PROBING THE ANTIBIOTIC TARGET MURA FROM *S. AUREUS* AND *B. SUBTILIS*

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Abstract

The survival of all microorganisms depends on the functionality of the enolpyruvyl transferase family of enzymes. MurA (UDP-N-acetylglucosamine enolpyruvyl transferase, EC 2.5.1.7) and EPSPS (5-enolpyruvyl-shikimate-3-phosahate synthase, EC 2.5.1.19) are the only known enzymes in this family. MurA catalyzes the first committed step in the biosynthesis of cell wall peptidoglycan. EPSPS is the enzyme that catalyzes the sixth step of the shikimate pathway, leading to the synthesis of essential aromatic compounds found in plants, fungi and microorganisms. Because both pathways are absent in mammals, enolpyruvyl transferases are attractive targets for the development of antimicrobial agents.

While *Escherichia coli* and all other gram-negative bacteria possess only one copy of the MurA gene, analyses of the genomes of several gram-positive bacteria reveal the existence of two MurA genes, termed MurA1 and MurA2. For *Streptomyces pneumoniae,* it has been demonstrated that both MurA1 and MurA2, encode active enzymes. Kinetic characterization of these enzymes failed, however, to demonstrate any significant difference between the two. In addition, both are inhibited by the only naturally occurring antibiotic that inactivates MurA, fosfomycin. The research presented here focuses on the cloning, expression, purification and kinetic characterization of the MurA enzymes from two gram-positive pathogenic organisms: *Staphylococcus aureus* and *Bacillus subtilis*.

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List of Abbreviations

Ala, A	Alanine
Amp	Ampicillin
Arg, R	Arginine
APS	Ammonium persulfate
BSA	Bovine serum albumin
B. subtilis	*Bacillus subtilis*
$CaCl_2$	Calcium chloride
Cm	Chloramphenicol
CV	Column volume
Cys, C	Cysteine
DMSO	Dimethysulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. cloacae	*Enterobacter cloacae*
EDTA	Ethylenediaminetetraacetic acid
EP-UNAG	Enolpyruvyl uridine diphosphate N-acetylglucosamine
EPSP	5-enolpyruvylshikimate-3-phosphate
EPSPS	5-enolpyruvyl-shikimate-3-phosphate synthase
EtOH	Ethanol
FPLC	Fast protein liquid chromatography
FOS	Fosfomycin

Glu, E	Glutamic acid
Glucoside	n-Octyl-β-D-glucoside
GOX	Glucose oxidase
Gm	Gentamycin
GST	Glutathione-S-transferase
HCl	Hydrochloric acid
HEPES	N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
IC	Inhibition constant
Ile, I	Isoleucine
IPTG	Isopropyl-β-D-thiogalactoside
Kb	Kilobase
kDa	Kilodalton
LB	Luria-Bertani
Leu, L	Leucine
MES	2-[N-Morpholino] ethanesulfonic acid
$MgCl_2$	Magnesium chloride
MurA	Uridine diphosphate N-acetylglucosamine enolpyruvyl transferase
MurB	UDP-N-acetylenolpyruvylglucosamine reductase
NaCl	Sodium chloride
NADPH	Nicotinamide adenosine dinucleotide phosphate
OD	Optical density
PCR	Polymerase chain reaction
PEP	Phosphoenol pyruvate

Phe, F	Phenylalanine
Pi	Inorganic phosphate
Q-seph	Q-sepharose
rpm	Revolutions per min
S3P	Shikimate-3-phosphate
S. aureus	*Staphylococcus aureus*
Ser, S	Serine
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
S. pneumoniae	*Streptomyces pneumoniae*
TAE	Tris, Acetic acid and EDTA buffer
TEMED	N,N,N',N'-Tetra-methylethylenediamine
Tris	Tris hydroxymethyl aminomethane
UDP	Uridine diphosphate
UNAM	UDP-N-acetylmuramic acid
UNAG	UDP N-acetylglucosamine
UV	Ultraviolet
(v/v)	Volume/volume
(w/v)	Weight/volume

