2016). Regardless, Lipid II via FtsW/MurJ and its homologs is translocated across the cytoplasmic membrane and allows growth of the PG sacculus to occur.
Figure 1.3. Schematic representation of PG biosynthesis. The assembly of the stem peptide is catalyzed by four substrate specific amino acid transferases referred to as the Mur ligases (Mur C, D, E and F). MraY catalyzes the transfer of UDP MurNAc-pentapeptide to the undecaprenyl phosphate (C_{55}P) to form Lipid I. Then, MurG catalyzes the addition of a GlcNAc moiety from UDP-GlcNAc to Lipid I, to form Lipid II. The last stage of PG biosynthesis requires the translocation of Lipid II to the outer face of the cytoplasmic membrane which is believed to be flipped by MurJ and/or FtsW. This figure is adapted from (Typas et al., 2011).
Once Lipid II is outside the cell, the extension of the glycan backbone (transglycosylation) and the formation of the peptide cross-linkages (transpeptidation) reactions must occur to form mature PG. The enzymes that preform this role include high-molecular-weight and low-molecular weight penicillin binding proteins (PBPs) (Spratt, 1977). The transglycosylase domain of the high-molecular-weight PBPs forms the β-(1→4)-glycosidic bond between the MurNAc and GlcNAc residues, while the transpeptidase activities of both the high- and low-molecular weight PBPs cross link the stem peptides (Spratt, 1977). Additionally, some low molecular weight PBPs possess endopeptidase and/or carboxypeptidase activities that regulate the extent of crosslinking (Spratt, 1977). Along with PBPs, another important class of enzymes that are essential for the dynamic remodeling nature of PG are muralytic enzymes such as the LTs. PG maintenance and turnover are dynamic processes that rely on the efforts of a variety of multiple enzymes.

1.3 Peptidoglycan Lytic Enzymes

The PG sacculus is a dynamic structure that is constantly being synthesized and degraded maintaining a delicate balance to maintain cell viability. For example, in *Escherichia coli* there will be as much as 50% PG turnover per generation (Goodell, 1985). The products from PG turnover are recycling back into the cell and enter lipid II biosynthesis, they can be excreted into the extracellular environment and can function as a virulence factor or is used as signal to induce the cellular expression of β-lactamase expression (Fisher and Mobashery, 2014; Sinha and Rosenthal, 1980). A variety of enzymes are responsible for the remodeling activities and degradation of the sacculus (Fig 1.4). The N-acetylmuramoyl L-alanine amidases cleave the amide bond between the L-Ala residues of the stem peptide and the MurNAc residue, while carboxypeptidases and endopeptidases cleave the amide bonds between amino acids within the
stem peptide (Fig 1.4). Along with cleaving and modifying the stem peptide of PG, enzymes such as β-N-acetylglucosaminidases, LTs and lysozymes cleave the glycan backbone.

**Figure 1.4. PG lytic enzymes active on the stem peptide.** The PG structure shown is that of a Gram-negative organism. The peptidases are classified for the types of amino acid that they cleave (L or D) and where they cut (endo or carboxyl). R and R₁ represent MurNAc and GlcNAc residues, respectively.

**1.4 Lytic Transglycosylases**

The LTs represent an endogenous class of enzymes that are essential for PG remodeling and cleavage. They are ubiquitous in Gram-negative bacteria, with the exception of *Mycoplasmas*. Also, there is increasing evidence indicating LTs are also required in many Gram-positive bacteria. Thus, LTs have been implicated with PG remodeling during *Bacillus* spore germination (SleB protein), resuscitation of dormant *Mycobacterium tuberculosis* (Rfps proteins), and cell septation in *Staphylococcus aureus* (IsaA and SceD) (Cohen-Gonsaud *et al.*, 2005; Li *et al.*, 2012; Stapleton *et al.*, 2007).
LTs cleave PG at the β-(1→4)-glycosidic bond between MurNAc and GlcNAc residues, which represents the same substrate specificity as lysozymes/muramidases (Scheurwater et al., 2008) (Fig. 1.5). In contrast to lysozymes, however, LTs are not hydrolases because their lytic reaction leads to the concomitant formation of an intramolecular 1,6-anhydromuramoyl-reaction product, a reaction that does not require the addition of a water (Fig. 1.5) (Höltje et al., 1975).

**Figure 1.5 Comparison of LT and HEWL cleavage reactions.** (A) Open blue arrows represent the LT cleavage of the β-(1,4)-glycosidic bond forming an intracellular 1,6-anhydroMurNAc reaction and GlcNAc residue. (B) Closed grey arrows represent the hydrolase reaction that cleave the β-(1,4)-glycosidic bond with the addition of water to form GlcNAc and a reducing MurNAc residue. R₁ and R₂ represent adjacent GlcNAc and MurNAc residues, while R₃ represents the stem peptide attached to the lactyl group at the C-3 of MurNAc residue.
1.4.1 Classification of LTs

A classification scheme was developed to organize the LTs, initially, into four distinct families based on sequence similarities and identified consensus motifs (Fig. 1.6) (Blackburn and Clarke, 2001). Family 1 is a large superfamily made up of 5 subfamilies (1A-1E) that share partial sequence similarity to goose-type (G-type) lysozymes (Scheurwater et al., 2008). The members of Family 1 were implicated with the insertion of secretion systems into the PG sacculus (Scheurwater et al., 2008). However, recent work done by Roure et al., (2012) found that membrane-bound lytic transglycosylase (Mlt) D and soluble lytic transglycosylase (Slt) F, which are both Family 1 LTs, were involved with flagella formation and assembly in Helicobacter pylori. In addition, Family 3 LTs were initially believed to be the only family involved with flagella formation and assembly and therefore, this is not the case. These enzymes are classified into family 1 by a conserved catalytic Glu residue, and an invariant Ser (Blackburn and Clarke, 2001). Family 2 consists of enzymes that are produced by all members of the Pseudomonadaceae and Enterobacteriaceae families and are implicated with septation formation and cell division. Members of this family are classified by a conserved catalytic Asp residue (Blackburn and Clarke, 2001). The presence of conserved catalytic Glu and an invariant Thr is the basis for the inclusion of a LT into Family 3 (Scheurwater et al., 2008). Family 4 enzymes, which are characterized by conserved catalytic Ser and Glu are exclusively encoded by lambda bacteriophages as they are involved with phage induced cell lysis (Blackburn and Clarke, 2001; Scheurwater et al., 2008). With the discovery of new LTs involved in new roles within the cell, an updated classification scheme was generated (Herlihey and Clarke, 2017). This updated classification included the addition formation of an additional subfamily in family 1F, as well as
the inclusion of two additional families, family 5 and 6 (Herlihey and Clarke, 2017) (Fig. 1.6). The new subset of Family 1F was identified with the discovery of SltF, an LT encoded in flagellated α-Proteobacteria (Herlihey et al., 2016). Classification of this subset is distinguished by the presence of a Ser or Thr residue immediately after the catalytic residue in motif I (Herlihey and Clarke, 2017). Family 5 and 6 were identified with the respective discovery of E. coli MltG and RlpA from P. aeruginosa. Family 5 is classified by the a conserved catalytic Glu and invariant Thr in motif IV, while family 6 is identified by a conserved catalytic Asp residue found in motif III (Herlihey and Clarke, 2017).

Bacteria are found to encode a variety of different LTs from the various families mentioned. The presence of multiple LTs within a cell system may permit the elimination of one or more LTs without eliciting a lethal effect (Blackburn and Clarke, 2002). E. coli, a well-studied organism regarding LTs and their roles, encodes eight LTs: Slt70 (Family 1A), MltG (Slt superfamily: Family 1), MltC (Family 1B), MltE (Family 1C), MltD (Family 1D), MltF (Family 1E), MltA (Family 2) and MltB (Family 3) (Lee et al., 2013; Yunck et al., 2016). In comparison, P. aeruginosa encodes 11 LTs from various families. Using bioinformatics methods, Blackburn and Clarke, (2001) identified eight LT genes from P. aeruginosa and four different Family 3 isozymes, which are MltB, SltB1, SltB2 and SltB3 (Blackburn and Clarke, 2002). The ninth LT, RlpA (rare lipoprotein A), was recently identified by (Jorgenson et al., 2014). RlpA has weak structural similarity to MltA from E. coli, and no activity on wild-type P. aeruginosa PG (Jorgenson et al., 2014). However, RlpA was shown to degrade PG devoid of peptide stems, which is believed to contribute to its role with daughter cell separation (Jorgenson et al., 2014). It was initially believed that P. aeruginosa did not encode Family 1E enzymes (Blackburn and Clarke, 2001). However, the crystal structure of a P. aeruginosa protein, PA2865, has been
### Figure 1.6 Family organization of LTs and structures

Residues in plain and bold of the consensus motifs are numbered in roman numerals, which are present in greater than 80% and invariant amongst the sequences of each respective family. Red type which are labelled with the asterisks identify the putative catalytic residues. The number in parentheses denote the number of residues between each motif. LT structures are resented in a cartoon configuration of members of Family 1A (Slt70 from *E. coli*, PDB 1QTE), Family 2 (MltA from *Neisseria gonorrhoeae*, PDB 2G6G), Family 3 (Slt35 from *E. coli* PBD 1QUS), Family 4 (φλ LT, PDB 1D9U), Family 5 (MltG from *E. coli*, PB 2R1F). This figure is adapted from (Herlihey and Clarke, 2017).

<table>
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<tr>
<th>Family</th>
<th>Consensus Motifs</th>
<th>LT Structures</th>
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<tbody>
<tr>
<td>1A</td>
<td>R5E-I-I-A-S</td>
<td>Slt70</td>
</tr>
<tr>
<td>1B</td>
<td>-I-E-I-A-S</td>
<td>MltA</td>
</tr>
<tr>
<td>1C</td>
<td>-I-E-I-A-S</td>
<td>Slt35</td>
</tr>
<tr>
<td>1D</td>
<td>YQ S6W-A ST</td>
<td>φλ LT</td>
</tr>
<tr>
<td>1E</td>
<td>-I-E-I-A-S</td>
<td>MltG</td>
</tr>
<tr>
<td>2</td>
<td>D-I-E-I-A-S</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>I-E-I-A-S</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>-I-E-I-A-S</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>-I-E-I-A-S</td>
<td></td>
</tr>
</tbody>
</table>
solved (PBD 4OWD). It is similar to \textit{E. coli} MltF, and thus it represents the tenth LT found in \textit{P. aeruginosa} (Reddem and Thunnissen, 2015). The last known LT in \textit{P. aeruginosa} is MltG, which has only been shown in \textit{E. coli} but a hypothetical homolog appears to exist in \textit{P. aeruginosa} (Yunck \textit{et al}., 2016). The classification of LT families represents an emerging and growing field. The identification of potentially new members of the existing families and even new classification of families are expected to emerge in further research.

\textbf{1.4.2. Structure of LTs}

Perhaps not surprisingly given the complexity of their substrate PG, the LTs share structure similarity to lysozymes, despite not having significant sequence identity (van Straaten \textit{et al}., 2005). LTs and lysozymes both have deep grooves near the active site that accommodate the sugar residues of the PG backbone (van Straaten \textit{et al}., 2005). However, LTs contain hydrophobic residues around the active cleft, which preclude the access of water to the catalytic residues. It is important to consider that there are some disparities between LT structures amongst the different families. This would be expected as each LT family is implicated with different biological roles within the cell. Families 1A, 3 and 4 are classified to have an \(\alpha, \beta\)-hydrolase fold, which is consistent with a “lysozyme-like” fold (Scheurwater \textit{et al}., 2008). In contrast, Family 2 enzymes possess a \(\beta\)-barrel structure, similar to an endoglucanase V fold (van Straaten \textit{et al}., 2005). Along with the variety of structures amongst LT families, there are differences with substrate specificity. For example, Slt70 from \textit{E. coli} only cleaves PG with peptide stems linked to the glycan backbone, whereas MltA does not require the presence or peptides for cleavage (Ursinus and Holtje, 1994).
1.4.3 Mechanism of Action

As mentioned previously, LTs cleave the β-(1→4)-glycosidic bond between the MurNAc and GlcNAc residues to form a 1,6-anhydromuramoyl reaction product (Ursinus and Holtje, 1994). Initially, it was postulated that the mechanism of action for LTs functioned like other glycosidases, which would use a single or double-displacement reaction mechanism (Blake et al., 1967). Hen egg-white lysozyme (HEWL) possesses two catalytic residues to perform its double-displacement (retaining) reaction mechanism with the addition of water (Blake et al., 1967). The protonated Glu35 acts as a catalytic acid/base while the deprotonated Asp52 serves as a nucleophile to stabilize the putative oxocarbenium transition state that is formed (Vocadlo et al., 2001). In contrast to lysozymes, LTs catalyze the cleavage of the β-(1→4)-glycosidic bond involving only a single catalytic residue (Blackburn and Clarke, 2002). Currently, with the LTs presently studied, Glu and Asp are the known residues to function as the catalytic acid/base (Scheurwater et al., 2008).

The single catalytic acid/base residue initially acts as a general acid, donating its proton to the glycosidic oxygen (Fig. 1.7) (Blackburn and Clarke, 2002). As bond cleavage occurs, to the oxocarbenium transition state is stabilized by the formation of an oxazolinium intermediate involving the N-acetyl group of the muramoyl residue (Blackburn and Clarke, 2002). The deprotonated catalytic residue [(E) or (D)] then acts as a general base to abstract the C-6 hydroxyl proton of the MurNAc residue, thus promoting an intramolecular nucleophilic attack at C1 position, collapsing the oxazolinium intermediate and leading to the concomitant formation of the 1,6-anhydro reaction product (Fig. 1.7) (Reid et al., 2004). Experimental evidence for this proposed mechanism was provided by inhibition studies using N-acetylglucosamine thiazoline (NAG-thiazoline) which is an analog of the putative oxazolinium intermediate (Reid et al., 2004).
Recently, Dik et al., (2017) proposed an alternative mechanism can occur without the formation of the covalent oxazolinium intermediate. The neighboring MurNAc N-acetyl group may be involved with substrate assisted catalytic by stabilizing the C1 by bonding with the C2. In addition, recent work by Lee et al., (2013) have reported that Slt70, MltC, MltD and MltE from *E. coli* and CwlQ from *B. subtilis* have secondary minor hydrolytic-like activity (Sudiarta et al., 2010). It is postulated that the hydrolytic activity arises due to the similar structure shared between LTs and lysosomes and presumably occurs as a water molecule slips into the active site to replace the participation of the C-6 hydroxyl residue (Lee et al., 2017).

**Figure 1.7 Proposed mechanism catalyzed by LTs.** The catalytic Glu (catalytic Asp with Family 2 LTs) serves initially as an acid to protonate the glycosidic linkage to be cleaved leading to the formation of a muramoyl oxazolinium-ion intermediate, and then as a base to abstract the C-6 hydroxyl proton of the oxazolinium ion promoting its collapse and the concomitant formation of the 1,6-anhydromuramoyl product. R, R₁, R₂ represents adjacent GlcNAc, MurNAc and stem peptides.

### 1.4.4 Biological Functions

LTs are space-making enzymes for bacteria to accommodate a number of physiological functions within the cell. They are essential for the insertion of protein complexes that extend through the PG sacculus (Koraimann, 2003). These protein complexes include flagella, pili and secretion systems (Koraimann, 2003). In addition, the main biological function of LTs involves the turnover and recycling of PG metabolites. The 1,6-anhydromuropeptides that are generated when LTs cleave PG are transported back into the cytoplasm for reuse (Vollmer et al., 2008b). These anhydromuropeptides have also been shown to contribute to be important virulence
factors. For example, the GlcNAc-anhydroMurNAc tetrapeptide released by *Bordatella pertussis* (whooping cough), has been shown to induce cell damage in the respiratory tract of those suffering from *B. pertussis* infections (Luker *et al.*, 1993). In addition, the 1,6-anhydroMurNAc fragments released by *Neisseria gonorrhoeae* have been shown to kill the ciliated cells that line the Fallopian tube (Melly *et al.*, 1984). This metabolite also induces β-lactamase expression in Gram-negative *Enterobacteriaceae* spp. and *P. aeruginosa* (Lee *et al.*, 2015). β-Lactamases are enzymes that provide resistance to commonly used β-lactam antibiotics, such as penicillin’s and cephamecin. AmpC expression in *P. aeruginosa* is linked to PG recycling by membrane-bound and soluble LTs (Fisher and Mobashery, 2014; Lamers *et al.*, 2015). These LTs are responsible for generating the anhydromuropeptides that induce AmpC expression (Fisher and Mobashery, 2014). AmpC catalyzes the hydrolytic degradation of the β-lactam core to cephalosporins and thus contributes to β-lactamase resistance (Lamers *et al.*, 2015). There is no doubt that LTs play an important role with biological functions; pathogenesis and β-lactam resistance and therefore an understanding of how to control and regulate LT activity would be essential.

1.5. Control of Lytic Transglycosylases

An understanding of what factors control LT activity is gradually developing. This is important given how many processes and pathways LTs are involved in. LTs are classified as autolytic enzymes, which signifies that if their activity were allowed to proceed uncontrolled, complete cellular lysis would occur (Blackburn and Clarke, 2001). To date, there are three mechanisms postulated for how LT are controlled have been discovered. The first mechanism involves the chemical modification, O-acetylation, of their substrate PG. O-Acetylation of PG involves the addition of an acetyl group to the C-6 hydroxyl of the MurNAc residue (Clarke and Dupont, 1992). An unmodified C-6 hydroxyl is a strict requirement for LT activity to permit the
formation of the 1,6-anhydromuramyl reaction product (Moynihan and Clarke, 2011). Consequently, PG O-acetylation totally precludes LT activity. There are two enzyme systems known for the O-acetylation of PG: O-acetyltransferase A (OatA) in Gram-positive bacteria, and the PG O-acetyltransferase A/B (PatA/PatB) complex that are found in some Gram-negative bacteria (Moynihan and Clarke, 2010, 2013).

PG O-acetylation appears to occur in all pathogenic Gram-positive bacteria. However, this modification is not universally present in Gram-negative bacteria despite the fact that they all produce LTs (Pfeffer et al., 2012). This presents a problem regarding the understanding of how LTs are controlled in Gram-negative pathogens, such as E. coli and P. aeruginosa. Neither of these species encode an OAP (O-acetylation of peptidoglycan) gene cluster, and consequently they do not produce O-acetylated PG (Pfeffer et al., 2012). A second mechanism of LT control that may be used in these bacteria is allosteric regulation. Recently, work done by Domínguez-Gil et al., (2016), provided structural evidence that showed a large conformational change in P. aeruginosa MltF upon binding of a PG-derived effector. This PG derived effector (tetrapeptide) binds to the N-terminal ABC transporter-like regulatory module and induces a large conformational change (40 Å) over the whole protein which opens up the active site and allows access for substrate to bind (Domínguez-Gil et al., 2016).

1.5.1 Proteinaceous Inhibitors of Lytic Transglycosylases

An alternative method of control for E. coli and P. aeruginosa, and other Gram-negative bacteria that do not O-acetylate their PG, appears to be the production of proteinaceous inhibitors (Clarke et al., 2010). The first proteinaceous inhibitor, Ivyc, was discovered as an inhibitor of C-type lysozyme (Monchois et al., 2001). It was discovered in E. coli to be the product of the ykfE gene and it is known now to be produced by a wide variety of Gram-negative bacteria as a stress
response to PG damage (Deckers et al., 2004). Originally this proteinaceous inhibitor was thought to only inhibit lysozymes and protect the host cell from innate immune defenses. However, it is not a secreted protein but rather it is localized to the periplasm making it physiologically ineffective as an inhibitor of the lysozymes of innate immunity systems (Abergel et al., 2007; Deckers et al., 2004). Moreover, a paralog of Ivy, Ivyp2, produced exclusively by a number of Pseudomonads was found to be inactive against lysozymes (Clarke et al., 2010). The inactivity of Ivyp2 against lysozyme and the localization of Ivy proteins in the periplasm led Clarke et al., (2010) to propose that the true physiological function of these proteins is to control LT activity. This proposal was supported by the observation that both IvyP1 and IvyP2 from P. aeruginosa inhibit sMltB from this bacterium (Clarke et al., 2010). This work provided the initial insight indicating that the true physiological function of these proteinaceous inhibitors is not to inhibit lysozyme but rather to control and regulate LT activity within Gram-negative bacteria that do not O-acetylate their PG.