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1. Introduction

1.1. The Need for New Antibiotics

According to the World Health Organization, infectious disease caused by bacterial pathogens continues to be a major cause of morbidity and mortality worldwide. Pathogenic bacteria are linked to over 60% of the total deaths in underdeveloped countries and are the third leading cause of death throughout Europe. In industrialized countries, pathogens that were formerly found only in hospital environments are now being found in communal settings [1]. The majority of the bacterial species causing these infections have acquired resistance to one or more antibiotics. Antibiotic resistance can emerge rapidly, is highly adaptable and can progress through bacterial populations with relative ease. Pathogens can become resistant to antibiotics through modification of their own genes or acquisition of resistance genes from other bacteria [2, 3]. This ability to exchange genetic material across species and genera has resulted in the accumulation of multidrug-resistant phenotypes in species where infectious disease management is especially challenging. These species include methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *pneumoncoccus*, vancomycin-resistant *Enterococcus faecalis*, and multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* [4].

Advances in DNA sequencing technology have made it possible to elucidate an organism's entire genome. The availability of various bacterial genomic sequences gives us the opportunity to compare them at various levels and understand their similarities and differences. These comparative genetics studies, in conjunction with structural and functional genomics, will provide valuable information for the generation and identification of novel drug products [5].

1.2. The Enolpyruvyl Transferase Family of Enzymes

The survival of all microorganisms depends on the functionality of the enolpyruvyl transferase family of enzymes [6]. MurA (UDP-N-acetylglucosamine enolpyruvyl transferase, EC 2.5.1.7) and EPSPS (5-enolpyruvyl-shikimate-3-phosahate synthase, EC 2.5.1.19) are the only known enzymes in this family. MurA catalyzes the first committed step toward the biosynthesis of peptidoglycan, the main component of the bacterial cell wall [7, 8]. EPSPS catalyzes the sixth step of the shikimate pathway toward the synthesis of essential aromatic compounds found in plants, fungi and microorganisms [9-11]. Because both pathways are absent in mammals, enolpyruvyl transferases are attractive targets for the development of novel antimicrobial agents [6, 12, 13].

1.2.1. Reactions Catalyzed by MurA

The reactions catalyzed by the enolpyruvyl transferases proceed through the transfer of the enolpyruvyl moiety of phosphoenol pyruvate (PEP) to the hydroxyl group of a second substrate. Unlike most PEP-dependent enzymes, which use PEP as a phosphoryl donor through the cleavage of the high-energy phosphorous oxygen bond, enolpyruvyl transferases cleave the carbon-oxygen bond of PEP to transfer the enolpyruvyl moiety to a second substrate releasing inorganic phosphate (P_i) [14] .

MurA catalyzes the transfer of the enolpyruvyl moiety of PEP to UDP-N-acetylglucosamine (UNAG) to form enolpyruvyl-UDP-N-acetylglucosamine (EP-UNAG) and P_i (Fig. 1). EP-UNAG is a precursor of N-acetylmuramic acid, which alternates with N-acetylglucosamine to form the glycan chains that constitute the peptidoglycan layer of the cell wall [13, 15].

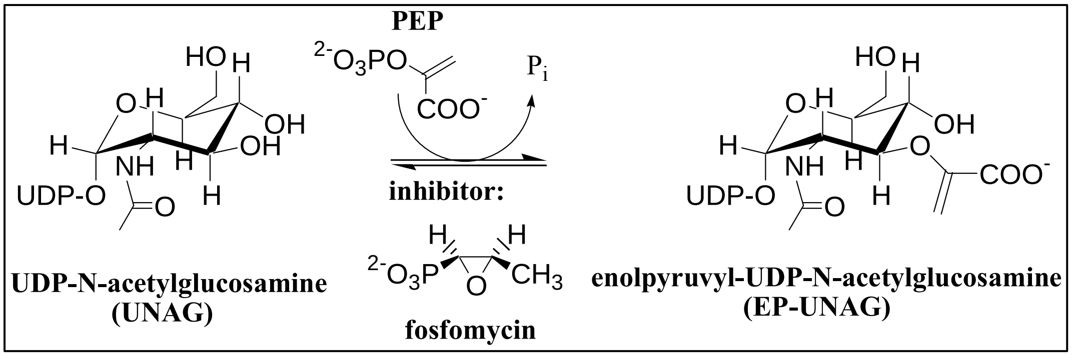

Figure 1: The reaction catalyzed by MurA.

1.2.2. Mechanism of Action of MurA

MurA has received attention because it is the molecular target of the antibiotic fosfomycin [16-18]. Mechanistic and structural data for MurA show that the reaction follows an ordered mechanism in which UNAG interacts with free enzyme prior to the binding of PEP or inhibitor (Fig. 2). The reaction pathway proceeds by the

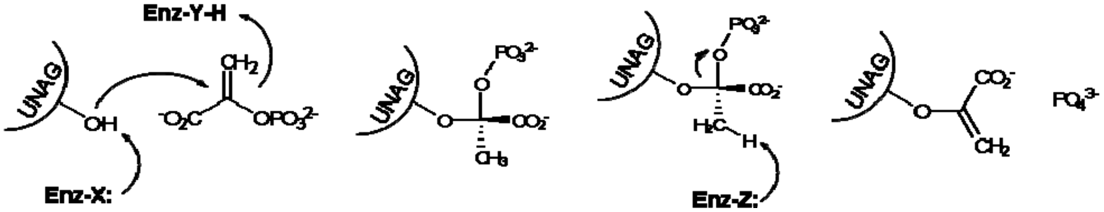

Figure 2: The addition-elimination mechanism for MurA.

addition of a proton to PEP, yielding a PEP oxocarbenium ion. The 3’-hydroxy group of UNAG is then deprotonated, resulting in a nucleophile that attacks the C-2 position of the oxocarbenium ion of PEP leading to a tetrahedral intermediate of the substrate [14, 19, 20]. A proton is then abstracted from the methyl group of the tetrahedral intermediate resulting in the formation of the vinyl ether product and P_i [20-23].

1.2.3. Catalytic Cycle of MurA

The proposed catalytic cycle of the enolpyruvyl transferases, is shown in Fig. 3.

$$\mathbf{E + S_1 \leftrightarrow E\ S_1 + S_2 \leftrightarrow E\ S_1\ S_2 \leftrightarrow EP_1P_2 \rightarrow E + P_1 + P_2}$$

Figure 3: The catalytic cycle of MurA.

The free enzyme (E) reversibly binds the first substrate (S_1), forming a binary complex (ES_1) that undergoes a conformational change resulting in the formation of an active site for the second substrate. The second substrate (S_2), can then form a ternary complex (ES_1S_2) that is followed by the formation of two reaction products (EP_1P_2). Lastly, product release ($E + P_1 + P_2$) allows the enzyme to return to its original conformation [15, 24, 25].

Following formation of the rapidly reversible MurA-UNAG complex, the loop region of MurA (residues 111-122), undergoes a conformational change that allows for the binding of PEP or fosfomycin in the active site [26, 27]. Fosfomycin inhibits MurA by forming an irreversible [15] covalent attachment with the thiol group of the Cys115 residue [19, 28-30] in the loop region preventing PEP from binding to the active site [22, 31]. Although fosfomycin covalently links to the thiol group of

Cys115, it is not merely a group-specific reagent, because no other enzyme is known to be modified by this epoxide [16, 28, 30, 32].

1.3. Purpose of Research

Interference with cell wall biosynthesis is well-established as an excellent mechanism for killing both gram-positive and gram-negative bacteria, and enzymes of bacterial cell wall synthesis have historically been important targets for antibacterial agents [13, 23]. A series of enzymes, MurA to MurF, are required to synthesize peptidoglycan. Inhibition of any one of these enzymes leads to cell lysis and death; however, aside from the inhibition of MurA by fosfomycin, enzymes in this pathway are not targeted by any known antibacterial agents [12, 13, 28, 33].

While *E. coli* and all other gram-negative bacteria possess only one copy of the MurA gene, analysis of the genomes of several bacterial species revealed two copies of the MurA gene [34]. Found primarily in gram-positive bacteria exhibiting low-G+C content, the duplicate genes were termed MurA1 and MurA2. When compared to the protypical gram-negative MurA, the MurA1 sequence typically exhibits higher sequence conservation than the similarly-sized MurA2. Comparison to the structure of *E. coli* MurA shows that the major structural features and residues involved in ligand interactions are highly conserved, suggesting that it is unlikely that one of the genes is a nonfunctional copy.