1.5.2 Membrane Bound lysozyme inhibitor of C-type lysozyme (MliC)

The discovery of the Ivy proteins provided a basis for the search of other proteinaceous inhibitors of lysozymes. This led to the identification of a membrane bound lysozyme inhibitor of C-type lysozyme, MliC (Callewaert et al., 2008). MliC, like the Ivy proteins, is produced by Gram-negative bacteria such as E. coli and P. aeruginosa and it was first discovered in E. coli, as ydhA (Callewaert et al., 2008; Revington et al., 2006). MliC homologs are similar to proteins containing the conserved domain COG3895 (Tatusov et al., 2003). Homologs of MliC appear to be widespread amongst the members of the Proteobacteria with the exception of ε-Proteobacteria (Callewaert et al., 2008). Furthermore, the majority of COG3895 members are small proteins.
that are predicted to be periplasmic proteins or lipoproteins that are localized to the inner leaflet of the OM of Gram-negative bacteria (Callewaert et al., 2008; Yum et al., 2009).

MliC from E. coli and P. aeruginosa were demonstrated experimentally to inhibit C-type lysozyme (Callewaert et al., 2008). Yum et al., (2009) provided further insight into the function of MliC as a lysozyme inhibitor by describing the crystal structure of the P. aeruginosa protein in complex with HEWL (Fig 1.8 A). The structural features of MliC display an 8-stranded β-barrel which is stabilized by a disulfide bond (Fig. 1.8 A-C) (Yum et al., 2009). They were able to demonstrate that the insertion of the invariant loop of MliC into the active site cleft of lysozyme plays a key role with its inhibition (Fig 1.8 B) (Yum et al., 2009). Key conserved regions (residues 86-92 and 97-104) of MliC were shown to be inserted into the active site of HEWL. These residues (Ser89 and Lys103) were shown to form hydrogen and ionic bonds with the catalytic residues (Asp52 and Glu35) of HEWL which led to its inhibition (Fig 1.8 C) (Yum et al., 2009).

1.6 Rationale of Study and Thesis Objectives

Although, MliC has been classified as a lysozyme inhibitor, it is possible that the physiological role is also to control LT activity. As previously mentioned, lysozymes and LTs share similar overall structure (α, β-hydrolase fold), which contains a deep active site cleft. It is therefore conceivable that the invariant loop of MliC can also inhibit LTs using the same inhibition mechanism. Thus, Ser89 and Lys103 of the invariant MliC loop would be able to form hydrogen and ionic bonds with the catalytic glutamic acid or aspartic acid of the corresponding LT. Furthermore, the localization of MliC within the cell indicates that it is more likely to come into contact with LTs and not lysozymes. This is due to the fact that proteinaceous inhibitors are localized to the inner leaflet of the OM (MliC) or are localized in the periplasm (Ivyp1 and
Ivyp2) (Clarke et al., 2010; Yum et al., 2009). Also, in *P. aeruginosa*, the outer membrane and the LPS prevent physical barriers from lysozyme from accessing the cell’s PG. It is reasonable to suggest that *P. aeruginosa* contains multiple proteinaceous inhibitors, like Ivyp1, Ivyp2 and MliC, whose role is to control the multiple LTs for a number of biological processes.

The research presented in this thesis tests my hypothesis that the physiological role of MliC is to serve as an inhibitor of LTs for their control within the periplasm of producing Gram-negative bacteria. The inhibitory potential of *P. aeruginosa* MliC was tested against MltA, MltB, and MltF from this same bacterium as a model system.
Figure 1.8 The interaction of HEWL and MliC. A. Cartoon representation of MliC forms dimers and each monomer directly inhibits one HEWL molecule (PDB 3F6Z). B. MliC monomer interacting with one HEWL molecule. MliC inhibits HEWL with a protruding loop into the active site (Black dashed lines). C. Ser 89 forms a hydrogen bond with the catalytic Asp 52 from HEWL while Lys 103 forms a stabilizing salt bridge with Asp 52 and Glu 35 of HEWL. The active site of HEWL and the loop of MliC are shown in a stick configuration (PBD 3F6Z).
Chapter 2. Material and Methods

2.1 Chemicals and Reagents

DNase I, RNase A, EDTA-free protease inhibitor tablets, isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Roche Diagnostics (Laval, QC, Canada). Nickel(II)-nitrilotriacetic acid (Ni\(^{2+}\)-NTA)-agarose and TALON cobalt resin were obtained from Qiagen (Valencia, CA) and Takara Bio USA Inc. (Mountain view, CA), respectively. Source 15S chromatography column, Glutathione Sepharose 4B™, and Benzamidine Sepharose 6B® were purchased from GE Healthcare (Chicago, Illinois). Fischer Scientific provided acrylamide, glycerol and LB (10g/L tryptone peptone, 5g/L yeast extract, 10g/L sodium chloride) and SB (32g/L tryptone peptone, 20g/L yeast extract, 5g/L sodium chloride) (Nepean, ON, Canada). All other reagents and chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada).

2.1.1 Preparation of FITC labeled M. luteus PG

The preparation of FITC labeled M. luteus was performed as described by Maeda, (1980). A 24 mg sample of purified M. luteus PG was reacted with 11 mg of FITC isomer I in a tube with approximately 2.5 mL of 0.5 M sodium bicarbonate-carbonate buffer pH 9.3. The reaction was incubated at 37 °C for 4 hours and protected from light. After incubation, the reaction mixture was washed three times with each of the following buffers/solvents: 0.5 M sodium bicarbonate-carbonate, de-ionized water, acetone and ethanol. Samples were then dried in vacuo, and stored at 4 °C until needed.

2.2 Bacterial strains and growth

The source of bacterial strains and plasmids that were used in this study, along with their genotypic description are listed in Tables 2.1 and 2.2, respectively.
Table 2.1 List of bacterial strains and genotypes used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> BL21 (λDE3) pLysS</td>
<td>F-ompT hsdS(_B) (r(_B)-m(_B)-) gal dcm ren131 (DE3)pLysS(Cm(^r))</td>
<td>Qiagen/Novagen</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>flha2 lac(del) phoA glnV44 φ80' lacZ(del) M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</td>
<td>Invitrogen</td>
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<tr>
<td><em>E. coli</em> T7 SHuffle</td>
<td>F' lac, pro, lacI(^q)/Δ(ara-leu)7697 araD139 flhuA2 lacZ::T7 gene1 Δ(phoA)PvuII phoR ahpC* galE</td>
<td>New England Biolabs Inc.</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PAO1</td>
<td>Genome sequence strain</td>
<td>Lam laboratory strain (CITE)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PAO1 ΔmliC</td>
<td>ΔmliC knockout</td>
<td>C. Vandenende (unpublished)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PAO1 Δivyp1</td>
<td>Δivyp1 knockout</td>
<td>C. Vandenende (unpublished)</td>
</tr>
<tr>
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<td>Δivyp2 knockout</td>
<td>C. Vandenende (unpublished)</td>
</tr>
<tr>
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<td>ΔmliCΔivyp1 knockout</td>
<td>C. Vandenende (unpublished)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PAO1 ΔmliCΔivyp2</td>
<td>ΔmliCΔivyp2 knockout</td>
<td>C. Vandenende (unpublished)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PAO1 ΔmliCΔivyp1,Δivyp2</td>
<td>ΔmliCΔivyp1,Δivyp2 knockout</td>
<td>C. Vandenende (unpublished)</td>
</tr>
<tr>
<td><em>M. luteus</em> ATCC 4698</td>
<td>Type Strain, freeze-dried</td>
<td>Sigma-Aldrich</td>
</tr>
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**Table 2.2 List of plasmids and derivatives used in this study**

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Descriptions</th>
<th>Source of Reference</th>
</tr>
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<tr>
<td>pET28a(+)</td>
<td>IPTG-inducible T7 expression vector; N- and C-terminal His₆-tag, Kn²</td>
<td>Novagen</td>
</tr>
<tr>
<td>pGET-4T-1</td>
<td>Arabinose inducible, Tac expression, N-terminal GST-tag, thrombin cleavage site, Amp²</td>
<td>GE Healthcare Life Sciences</td>
</tr>
<tr>
<td>pNBAC54-1</td>
<td>pET28a(+) derivative with recombinant <em>P. aeruginosa</em> mltB encoding sMltBΔ2-17, C-terminal His₆-tag; Kan²</td>
<td>N. Blackburn (2002)</td>
</tr>
<tr>
<td>pACCV4</td>
<td>pET28a(+) derivative with recombinant <em>P. aeruginosa</em> mltA encoding sMltAΔ2-25, C-terminal His₆-tag; Kan²</td>
<td>C. Vandenende (unpublished protocol)</td>
</tr>
<tr>
<td>pACRW-2-<em>mliC</em> (PAO1)</td>
<td>pET28a (+) derivative with recombinant <em>P. aeruginosa</em> mliC encoding MliCΔ2-30</td>
<td>C. Vandenende (unpublished)</td>
</tr>
</tbody>
</table>

*E. coli* strains BL21 (λDE3) pLysS and *E. coli* T7 SHuffle were maintained in LB broth or on LB agar at 37 °C, supplemented with kanamycin sulphate (50 µg/mL), chloramphenicol (34 µg/mL) or ampicillin (100 µg/mL) as required. Solid media was prepared by added 15g/L agar to LB. For protein overproduction and purification, *E. coli* BL21 (λDE3) pLysS containing pNBAC54-1 (*mltB*) and pACCV4 (*mltA*) were grown in SuperBroth (SB) at 37 °C supplemented with the appropriate antibiotics with agitation. *E. coli* T7 SHuffle strain containing the pGEX-4T-1 + pACRW-2-*mliC* (PAO1) vector was grown in SB at 37°C supplemented with the appropriate antibiotics. All strains were stored in 25 % glycerol at -80 °C. Cloning and growth of sMltF was completed by C. Vandenende of the Clarke laboratory (unpublished protocol).