In the gram-positive bacteria *Streptomyces pneumoniae,* it was demonstrated that the MurA1 and MurA2 genes encode two active enzymes that are neither close to each other in the genome nor clustered with any other peptidoglycan synthesis enzymes [34]. Kinetic characterization of both enzymes, however, failed to demonstrate any significant differences between the two, and both are inhibited by fosfomycin. This suggests that both enzymes are active tranferases, able to catalyze the transfer of enolpyruvate from PEP to UNAG. In addition, gene knockout experiments revealed that single deletions of either gene did not cause a loss of viability, but deletion of both genes was lethal. Therefore, it is critical that a novel MurA inhibitor possess inhibitory activity against both MurA1 and MurA2 to ensure antibacterial activity against gram-positive cocci [6].

To design new antibiotics, we need to improve our knowledge of the structural and functional genomics of pathogenic bacteria. To date, little information is known about the pair of MurA enzymes found in many gram-positive bacterial pathogens. The research presented here focuses on the expression, purification and kinetic characterization of the MurA enzymes from two such organisms: *Staphylococcus aureus* and *Bacillus subtilis*.

2. Materials and Methods

2.1. General Materials

2.1.1. Competent Cells

Competent cell strains used were: One Shot® BL21(DE3) (Invitrogen, Carlsbad, CA), BL21(DE3)pLysS (Invitrogen), BL21-CodonPlus(DE3)-RIL (Stratagene, La Jolla, CA), ArcticExpress(DE3) (Stratagene) and DH5α (Invitrogen).

2.1.2. Chemicals and Equipment

A Labconco Water Pure Plus System was used to purify all H_2O used for research. All chemicals and equipment were purchased from Sigma (St. Louis, MO) or Fisher (Springfield, NJ) unless otherwise noted.

2.1.3. Solutions

2.1.3.1. Media

All media was autoclaved and stored at 4 ˚C. Antibiotic stocks were added to the media prior to use.

<u>LB Media:</u>

10 g tryptone

7.5 g yeast extract

5 g NaCl

Fill to 1 L with H_2O

LB Agar:

20 g granulated agar

Fill to 1 L with LB media

To prepare plates, the agar was heated in the microwave until liquid and then allowed to cool to roughly 30 °C before antibiotic stocks were added. The LB-antibiotic agar was then poured into the desired number of Petri dishes in a Laminar flow hood.

2.1.3.2. Antibiotics

Ampicillin stocks at 100 mg/ml in H_2O, gentamycin stocks at 20 mg/ml in H_2O and chloramphenical stocks at 25 mg/ml in EtOH were sterile filtered with a 0.45 µm filter and stored at -20 ˚C. Amp and Gm stocks were diluted 1 : 1000 (final [Amp] = 100 µg/ml; final [35] = 20 µg/ml) and Cm stocks were diluted 1 : 2000 (final [Cm] = 12.5 µg/ml) for all purposes.

2.1.3.3. DNA Electrophoresis

A 50X concentrated solution of TAE was stored at room temperature and diluted to 1X prior to use. The following protocol was used to prepare the DNA gel: 500 mg of agarose was heated in 50 ml of 1X TAE until dissolved, 1 µl of ethidium bromide (5 mg/ml) was added to the solution and the gel was cast using a DNA mini-submarine gel electrophoresis unit (GE Healthcare, Piscataway, NJ). The DNA was visualized with UV light following electrophoresis.

<u>50X TAE:</u>

242 g Tris (pH 8.5)

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA (pH 8.0)

Fill to 1 L with H_2O

Molecular ladder was prepared by adding 5 µl DNA marker (Cambrex Bio Science, Rockland, ME) and 2 µl 10X Bluejuice (Invitrogen) to 3 µl H_2O. The DNA ladder corresponded to 10, 7, 5, 4, 3, 2.5, 2, 1.5 and 1 Kb markers.

2.1.3.4. SDS Electrophoresis

Running buffer was prepared as a 10X concentrated solution and diluted to 1X prior to use. Coomassie stain was filtered with a Whatman 5 filter and stored in a dark bottle. Acrylamide/Bis Solution, 30% 19:1 crosslinker ratio (Biorad, Hercules, CA), APS, TEMED (Biorad) and Tris buffers were stored at 4 ˚C. All other solutions were stored at room temperature. SDS-PAGE gels were made according to the following protocol and stored at 4 ˚C for up to 2 weeks. The resolving portion of the gel was cast and allowed to solidify before casting the stacking portion. Gels were stained for a minimum of 15 minutes and destained for several hours in the solutions listed below.