The *P. aeruginosa* PAO1 gene knockout strains of ΔmliC, Δivyp1, Δivyp2, ΔmliCΔivyp1, ΔmliCΔivyp2, ΔmliCΔivyp1Δivyp2 were constructed by C. Vandenende of the Clarke laboratory according to the method described in Choi and Schweizer, (2005).
2.3 Growth Curves

*P. aeruginosa* PAO1 strains harboring the following gene knockouts: ΔmliC, Δivyp1, Δivyp2, ΔmliCΔivyp1, ΔmliCΔivyp2, ΔmliCΔivyp1Δivyp2 were grown overnight in 5 mL of LB broth. Cultures were standardized to the same OD$_{600}$ nm the following day in the same media. Then, a 1/1000 dilution was made into 200 µL of sterile LB and the OD$_{600}$ nm was measured every 20 minutes for 20 hours in triplicate using a Biotek® Synergy H1 micro plate reader at 37 °C with agitation

2.4 DNA Techniques

2.4.1 Competent cell preparation

Chemically-competent cells were prepared using a variation of the method of Cohen *et al.*, (1972). A 5 mL overnight culture was sub-cultured 1/10 in 50 mL LB broth and grown at 37 °C with agitation for 1-2 hours or until mid-exponential phase (OD$_{600}$ nm of approximately 0.6). Cells were collected by centrifugation (5000 x g, 10 minutes) and re-suspended in 50 mL in cold solution α (0.1 M MgCl$_2$). Cells were collected by centrifugation again and re-suspended in 10 mL solution β (0.1 M CaCl$_2$). Cells were collected by centrifugation for a third time and re-suspended in 3 mL of 0.1 M CaCl$_2$ + 25 % glycerol (v/v) and prepared into 300 µL aliquots stored at -80 °C.

2.4.2 Transformation of *E. coli*

Chemically competent *E. coli* cells were transformed with 0.5 µL plasmid DNA (approx. 200 ng) and incubated on ice for 30 minutes. After this step, 1 minute of heat shock at 42 °C was performed followed immediately by a 5-minute incubation on ice. LB broth (500 µL) was added to cells prior to incubation at 37 °C for 60 minutes with agitation. After incubation, 0.1 mL was plated onto solid LB agar supplemented with the appropriate antibiotics and incubated at 37 °C
overnight. An isolated colony was used to inoculate 5 mL LB with appropriate antibiotics and incubated at 37 °C overnight with agitation. Glycerol stocks were prepared by combining 500 µL of culture and 500 µL of sterile 50 % glycerol (v/v) and stored at -80 °C immediately.

2.5 Protein Production and Purification

2.5.1 Over-Production and Purification of sMltB from P. aeruginosa

The production conditions and purification protocols for sMltB previously described in Blackburn and Clarke, (2002) were used with slight modifications. E. coli BL21 (λ DE3) pLysS cells were freshly transformed with the plasmid DNA and plated onto LB plates supplemented with the appropriate antibiotics. An isolated colony was used to inoculate 10-20 mL of fresh LB and grown overnight at 37 ºC. One liter of SB supplemented with kanamycin sulfate (50 µg/mL) and chloramphenicol (34 µg/mL) was inoculated with 10-15 mL of starter culture. Cultures were incubated at 37 ºC until early exponential phase (OD$_{600}$ of ~ 0.6). Cells were cooled at 4 ºC for 15 minutes prior to the addition of IPTG (freshly prepared in water and filter sterilized) to a final concentration of 0.1 mM. Induced cells were incubated for 3-4 hours at 15 ºC and collected by centrifugation (5000 x g, 15 min, 4 ºC) and the pellets were frozen at -20 ºC until needed.

For cellular lysis and protein purification, the cells were thawed and suspended in 25 mL of sMltB IMAC buffer (50 mM sodium phosphate, pH 8.0, 300 mM sodium chloride, 10 % (w/v) glycerol and 0.1 % Triton X-100). RNase A, DNase I, and EDTA-free protease inhibitor cocktail tablets, were added to the cell suspension and allowed to incubate on ice for 10-15 minutes. Cells were lysed via sonication [3x (5 min, 10/15 pulse, 50 % amplitude)] using a Sonic Vibra Cell™ high intensity ultrasonic processor fitted with a macroprobe. Cellular debris was removed by centrifugation (27000 x g, 20 min, 4 ºC). The supernatant was incubated with 0.5 mL of Ni-NTA agarose in a disposable chromatography column and incubated at 4 ºC for 1 hour with agitation.
The columns were washed with 150 mL of wash buffer (sMltB IMAC buffer pH 8.0 and 7.0). Subsequent washed were conducted with 5 mL of sMltB IMAC buffer at pH 6.0 and pH 5.0. Purified proteins were eluted in approximately 8 mL of sMltB elution buffer pH 4.5.

Following protein purification by IMAC, sMltB was dialyzed against 50 mM sodium phosphate pH 8.0 at 4 °C and further purified by anion exchange chromatography on a Source 15S column. sMltB was loaded onto the column previously equilibrated with dialysis buffer and recovered with a linear gradient of 0 to 1 M sodium chloride over 60 minutes at a flow rate of 1 mL/min. Under these conditions sMltB eluted at 20 % buffer B (20 mM sodium chloride) and was subsequently dialyzed against 10 mM sodium acetate, 100 mM sodium chloride buffer pH 5.8.

2.5.2 Over-Production and Purification of sMltA from P. aeruginosa

*E. coli* BL21 (DE3) pLysS cells were freshly transformed with the plasmid DNA and plated onto LB agar supplemented with the appropriate antibiotics. An isolated colony was selected to inoculate 5 mL tubes of sterile LB and grown overnight at 37 °C. One litre of SB supplemented with kanamycin sulfate (50 µg/mL) and chloramphenicol (34 µg/mL) was inoculated with 10-15 mL of starter culture and incubated at 37 °C until early exponential phase (OD₆₀₀nm of ~ 0.6). Cells were induced with the addition of IPTG (freshly prepared in water and filter sterilized) to a final concentration of 0.1 mM. Induced cells were incubated for 3-4 hours at 37 °C with agitation and collected by centrifugation (5000 x g, 15 min, 4 °C) and the pellets were collected and frozen at -20 °C until needed.

For cellular lysis and protein purification, the cells were thawed and suspended in 25 mL of sMltA IMAC buffer (25 mM sodium phosphate, pH 8.0, 300 mM sodium chloride. A 10 % (w/v) glycerol). RNase A, DNase I, and EDTA-free protease inhibitor cocktail tablets, were
added to the cell suspension and allowed to incubate on ice for 10-15 minutes. Cells were lysed via sonication (10 min, 10/15 pulse, 50 % amplitude)] using a Sonic Vibra Cell™ high intensity ultrasonic processor fitted with a macro-probe. Cellular debris was removed by centrifugation (27000 x g, 20 min, 4 °C). The supernatant was incubated with 0.5 mL of cobalt (Co²⁺) resin in a disposable chromatography column and incubated at 4 °C for 1 hour with agitation. The columns were washed with 150 mL of wash buffer (sMltA IMAC buffer pH 8.0 and 7.0). Purified proteins were eluted in approximately 5 mL of sMltA IMAC buffer pH 6.0. Following protein purification by IMAC, sMltA was dialyzed against 1.5 L of 10 mM sodium acetate, 10 mM magnesium chloride buffer pH 5.0 + 10 % glycerol at 4 °C. Protein was concentrated using an Amicon Ultra-15 centrifugal filters (MWCO 10 kDa). After sMltA was concentrated, the protein was used immediately or stored at -80 °C until needed.

2.5.3 Over-Production and Purification of sMltF from *P. aeruginosa*

The overproduction and purification of sMltF was optimized and performed by C. Vandenende of the Clarke laboratory. *E. coli* T7 SHuffle cells containing a plasmid encoding sMltF and a His₆ tag were resuscitated from a glycerol stock and grown overnight in LB supplemented with ampicillin (100 µg/mL). One litre of SB supplemented with the appropriate antibiotics with ampicillin (100 µg/mL) was inoculated with 15 mL of starter culture and grown at 37 °C until the exponential phase (OD₆₀₀ nm of ~0.8). Cells were incubated at 4 °C for 30 min and then induced with 1 µM (final concentration) of IPTG at 30 °C overnight. Cells were collected by centrifugation (5000 x g, 15 min, 4 °C) and the pellets were suspended in 15 mL of sMltF IMAC buffer (50 mM sodium phosphate buffer pH 8.0, 300 mM sodium chloride containing 10 % (w/v) glycerol) and frozen at -20 °C until needed.
For cellular lysis and protein purification, the cells were thawed and EDTA-free protease inhibitor cocktail tablets were added to the cell suspension and allowed to incubate on ice for 10-15 minutes. Cells were lysed via sonication [3x (2.5 min, 10/10 pulse, 50 % amplitude)] using a Sonic Vibra Cell™ high intensity ultrasonic processor fitted with a macro-probe. Cellular debris was removed by centrifugation (27000 x g, 20 min, 4 ºC). The supernatant was filtered through a 0.45 µm syringe filter and incubated with 0.25 mL of cobalt (Co²⁺) resin in a disposable chromatography column and incubated at 4 ºC for 1 hour with agitation. The columns were washed with 50 mL of sMltF IMAC buffer pH 8.0 then 50 mL sMltF IMAC buffer pH 7.0 containing 0.1 % Triton X-100. Purified proteins were eluted in approximately 2.5 mL of sMltF IMAC buffer containing the addition of 0.1 % Triton X-100 at pH 5.0 and pH 4.5.

2.5.4 Over-Production and Purification of MliC from *P. aeruginosa* using a GST-tag.

*E. coli* T7 SHuffle cells were freshly transformed with plasmid DNA into competent cells onto LB agar supplemented with ampicillin (100 µg/mL). An isolated colony was used to inoculate 5 mL tubes of sterile LB and grown overnight at 37 ºC. One litre of SB supplemented with the appropriate antibiotics with ampicillin (100 µg/mL) was inoculated with 10-15 mL of starter culture and grown at 37 ºC until the exponential phase (OD₆₀₀ nm of ~0.8). Cells were induced with the addition of IPTG (freshly prepared and filter sterilized) to a final concentration of 0.1 mM. Induced cells were incubated for 3-4 hours at 37 ºC and collected by centrifugation (5000 x g, 15 min, 4 ºC). The pellets were collected and frozen at -20 ºC until needed.

The cells were thawed and suspended in 25 mL of 1X PBS pH 7.4 and 10 % glycerol (v/v). RNase A, DNase I and EDTA-free protease inhibitor cocktail tablets were added to the cell suspension and allowed to incubate on ice for 10-15 minutes. Cells were lysed by sonication [3x (5 min 10/10 pulse, 50 % amplitude)] using a Sonic Vibra Cell™ high intensity ultrasonic
processor fitted with a macro-probe. Cellular debris was removed by centrifugation (27000 x g, 20 min, 4 °C). The supernatant was incubated with 5 mL of Glutathione Sepharose 4B resin (GE Healthcare) in a disposable chromatography column and incubated at ambient temperature for 1 hr with agitation. The column was washed 4-6 times with 150 mL of 1X PBS pH 7.4 and 10 % glycerol (v/v). GST-MliC was eluted in three, 5 mL elution fractions in 10 mM glutathione in 50 mM Tris-HCl, that was freshly prepared each time. Elution fractions were dialyzed against 2L of 1X PBS pH 7.4 and 10 % glycerol (v/v) with two buffer changes (one for 1 hour at 4 °C and the other for 16 hours at 4 °C).

Following dialysis, recombinant GST-MliC protein was cleaved by thrombin protease (GE Healthcare) for 20 hours at 25 °C. Following digestion, cleaved protein (MliC) was passed again through Glutathione Sepharose 4B resin in-tandem with immobilized Benzamidine resin, to remove any un-cleaved fusion protein, soluble GST and thrombin protease. The elution was then further purified by gel filtration using a SuperDex 75 chromatography column in 1X PBS pH 7.4 and 10 % glycerol (v/v). Protein was used immediately or stored at -80 °C until required.

2.5.5 Protein concentration

Amicon Ultra-15 Centrifugal Filter units (EMD Millipore Corp., Billerica, MA) were used to concentrate protein samples after dialysis. A 3 kDa MWCO was used for concentration of MliC and a 30 kDa MWCO filter was used for sMltA concentration. sMltB did not require concentration after anion exchange. Dialyzed protein (5-10 mL) was added to the upper reservoir of the filter unit and the protein was concentrated by centrifugation (5000 x g, 10-15 min, 4 °C) using a swinging bucket centrifuge. This process was repeated as necessary until the volume in the upper reservoir decrease by 2-5 mL.
2.6 Protein Analyses

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

SDS-PAGE analyses was conducted on protein samples from every protein purification by the method described by Laemmli, (1970). For the analysis of protein each purification, 15 % separating gels and 4 % stacking gels were used. Protein samples from various points along the purification process were mixed with in SDS sample buffer (200 mM Tris-HCl, pH 6.8, 40 % glycerol, 2 % SDS, 0.04 % Coomassie Brilliant Blue G-250). Before loading the samples into the gel, they were boiled for 15 minutes at 95 °C. Then, samples were loaded in the gel (5 µL) alongside with 5 µL of pre-stained ladder (Thermo Scientific) and the gels were run at 150 V for 60 minutes. Gels were washed with dH₂O for 20-30 minutes, stained with Coomassie blue stain G-250 for 30-60 minutes and were de-stained for 30-60 minutes. The gels were scanned immediately using Image Lab™ software (Bio-Rad Laboratories, Inc.).

2.6.2 Western immunoblotting

Western immunoblotting was performed after each purification in order to confirm the presence of the protein of interest. Following SDS-PAGE, resolved proteins were transferred to a nitrocellulose membrane (0.45 µm) in transfer buffer (10 mM NaHCO₃, 3 mM Na₂CO₃, 1 % SDS and 20 % methanol) using a BioRad mini-electroblotting apparatus (BioRad Laboratories, Inc. Mississauga, Ontario) for 1 hour at 80 V. After transfer, the nitrocellulose membrane was washed twice in TBS buffer (10 mM Tris-Cl, pH 7.5, 150 mM NaCl) for 10 minutes at room temperature and then incubated with blocking buffer (3 % BSA in 1X TBS) for 1 hour. (Following blocking,) The membrane was washed once for 5 minutes with 1X TTBS (20 mM Tris-Cl, pH 7.5, 500 mM NaCl, 0.05 % (v/v) Tween-20, 0.2 % (v/v) Triton X-100) and incubated with a 1:1000 dilution of mouse anti-His₆ antibody (Santa-Cruz Biotechnology, Santa Cruz CA)
for 1 hour at ambient temperature. Following the incubation of the primary antibody, the membrane was washed twice with 1X TTBS for 5 minutes, plus an additional wash with 1X TBS for 5 minutes. The membrane was incubated with the secondary antibody (1:2000 dilution of goat anti-mouse IgG) for 1 hour and then the membrane was washed four times for 5 minutes in 1X TTBS. The blot was stained with 1-2 mL of 1-Step NBT-BCIP (Roche Molecular Biochemical, Laval, PQ) for 5-10 minutes or until bands were visualized and rinsed with dH₂O.

2.6.3 Protein quantification

Protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific). Dilutions of bovine serum albumin (BSA) were made in the same buffer conditions as the protein of interest was in, and these dilutions were used to create a standard protein concentration curve. For each sample and standard, 25 µL samples were added to a 96-microtitre plate in triplicate and following by 200 µL of freshly prepared working reagent at a 50:1 mixture of BCA: (cupric sulphate). The plate was then incubated at 37 °C for 30 minutes and the absorbance was read immediately at 595 nm using Biotek® Synergy H1 micro plate reader.

2.7 Enzyme Activity Assays

2.7.1 Measurement of lytic activity

The turbidimetric assay of Hash, (1967) was used to monitor the time course of PG solubilisation either by HEWL, sMltA, sMltB or sMltF. Whole cells from Micrococcus luteus were used as the substrate for the muralytic enzymes and were re-suspended to a final concentration of 0.4 mg/mL in the appropriate assay buffer for each enzyme. Suspensions were subjected to sonication to provide a homogenous distribution of the cells. Enzyme was added to the substrate suspensions in a concentration range from 0.4 µM-3 µM, and the decrease in
turbidity was monitored continuously at OD_{600 nm} for a period of 15 to 60 minutes. All reactions were done in a volume of 200 µL in a microtiter plate and incubated at 25 °C or at 37 °C (sMltB) with a Biotek plate reader. The rates were normalized as a percent reduction of OD_{600 nm}. Statistical analysis of the data was completed using Microsoft Excel and GraphPad Prism 5.

2.7.2 Inhibition assays

Inhibition of the muralytic enzymes of interest was assessed using the turbidimetric assay described above using *M. luteus* whole cells as substrate HEWL, sMltA, sMltB, or sMltF at 0.4 µM, 2 µM, 3 µM, molar concentrations was pre-incubated with varying concentrations of MliC for 10 minutes on a nutator at ambient temperature prior to the addition of *M. luteus* whole cells. PG solubilisation of each muralytic enzyme was monitored over 20-30 minutes at 25 °C or 37 °C.

2.7.3 Fluorescent Activity and Inhibition Assays

As an alternative method of monitoring PG solubilisation of muralytic enzymes, the fluorescence-based assay described by Maeda, (1980) was used. The substrate in this assay was purified *M. luteus* PG that was labeled with fluorescein isothiocyanate isomer I (FITC) at the amine group of the stem peptide, as described above. The FITC-labeled substrate was exposed to a brief (10 second) sonication to re-suspend into the appropriate assay buffers. Enzyme and/or proteinaceous inhibitor was added at the desired concentrations and the relative increase of fluorescence was monitored fluorometrically using emission and excitation wavelengths of 525 nm and 495 nm, respectively, over 10-30 minutes. Each assay was performed in triplicate and the data was normalized to the initial relative fluorescent units (RFUs) reading and then reported as an increase of RFUs per minute.
Chapter 3. Results

The goal of this research was to test the hypothesis that MliC functions as an inhibitor of LTs present within the periplasm of *P. aeruginosa*. In order to test the hypothesis, MliC, sMltA and sMltB were purified to homogeneity for activity assays, which included the development of a fluorescence-based assay. This was followed by investigating the inhibition of sMltA and sMltB by MliC.

3.1 Production and Purification of GST-MliC Overproduced in *E. coli* T7 SHuffle cells

The *P. aeruginosa* PAO1 mliC gene was previously cloned into pGEX-4T-1 expression vector for the production of an N-terminal GST-tagged MliC with a thrombin cleavage site (C.V. unpublished). In addition, the lipidation site and signal peptide were removed to allow for expression of the relatively soluble protein in the cytoplasm. Expression of *mliC* in *E. coli* T7 SHuffle lead to the overproduction of GST-MliC. Cells were lysed by sonication and GST-MliC was purified by affinity chromatography using immobilized glutathione (Fig. 3.1A). Three washes with 1X PBS containing 10 % glycerol (v/v) removed contaminants from the resin. GST-MliC eluted from the resin in 10 mM glutathione. The expected size of GST-MliC was 40 kDa (theoretical masses of GST and MliC separately are 26 kDa and 14 kDa respectively). The apparent mass of the GST-MliC fusion protein was 37.5 kDa as determined by SDS PAGE (Fig. 3.1A). Elution fractions were dialyzed against 10 % glycerol (v/v) in PBS pH 7.4 initially for 1 hour at ambient temperature, and then overnight at 4 °C.

After dialysis, GST-MliC was treated with thrombin protease for 20 hours at 25 °C to remove the GST moiety (Fig. 3.1B). The pooled thrombin reactions were passed through immobilized glutathione and benzamidine Sepharose 4 Fast Flow resin in-tandem to remove the thrombin protease, cleaved GST protein and any un-cleaved fusion protein (Fig. 3.1B). The last
puriﬁcation step involved size-exclusion chromatography using a Superdex 200 column. The resulting MliC preparation appeared to be homogeneous by SDS PAGE analysis, and hence suitable for the turbidimetric assay (Fig. 3.1C). Samples were concentrated and stored at -80 °C until required.