<u>Resolving Portion SDS gel: (1 gel)</u>

4 ml 30 % Acrylamide/Bis solution

100 µl 10 % (w/v) SDS

2.5 ml 1.5 M Tris (pH 8.8)

3.36 ml H_2O

80 µl 10 % (w/v) APS

10.0 µl TEMED

Stacking Portion SDS gel: (1 gel)

650 µl 30 % Acrylamide/Bis solution

50 µl 10 % SDS

1.25 ml 0.5 M Tris (pH 6.8)

3.05 ml H_2O

40 µl 10 % APS

5 µl TEMED

10X SDS Running Buffer:

30 g Tris (pH 8.5)

144 g glycine

10 g SDS

Fill to 1 L with H_2O

Coomassie Stain:

1.25 g Coomassie brilliant blue R-250

500 ml 95 % EtOH

450 ml H_2O

50 ml 100 % acetic acid

<u>Destain:</u>

500 ml EtOH

100 ml acetic acid

400 ml H_2O

<u>5X Loading Buffer:</u>

1.82 g Tris (pH 8.5)

5 g SDS

12.5 ml β-mercaptoethanol

25 ml glycerol

1.28 ml HCl

0.028 g bromophenol blue

Fill to 50 ml with H_2O

<u>SDS Low Range Marker:</u>

Marker was prepared by adding 50 μl concentrated marker (Biorad) and 100 μl 5X loading dye to 350 μl H_2O and heating the solution for 5 min at 100 ˚C. Marker was stored at -20 ˚C. The molecular weights in the marker corresponded to 97.4, 66.2, 45, 31, 21.5 and 14.4 kD.

2.1.3.5. FPLC Buffers

All buffers for protein purification were prepared using purified H_2O cooled to 4 °C. Following filtration with a 0.45 μm filter, the pH was adjusted with 12 N HCl and the buffers were stored at 4 ˚C.

Buffer A:

50 mM Tris (pH 7.8)

2.5 mM DTT

1 mM EDTA

Buffer A + 1.0 M NaCl:

50 mM Tris (pH 7.8)

1.0 M NaCl

2.5 mM DTT

1 mM EDTA

Buffer A + Glutathione:

50 mM Tris (pH 7.8)

10 mM reduced glutathione

2.5 mM DTT

1 mM EDTA

Extraction Buffer:

50 mM Tris (pH 7.8)

2.5 mM DTT

1 mM EDTA

0.1 % (v/v) Tween 20

PreScission Buffer A:

50 mM HEPES (pH 8.0)

150 mM NaCl

2 mM DTT

1 mM EDTA

PreScission Buffer A + Glutathione

50 mM HEPES (pH 8.0)

150 mM NaCl

2 mM DTT

1 mM EDTA

10 mM reduced glutathione

2.2. General Methods

2.2.1. Preparation of Competent Cells

Commercially available competent cells were used to make lab stocks of new $CaCl_2$ competent cells. BL21(DE3) and DH5α cells were grown in media containing no antibiotic, PLysS(DE3) and RIL(DE3) cells were grown in media containing chloramphical and ArcticExpress(DE3) cells were grown in media containing gentamycin. A Beckman J2-21 centrifuge programmed to spin for 10 min at 6,000 rpm and 4 ˚C was used for all centrifugation steps. All cells were incubated at 37 ^{o}C while shaking at 250 rpm. Overnight cultures were grown in 5 ml LB media and then transferred to 250 ml LB media. Upon reaching an OD_{600} of 0.5 - 0.7, the cells were harvested by centrifugation. The supernatant was decanted and the pellets were washed with 100 ml of sterile 0.1 M $MgCl_2$ and then the cells were harvested by centrifugation. The supernatant was decanted and the pellets were resuspended in

100 ml of sterile 0.1 M $CaCl_2$. The resuspended solution was incubated on ice for 20 min and then the cells were harvested by centrifugation. The supernatant was decanted and the cells were resuspended in 20 ml of sterile 0.1 M $CaCl_2$. Following resuspension, the cells were incubated for 30 min on ice before 5 ml of sterile 50 % (v/v) glycerol was added. The cells were aliquoted into microcentrifuge tubes and stored at -80 ˚C.