Figure 3.1 Purification of MliC from *E. coli* T7 SHufﬂe cells. (A) SDS-PAGE with Coomassie Brilliant Blue staining of the puriﬁcation of GST-MliC using immobilized glutathione. CL; Crude lysate, FT; ﬂow through, W1,3: Washes 1 to 3, E1,3: 10 mM glutathione elution. (B) SDS-PAGE analysis of thrombin digestion and in-tandem afﬁnity chromatography. E1,3: pooled elution fractions from (A) after dialysis. TD1,2: thrombin digestion reaction 1 and 2. E1A,2A: Elution fractions from the immobilized glutathione and benzamidine resins in-tandem. GE: 10 mM glutathione elution from chromatography columns in tandem. (C) SDS-PAGE analysis of gel ﬁltration chromatography of MliC. CE: concentrated elution fractions from (B), SEC E1,4: Elution fraction from SEC using a HiLoad Superdex 200 column. PageRuler Prestained Protein Ladder was used for the ladder. Molecular masses of standards are reported in kDa. The apparent mass of GST-MliC is 40 kDa, MliC 14 kDa and GST 26 kDa.
3.2 Production and Purification of sMltB Over-produced in *E. coli* BL21 (λDE3) pLysS

The purification protocol for sMltB described by Blackburn and Clarke, (2002) was used, with modifications to purify sMltB. With sMltB, plasmid pNBAC54-1 encoding MltB lacking the its lipidation site and with an engineered histidine tag was previously cloned into *E. coli* BL21 (λDE3) pLysS cells. This protein following removal of the lipidation site is referred to as soluble-derivative of MltB (sMltB). sMltB was over-produced in *E. coli* BL21 (λDE3) pLysS and cells were lysed by sonication. After cell lysis, sMltB was purified by IMAC using a Ni-NTA resin by a pH gradient. Imidazole was not used for purified histidine-tagged LTs as imidazole inhibits their lytic activity. A pH gradient was performed in order to avoid potential downstream complications when monitoring LT activity. Washes were done with 150 mL of sMltB IMAC buffer (25 mM sodium phosphate, 300 mM sodium chloride, 10 % glycerol (v/v), 0.01 % Triton-X (v/v) at pH 8.0 and pH 7.0. Subsequent washes were performed in 5 mL of sMltB IMAC buffer pH 6.0 and pH 5.0. sMltB was eluted in a 5 mL fraction of IMAC buffer pH 4.5. (Appendix). Protein was observed to elute in IMAC buffer pH 6.0 and pH 5.0 washes, so all three fractions were collected (Fig 3.2A). An additional purification step was required due to the presence of contaminants (as determined by SDS-PAGE and Western immunoblotting). The expected molecular mass of sMltB is ~38 kDa, and it was experimentally determined to be 39.7 kDa (Fig.3.2B). sMltB was further purified by anion-exchange chromatography using Source 15S. After purification, protein was dialyzed against 2 x 1.5 L of 50 mM sodium acetate buffer, 100 mM sodium chloride 0.1 % Triton X-100, pH 5.8. This protocol routinely yielded 0.26 g/L per purification and was stored at -80 ºC in 1 mL aliquots or used immediately.
3.3 Production and Purification of sMltA Over-produced in *E. coli* BL21 (λDE3) pLysS

The *P. aeruginosa* PAO1 *mltA* gene was engineered previously to produce recombinant MltA with an N-terminal His6-tag and the removal of its putative lipidation site (Δ1-*mltAΔ1-37); this form of MltA was labelled sMltA (C.V unpublished). Expression of *mltA* within pET28a in *E. coli* BL21 (λDE3) pLysS led to the overproduction of the sMltA product that was purified to apparent homogeneity (as determined by SDS-PAGE analysis) by IMAC using immobilized Co^{2+} resin (Fig. 3.3A). Washes were performed with 150 mL of sMltA IMAC buffer (25 mM sodium phosphate, 300 mM sodium chloride, 10 % glycerol (v/v)) at pH 8.0 and pH 7.0. Protein was eluted in elution buffer (25 mM sodium phosphate, 300 mM sodium chloride pH 6.0, 10 % glycerol (v/v)). A pH gradient was used due to the potential complications discussed previously. Western blot analysis was used to confirm the presence of sMltA in each protein sample and its apparent mass was determined to be 43.2 kDa (Fig. 3.3B). After purification, the protein was
dialyzed against 2 x 1.5 L of 25 mM sodium acetate, 10 mM magnesium chloride, 100 mM sodium chloride pH 5.6 containing 10 % glycerol. This protocol routinely yielded 0.144 mg/L per purification and was the enzyme was stored at -80 ºC in 1 mL aliquots or used immediately.

![SDS-PAGE analysis of sMltA purified from E. coli BL21 (λDE3) pLysS](image)

**Figure 3.3** SDS-PAGE analysis of sMltA purified from *E. coli* BL21 (λDE3) pLysS. (A) SDS-PAGE with Coomassie Brilliant Blue staining and (B) Western blot using anti-His antibody. CL: Crude lysate; FT: IMAC flow-through; W₁: IMAC wash 1; W₂: IMAC wash 2; EL: IMAC elution; L: Ladder, PageRuler Prestained Protein Ladder. Molecular masses of standards are reported in kDa. The apparent mass of sMltA are 43.2 kDa.

### 3.4 Growth Curve Analysis of *P. aeruginosa* Proteinaceous Inhibitor Knockouts

Growth curves were generated in order to evaluate the ability of *P. aeruginosa* PAO1 knockout strains (ΔmliC, Δivyp1, Δivyp2, ΔmliCΔivyp1, ΔmliCΔivyp2, and ΔmliCΔivyp1Δivyp2) to grow in LB for 20 hours at 37 ºC. The growth of all knockout strains were unaffected and each displayed no statistical difference in morphological phenotype as the wild-type *P. aeruginosa* PAO1 strain under those specific conditions (Fig. 3.4).
Figure 3.4 Growth curves of each *P. aeruginosa* knockouts. The *P. aeruginosa* strains were cultured in LB media and the cellular growth was measured at OD_{600 nm} for 20 h at 37 °C with agitation (200 rpm). The red, black, yellow, blue, brown, cyan and purple lines represent wild-type PAO1, ΔmliC, Δivyp1, Δivyp2, ΔmliCΔivyp1, ΔmliCΔivyp2, ΔmliCΔivyp1Δivyp2 *P. aeruginosa* strains, respectively. Data was obtained in triplicate.

3.5 Establishing Conditions for PG Lysis Assays

3.5.1. Turbidimetric Assay

The turbidimetric assay performed by Clarke *et al.*, (2010) was used in this study to investigate MliC as a potential inhibitor of sMltB, sMltA and sMltF. This assay is based on that developed by (Hash, 1967). It is based on monitoring the decrease in the turbidity of insoluble PG over time as monitored by optical density measurements. In this study, the PG solubilisation of *M. luteus* whole cells by these muriylytic enzymes were monitored over a period of 15-30 minutes as the decrease in OD_{600 nm} measured. When this assay was performed in the presence of inhibitor, a decrease of the rate of PG solubilisation or abolishment of this activity would indicate inhibition of the muriylytic enzyme that was tested.

3.5.2 Fluorescence-Based PG Lytic Assay

PG was isolated from *M. luteus* cells and then modified under alkaline conditions with FITC isomer I as described in the Materials and Methods (see 2.1.1). This modified PG can be used in a fluorometric assay for PG lytic enzymes as the fluorescence of the FITC is quenched while retained within insoluble PG; an increase in fluorescence results from the solubilisation of
labelled PG fragments. To confirm that the purified PG had been successfully modified, it was analyzed spectrophotometrically. The presence of two maxima at approximately 470 nm and 500 nm in its UV absorbance spectrum indicated the presence of the fluorescein within the PG (Fig. 3.5), as demonstrated previously by Maeda, (1980).

![Absorbance spectra](image)

**Figure 3.5 Absorbance spectra of M. luteus PG and its fluorescein conjugated derivative.** Approximately 0.08 mg of PG (dashed line) and the FITC-PG derivative (solid line) were re-suspended in 200 µL of 50 mM PBS pH 7.3.

After validating that the fluorescein was conjugated to the purified PG, it was then tested in activity assays. Incubation of 0.08 mg/mL conjugated PG with 1 µM concentrations of both sMltA and sMltB led to an increase in fluorescence (Fig. 3.6) indicative of lytic activity. However, no lytic activity was detected with sMltF using this assay under the optimal conditions of pH 4.5 (see below) for this enzyme (Fig 3.11).

### 3.6 Confirmation of the Inhibition of HEWL Activity by MliC

The inhibition of HEWL activity by MliC was performed in order to validate the inhibition assay. Each assay was performed in triplicate and the data were reported as a percent reduction of OD$_{600\text{ nm}}$. Using its optimal conditions of pH 7.4, 0.4 µM HEWL demonstrated a 70
% reduction of OD$_{600}$ nm over the time course of 15 minutes (Fig. 3.7A). Purified MliC was able to inhibit this lytic activity in a dose-dependent manner (Fig. 3.7B). This assay was used to validate the inhibition assay and to confirm that MliC was properly folded as an “active” proteinaceous inhibitor. The lowest molar ratio that MliC inhibited the lytic activity of HEWL was 5:1, which is consistent with published data (Monchois et al., 2001).

![Figure 3.6 Determination of lytic activity of muralytic enzymes using the fluorescence assay.](image)

To both further validate the assay and confirm the inhibition of HEWL by *P. aeruginosa* MliC, the fluorescein-labelled PG was used as substrate. FITC-labeled *M. luteus* PG was re-suspended into lysozyme assay buffer (1X PBS pH 7.4). 0.4 µM HEWL was re-suspended in the same assay buffer and then pre-incubated with concentrations of MliC at the molar ratios of 1:1-6 for 10 minutes prior to the addition of FITC-labeled substrate. PG solubilisation by HEWL was monitored over 15 minutes at 25°C, and the increase of RFUs was measured over time (Fig. 3.8A). It was validated that MliC inhibits HEWL in a dose-dependent manner at similar molar ratios observed by the turbidimetric assay (Fig. 3.8B).
Figure 3.7 Inhibition of HEWL activity by MliC using the turbidimetric assay. (A) Representative curves of the turbidimetric assay determined using suspension of *M. luteus* whole cells as substrate (final concentration 0.4 mg/mL) in 50 mM PBS buffer. (A) HEWL (0.4 µM) in the absence and presence of 0.4 µM MliC, 0.8 µM MliC, 1.2 µM MliC, 1.6 µM MliC, and 2.0 µM MliC. (○) represents a negative control to accommodate settling of PG during the reaction. (B) The fractional rate of inhibited enzyme (V) over the initial rate (V₀) plotted versus the molar excess of MliC to HEWL. Error bards denote S.D (n=3).