2.2.2. Transformations

Competent cells were transformed by adding 50 to 250 ng of plasmid to 100 µl of cells. This mixture was incubated on ice for 30 min, heat-shocked for 45 sec at 42 ˚C and incubated again on ice for 2 min. After adding 900 µl of LB media, the mixture was incubated 1 h at 37 °C while shaking at 250 rpm. The cells were then centrifuged for 30 sec at 13,000 rpm using an Eppendorf 5417C centrifuge and 900 µl of supernatant was removed. The pellet was resuspended in the remaining broth, plated on LB agar plates containing the appropriate antibiotic and incubated overnight at 37 ˚C.

2.2.3. Plasmid Preparations

Plasmids were isolated and purified using the Mini-Prep kit (Qiagen, Valencia, CA), according to the specified protocol. DNA was eluted in 40 µl sterile H_2O.

2.2.4. DNA Electrophoresis

All DNA samples were prepared by adding 2 µl of 10X Bluejuice and 2 µl of DNA (50 - 250 ng) to 6 µl of H_2O. Electrophoresis of gels was performed at 120 mV for 45 min.

2.2.5. Sequencing

DNA sequences were determined using commercially available pGEX vector primers (GE Healthcare) at the University of Kansas Medical Center's biotech research support facility using a PE Biosystems Prism 377XL sequencer (Applied Biosystems, Foster City, CA).

2.2.6. Induction Studies

The positively sequenced plasmids of *S. aureus* and *B. subtilis* MurA1 and MurA2 were separately transformed into BL21(DE3), ArcticExpress(DE3), pLysS(DE3) and RIL(DE3) competent cell lines to look for soluble over-expression. All cell lines were analyzed by choosing three separate colonies from each transformation plate and incubating them individually in 5 ml LB-Amp media overnight at 37 °C and 250 rpm. The appropriate cell antibiotics (2.1.3.2) were included in the ArcticExpress(DE3), pLysS(DE3) and RIL(DE3) overnights. A 50 µl aliquot of the overnight culture was used to inoculate 5 ml of fresh LB-antibiotic media, and the culture was incubated at 37 ˚C while shaking at 250 rpm. After reaching an OD_{600} of 0.4 - 0.6, the temperature of the culture was lowered to 18 ^{o}C and a 1 ml aliquot was removed to serve as a control. Incubation continued for 20 -

30 min and then the cells were induced with IPTG to a final concentration of 0.5 mM. After induction, cells were incubated for 25 - 27 h at 18 ˚C while shaking at 250 rpm. A 1 ml aliquot was removed and all aliquots were centrifuged at 13,000 rpm for 1 min using an Eppendorf 5417C centrifuge. Following the removal of the supernatant, each pellet was resuspended with 200 µl extraction buffer and sonicated on ice (settings: duty cycle 100%, micro tip 4, pulsed sonication) for 2 x 20 s. Following sonication, the induced pellet solution was centrifuged for 1 min at 13,000 rpm and the supernatant (soluble protein) was transferred to a microcentrifuge tube. The pellet (cell debris and insoluble protein) was resuspended in 200 µl of extraction buffer. The control, supernatant, and pellet samples were mixed with 50 µl 5X loading dye, vortexed briefly, and heated for 5 min at 100 °C. The levels of over-expression were visualized with SDS-PAGE. In an attempt to increase the amount of soluble protein, the induction studies were repeated with adjustments to the time and temperature of incubation, the cell density at the time of induction and the final concentration of IPTG.

2.2.7. Glycerol Stocks

A 900 µl aliquot of an overnight cell culture was mixed with 160 µl 50% glycerol and stored at -80 ^{o}C for use in future experiments.

2.2.8. SDS Electrophoresis

All SDS-PAGE samples were prepared by adding 10 µl 5X loading buffer to 40 µl sample. The samples were heated for 5 min at 100 ˚C, and were loaded onto the gel by pipeting 15-20 µl into each lane. Electrophoresis of gels was performed using a mini PROTEAN® 3 cell (Biorad) at 160 V for 1 h.

2.2.9. Determination of Protein Concentration

Protein concentration was determined using Coomassie reagent (Pierce, Rockford, IL) according to the Bradford method [36]. The standards and unknowns were prepared using the following protocol: 1.7 µl of protein was added to 31.7 µl H_2O and 300 µl Coomassie reagent in a 96-well plate and the absorbance was measured at 596 nm using a SpectraMax 340PC plate reader. The UV absorption of each unknown was fit to a BSA standard curve at concentrations of 0.5, 1.0, 1.5 and 2.0 mg/ml to determine the protein concentration.