Figure 3.8 Inhibition of HEWL activity by MliC using the fluorescent assay. (A) Representative curves of the fluorescent assay determined using suspension of purified *M. luteus* PG tagged with FITC as substrate (final conc. 0.08 mg/mL) in 50 mM PBS buffer pH 7.4. (A) HEWL (0.4 µM) in the absence, 0.8 µM MliC, 1.6 µM MliC, and 2.4 µM MliC. The spontaneous release of FITC-PG was used to subtract from each reaction. (B) The fractional rate of inhibited enzyme (V) over the initial rate (V₀) plotted versus the molar excess of MliC to HEWL. Error bards denote S.D (n=3).
3.7 Optimization of sMltA Activity and Inhibition by MliC

The activity of purified sMltA was previously measured using the turbidimetric assay (Herlihey, 2017). The optimal assay buffer conditions were established to be 10 mM sodium acetate buffer, pH 5.2 containing 10 mM magnesium chloride. Under these conditions, 2 µM sMltA demonstrated approximately 3 % of PG solubilisation over 25 minutes (Fig. 3.9). As such, the average rate of solubilisation was determined to be 0.15 ± 0.024 % per minute at OD_{600 nm}. The inclusion of 10 µg/mL bovine serum albumin (BSA) in the same conditions appeared to help stabilize sMltA as it led to approximately 5 % of PG solubilisation over 25 minutes BSA alone did not cause any PG lysis (Fig. 3.6). Thus, the average rate of solubilisation was 0.28 ± 0.024 % per minute, which represented a ~31 % increase in the rate BSA was chosen as an appropriate additive as it has been shown to help retain the activity of other muralytic enzymes (Herlihey et al., 2014). Consequently, future activity assays and inhibition studies with sMltA were conducted in these optimized conditions.

![Figure 3.9 Optimization of sMltA activity using the turbidimetric assay.](image)

**Figure 3.9 Optimization of sMltA activity using the turbidimetric assay.** Solubilisation of *M. luteus* whole cells (final conc. 0.4mg/mL) in 10 mM sodium acetate buffer pH 5.2, containing 10 mM magnesium chloride. sMltA (2.0 µM) in the absence (□) and presence of 10 µg/mL of BSA (△). (●) represents a negative control to accommodate settling of PG during the reaction. Error bards denote S.D (n=3).
After the optimization of sMltA activity, these conditions were used to investigate the potential of MliC to inhibit the lytic activity of sMltA. As mentioned previously, sMltA belongs to the family 2 group of LTs, which possess a conserved catalytic aspartic acid residue. This makes sMltA unique as it is the only LT to possess a conserved catalytic aspartic acid residue, in contrast to a conserved glutamic acid that is widespread amongst the LTs of all other families. To investigate if MliC could inhibit sMltA, the lytic activity of sMltA was monitored in the presence of varying concentrations of MliC. Purified MliC was able to completely inhibit the lytic activity of sMltA at the lowest molar ratio of 1:1 under optimal conditions for sMltA activity (Fig. 3.10). These data suggest that MliC is a more potent inhibitor of sMltA compared to HEWL.

![Figure 3.10 Inhibition of sMltA activity by MliC](image)

Figure 3.10 Inhibition of sMltA activity by MliC. Representative curves of the turbidimetric assay determined using suspension of M. luteus whole cells as substrate (final conc. 0.4 mg/mL) in 25 mM sodium acetate buffer pH 5.6 containing 100 mM sodium chloride, 10 mM magnesium chloride and 10 % glycerol. A. sMltA (2 µM) + 10 µg/mL BSA in the absence (○) and presence of 2.0 µM MliC (●). (●) represents a negative control to accommodate settling of PG during the reaction.

The fluorescence-based assay was used to validate the inhibition ratio observed using the turbidimetric assay and M. luteus whole cells as substrate. FITC-labeled M. luteus PG was re-suspended sMltA assay buffer (10 mM sodium acetate, 10 mM magnesium chloride, pH 5.2). sMltA (2 µM) and 10 µg/mL BSA in 25 mM sodium acetate buffer, pH 5.2, containing 100 mM...
sodium chloride, 10 mM magnesium chloride, and 10 % glycerol was pre-incubated with concentrations of MliC at the molar ratios of 1:5 for 10 min prior to the addition of FITC-labeled substrate. PG solubilisation by sMltA was monitored for 15 minutes at 25 °C, and the increase of RFUs was measured over time. Surprisingly, inhibition of the lytic activity of sMltA was not observed at a 1:5 molar excesses of MliC (Fig 3.11).

3.8 Inhibition of the Lytic Activity of sMltB by MliC

sMltB activity was previously shown to be inhibited by soluble periplasmic inhibitors Ivyp1 and Ivyp2 (Clarke et al., 2010). MliC was investigated as a potential inhibitor of sMltB, a family 3 LT, and to determine if there is specificity between membrane-bound or soluble periplasmic inhibitors. Under optimal conditions for sMltB activity, sMltB displayed an 8 % reduction of turbidity over 20 minutes (Fig. 3.8A). MliC was able to inhibit the lytic activity of sMltB in a dose-dependent manner under the specific conditions optimal for sMltB activity (Fig. 3.12). The complete inhibition of sMltB was achieved by a 4:1 molar equivalent of MliC to
sMltB (Fig 3.12A). At the same ratio (4:1), 43 % and 55 % of the initial activity sMltB remained after its pre-incubation with Ivyp1 and Ivyp2, respectively (Clarke et al., 2010). These data reveal the potential specificity between soluble and membrane-bound proteinaceous inhibitors towards membrane-bound LTs, more specifically sMltB.

**Figure 3.12 Inhibition of sMltB activity by MliC.** Representative curves of the turbidimetric assay determined using suspension of *M. luteus* whole cells (final conc. 0.4 mg/mL) as substrate in 50 mM sodium acetate buffer pH 5.8, containing 0.1 % Triton X-100. (A) sMltB (2.0 µM) in the absence (■) and presence of 2.0 µM MliC (◇), 4.0 µM MliC (▲), 6.0 µM MliC (▼), and 8.0 µM MliC (▲). (▲) represents a negative control to accommodate settling of PG during the reaction. (B) The fractional rate of inhibited enzyme (V) over the initial rate (V₀) plotted versus the molar excess of MliC to sMltB.

### 3.9 Inhibition of the Lytic Activity of sMltF by MliC

Inhibition of sMltF activity by MliC from *P. aeruginosa* was assessed using the turbidimetric assay described. Optimal conditions of sMltF activity were established to be 10 mM sodium acetate buffer pH 4.5 containing 10mM magnesium chloride. Under these conditions, 2 µM sMltF catalyzed a 10 % reduction in turbidity of PG over 25 minutes (Fig 3.13). The presence of an equimolar concentration of MliC completely inhibited this lytic activity (Fig. 3.13).
Figure 3.13 Inhibition of sMltF activity by MliC. Representative curves of the turbidimetric assay determined using suspension of M. luteus whole cells (final conc. 0.4 mg/mL) as substrate in 10 mM sodium acetate buffer pH 4.5, containing 10 mM magnesium chloride. sMltF (2 µM) in the absence (■) and presence of 2.0 µM MliC (▲). (●) represents a negative control to accommodate settling of PG during the reaction.
Chapter 4. Discussion

This study presents evidence that the physiological role of MliC is to control the autolytic activity of LT’s present within the periplasm *P. aeruginosa* PAO1. The data reported are the first observations recorded that identified MliC as an inhibitor of LT activity and that this inhibition is more effective than that toward exogenous lysozyme. With respect to the LTs, MliC is a more potent inhibitor of sMltA and sMltF, than sMltB. Nonetheless, MliC was able to inhibit LTs from multiple families and those with two different catalytic residues. Interestingly however, knockouts of *mliC*, and combined with genes encoding other periplasmic inhibitors (*ivyp1* and *ivyp2*) in combination, resulted in no noticeable change in growth compared to wild-type *P. aeruginosa* PAO1.

The discovery of the first proteinaceous inhibitor of lysozyme occurred in 2001 when *Ivy*c was accidentally co-purified with HEWL that had been added to *E. coli* cells to aid in their lysis (Monchois *et al.*, 2001). As lysozyme represents an ancient anti-bacterial enzyme of the innate immune system, follow up research evaluated the role between this proteinaceous inhibitor and lysozyme. This led to the discovery of a second form of Ivy, Ivyp2, from *P. aeruginosa* and both MliC and PliC in a select group of other Gram-negative bacteria (Abergel *et al.*, 2007; Callewaert *et al.*, 2008; Yum *et al.*, 2009). Although these proteinaceous inhibitors have been promoted as inhibitors of lysozyme, there are some observations that challenge this classification as their primary function. The localization of these proteinaceous inhibitors by producing cells specifically to the periplasm presents a physical barrier for access to lysozyme. The soluble proteinaceous inhibitors (Ivyp1, Ivyp2 and PliC) remain free within the periplasm of Gram-negative bacteria, while MliC is localized to the inner leaflet of the outer-membrane (Choi *et al.*, 2011). This specific localization of potential defensive factors to the action of lysozyme is
curious given Gram-negative bacteria are not immediately susceptible to the action of this exogenous lytic factor of innate immune systems due to presence of the physical barrier imposed by the outer membrane. Certainly, other factors of the innate immune system may disrupt the outer membrane and thereby result in the release of the inhibitors, but why would cells wait for this destructive event, which alone can result in autolysis, for their release to counter lysozyme action? This raises a second question as to why proteinaceous lysozyme inhibitors are not produced by any Gram-positive bacteria where their PG is directly exposed and sensitive to lysozyme activity (Abergel et al., 2007)? Then there is the question of the function of the Ivy homolog IvyP2 which does not inhibit lysozyme at all (Abergel et al., 2007; Clarke et al., 2010; Liu et al., 2015). These questions prompted Clarke et al., (2010) to examine the pattern of production of the proteinaceous inhibitors amongst bacteria and they observed a strict correlation to those that do not produce O-acetylated PG. The O-acetylation of the C-6 hydroxyl group of the MurNAc residue blocks the muralytic activity of LTs (and exogenous lysozyme) and thus it has been proposed that this modification provides a level of control at substrate level for these autolysins (Moynihan and Clarke, 2011). However, bacteria that do not produce O-acetylated PG, such as *P. aeruginosa* and *E. coli*, would lack this level of autolysin control. The recognition of the direct correlation between producing the Ivy proteins and lack of PG O-acetylation led Clarke et al., (2010) to show that both Ivyp1 and Ivyp2 inhibit the activity of a model LT, sMltB, with high specificity. However, the question remained if this observed inhibition of sMltB was unique to this LT and the Ivy proteins or it represented a general property of each of the proteinaceous lysozyme inhibitors toward each LT? The work reported in this thesis begins to address these two questions with the demonstration of inhibitory activity of MliC from *P. aeruginosa* toward several LTs from this opportunistic pathogen.
MliC from *P. aeruginosa* was previously shown to inhibit HEWL (Callewaert *et al.*, 2008). A crystal structure of this interaction was determined and revealed that MliC has a β-barrel structure that is stabilized by a conserved disulphide bond and an anti-parallel β-strand interaction (Yum *et al.*, 2009). A five residue loop extends from the surface of MliC and extends into the active site of HEWL. The Ser89 and Lys103 residues that comprise this five residue loop forms a H-bond and a salt-bridge with the catalytic Asp52 and Glu35 of HEWL (Yum *et al.*, 2009). Ivy proteins also inhibit HEWL by an analogous loop insertion into the active site of HEWL (Abergel *et al.*, 2007). Given that lysozyme and the LTs act upon PG with the same substrate specificity (*viz.* the β,1-4 glycosidic bond between MurNAc and GlcNAc) and that all but the family 2 LTs share the similar αβ-hydrolase structural fold (*i.e.*., the lysozyme fold), it is tempting to speculate that MliC inhibits these autolysins in the same manner as lysozyme. Regardless though of its mode of action, the higher specificity of the interactions between MliC and the LTs compared to lysozyme supports the postulate that they a physiological role in controlling the autolytic activity of the LTs.

In this study, MliC was tested against 3 LTs, sMltB, sMltF, and sMltA, and it was shown to inhibit the activity of all of them. Family 1 and family 3 LTs are structurally conserved with lysozyme despite their sequence dissimilarity (van Asselt *et al.*, 2000). As discussed above, MliC is postulated to inhibit sMltF and sMltB in the same way it interacts with lysozyme due to the structural similarities between the enzymes. However, it was a little surprising that MliC inhibits the family 2 LT, sMltA, and it does so with higher specificity than observed for sMltB and exogenous lysozyme. As a family 2 LT, sMltA has a unique Asp for its catalytic residue and an endoglucanase V fold instead of the lysozyme-type fold observed in the other LT families (van Straaten *et al.*, 2005). This may suggest that MliC may be involved with inhibiting LTs from
different families that are implicated in different cellular processes. For example, family 1 LTs are implicated with PG recycling, and the insertion of PG-spanning complexes, while family 2 LTs are believed to be involved with cellular septation. Also, the data presented in this study suggest that MliC has greater specificity for membrane-bound LTs compared to the soluble periplasmic inhibitors, Ivyp1 and Ivyp2. Thus, with these latter two inhibitors, 11:1 and 13:1 molar equivalents are required to inhibit sMltB (Clarke et al., 2010), while only a 4:1 molar excess of MliC is sufficient to abolish this activity. This apparent specificity may be conferred, at least in part, by the localization of MliC to the outer membrane and hence in proximity to the membrane-bound LTs. On the other hand, recent observations made by Herlihey, (2017) support the postulate that Ivyp1 and Ivyp2 have specificity for soluble LTs; both Slt70 and SltB1 are inhibited by 1:1 molar equivalents of these two soluble inhibitors. Future inhibition studies with MliC and both Slt70 and SltB1 may bear out this postulate further where the inhibition would presumably be relatively weak.

It was unexpected to observe that cells lacking active forms of the respective proteinaceous inhibitors were still viable and did not display an phenotypic change compared to wild-type P. aeruginosa PAO1. This observation would appear to disprove the hypothesis that these inhibitors are required to control the autolytic activity of the LTs; if correct, their absence should have led to uncontrolled autolysis and rapid cell death. A plausible explanation is that there is further redundancy of these proteinaceous inhibitors involving yet another protein that can compensate for the those lacking in the engineered PAO1 mutants. Indeed, an analogous redundancy exists with the LTs themselves, where knockout mutants lacking four LTs in an engineered P. aeruginosa PAO1 mutant were still viable (Lamers et al., 2015). Hence, one or more unidentified proteinaceous inhibitors may exist in Gram-negative organisms that contribute
to the control of the LTs. One candidate could be the Tsi3 protein produced by *P. aeruginosa* to provide immunity against a putative muramidase, Tse3, that is a component of its Type VI secretion system (Li *et al.*, 2013; Lu *et al.*, 2014).

Another level of control of the LTs at the enzyme level involves allosterism, at least for MltF from *P. aeruginosa*. Recently, Domínguez-Gil *et al.*, (2016) showed that this LT can be allosterically activated by a PG-derived muropeptide. The addition of the muropeptide caused a conformational change in MltF from an inactive to an active state that became capable of lysing PG (Domínguez-Gil *et al.*, 2016). However, Domínguez-Gil *et al.*, (2016) also observed that once MltF was in its active confirmation, the enzyme would remain in the activated state, even in the absence of the ligand that caused the initial conformational change. Therefore, the activated form of MltF within the periplasm would still need to be regulated by a second level of inhibition that could be provided by MltF to prevent potentially disastrous autolysis.

This study evaluated the applicability of an alternative assay to assess LT activity and its inhibition. This was done because the established method posed some challenges when trying to assay the more-weakly active LTs. In general, the LTs have very low rates of activity when assayed *in vitro* using the turbidimetric assay compared to that of HEWL. The settling of suspended PG is a constant issue, especially when it may account for over half of the total clearing detected during the time course of the reaction involving the weakly active LTs. This becomes truly problematic when the “signal to noise” ratio cannot be increased by the simple increase in LT concentration due to their solubility limitations. Consequently, the level of error associated with this assay can be quite high. In order to address this problem, the fluorescence-based assay developed initially by Maeda, (1980) was tested for its usefulness to monitor weak LT activity. The advantages of a fluorescence assay are that it is more sensitive than the
turbidimetric assay, indeed it can provide the same level of detection of activity as radioactive assays (Maeda, 1980). This high sensitivity would permit the use of lower concentrations of the LTs which, in turn, require lower concentrations of the inhibitors to achieve the molar ratios required to be tested. As a test of the assay, the inhibition of HEWL by MliC was repeated. There was a slight increase in the molar ratio of MliC: HEWL required to effect complete inhibition but this can be explained by the fluorescence assay being more sensitive with determining lytic activity. However, inhibition of the lytic activity of sMltA by MliC was not observed using this assay. This may be explained by understanding the effect that pH has on the FITC-PG conjugate recognizing that the HEWL assay was conducted at pH 7.3, while that for MltA was at pH 5.2. FITC is very sensitive to pH changes, as fluorescein possesses four different ionization states depending on the pH (Margulies et al., 2005). These changes in ionization state lead to each having a unique absorbance spectrum (Margulies et al., 2005). In addition, the pH of this fluorophore’s environment can decrease its overall quantum yield thus lowering the sensitivity of the assay. For example, Zhu et al., (2005) demonstrated that a pH change from 6.9 to 8.4 increases the intensity of solutions containing fluorescein by 1.25 times. Hence, to benefit from the potential of a fluorescence-based assay to monitor the LTs that function individually over a broad range of pH, future work should investigate the use of a different fluorophore reporter other than FITC, one that is less sensitive to pH changes.
4.1 Conclusions and Future Directions

In conclusion, the work reported in this thesis provided the first experimental evidence that MliC can inhibit LTs and thus likely represents a level of control of these potentially lethal autolysins in the bacteria that do not produce O-acetylated PG. However, there are still many open questions regarding the interaction between MliC and the LTs tested in this study. It would be informative to be able to obtain kinetic parameters of the interactions between MliC and MltA, MltB and MltF. To begin to address this issue, preliminary experiments have been conducted to test the feasibility of using SPR to monitor binding between MltA and MliC. In addition, further inhibition studies with MliC and the remaining LTs that are present within *P. aeruginosa* would inform on any specificity of MliC for the membrane-bound LTs. There are 11 known LTs produced by *P. aeruginosa*, seven of which are thought to be membrane-associated. Thus, the inhibitory activity of MliC toward each of the other four Mlts together with the four soluble LTs should be examined. Structural details of these interactions need to be investigated. Presumably like the interaction with HEWL, the five residue loop of MliC protrudes into the catalytic cleft of the LTs to interaction with their catalytic residues. To investigate this, site directed replacements of the residues on MliC loop, Ser89 and Lys103, could be made and inhibition studies conducted to demonstrate weakened inhibition. In addition, crystallization trails with purified LT and inhibitor would help provide structural insight with regards to the method of inhibition. Finally, it would be interesting to determine if mutant strains lacking one or more of the of the proteinaceous inhibitors are more susceptible to antibiotics, especially to those that involve autolytic activity for their lethal effect, such as the β-lactams. Overall, the results presented in this begin to uncover the details on the interactions and relationships between proteinaceous inhibitors and PG lytic enzymes in *P. aeruginosa* and provide experimental
evidence that supports the hypothesis that they have a physiological function which is to control LT activity within the periplasm. By understanding these interactions, future research can be directed to determine if this regulatory mechanism presents a viable new target for the development of a new class of antibiotics.
References


Herlihey, F. (2017). Biochemical Characterization of the Activity and Control of the Autolysins Involved in Flagella Assembly in Gram-Negative Bacteria.


lactam sensitivity in *Pseudomonas aeruginosa*. Microbiologyopen n/a-n/a.


updated version includes eukaryotes. BMC Bioinformatics 4, 41.