2.3. *E. cloacae* MurA and *E. coli* MurB

To measure enzymatic activity, the MurA - MurB coupled assay was utilized [37]. The coupled reaction is started by MurA converting UNAG and PEP to enolpyruvyl-UNAG (EP-UNAG), which is subsequently reduced to UDP-N-acetylmuramic acid (UNAM) by MurB, using one equivalent of NADPH (Fig. 4). To compensate for the diaphorase activity of MurB, a glucose oxidase (GOX)/glucose system was exploited, resulting in a stable base line prior to initiation of the MurA reaction.

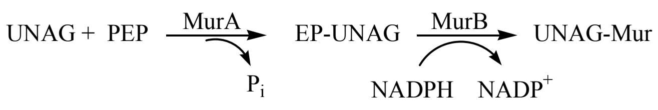

Figure 4: MurA-MurB coupled assay.

2.3.1. DNA

E. cloacae MurA in a pET-9d vector was provided by Dr. Ernst Schönbrunn. *E. coli* MurB in a pGEX-5X-1 GST fusion vector was provided by Dr. Florian Krekel.

2.4. *S. aureus* and *B. subtilis* MurA

2.4.1. DNA

The pGEX-6P-1 vector containing tac promoter was purchased from GE Healthcare. This particular vector was chosen to enhance solubility of the protein as well as simplifying the purification due to the GST tag. The *S. aureus* MurA1 and the *B. subtilis* MurA1 and MurA2 genes were provided by Scott Crupper. The *S. aureus* MurA2 gene was synthesized in the pGEX-6P-1 vector by GeneArt (Regensburg , Germany).

2.4.2. Primers

All primers for cloning were synthesized by MWG Biotech AG (High Point, NC), diluted to 100 pmol/µl with H_2O and stored at -20 ˚C. All primers are written in the 5' to 3' direction with the altered codon or the restriction sites used for restriction digests underlined.

S. aureus MurA1:

S33L #1- CCAATATTGACAGCATCTTTATTAGCTTCTGATAAACCGAGTAAATTAG

S33L #2- CTAATTTACTCGGTTTATCAGAAGCTAATAAAGATGCTGTCAATATTGG

I335F #1-
CCGAAACTGTTTTTGAAAACCGTTTTATGCATGTTGCAGAGTTCAAACG

I335F #2-
CGTTTGAACTCTGCAACATGCATAAAACGGTTTTCAAAAACAGTTTCGG

B. subtilis MurA1:

BamH I- ATACGCGGATCCATGGAAAAAATCATCGTCCGCGGC

Not I- GTCGTTTCTGACTTAAATGCATAAGCGGCCGCAAAAGGAAAA

B. subtilis MurA2:

EcoR I- ATACCGGAATTCATGGAAAAGTTGAATATTGCCGGCGGTGACTCGTT

Not I- GAACAGCTTCAAAATTCATAAGCGGCCGCTAAACTATGGCCGGCCG

A374S #1- GCGTGCCGGATCCTGCTTGGTGGTAGCCGG

A374S #2- CCGGCTACCACCAAGCAGGATCCGGCACGC

2.4.3. Polymerase Chain Reaction

The *B. subtilis* DNA and primers were used to set up PCR and restriction digest sites to ligate the genes into the pGEX-6P-1 vector. All reagents for PCR were from the Failsafe PCR kit (Epicenter, Madison, WI). The PCR mixture contained the following: 46 µl H_2O, 1 µl (2 - 10 ng) DNA template, 1 µl (100 pmol/µl) of each primer, 1 µl enzyme mix and 50 µl buffer B. The reaction was prepared on ice and divided into two tubes that were then placed in an Eppendorf Mastercycler, set for an initial denaturing period at 95 °C for 4 min. A cycle of denaturation at 95 °C for 45 s, annealing at 67 °C for 45 s and elongation at 72 °C for 2.5 min was repeated 33 times. The thermocycler was programmed to hold at 4 °C indefinitely following the completed program. After PCR was completed, 10 µl of 10X Bluejuice was added to each tube and the total volume of the sample was loaded onto an agarose gel.

2.4.4. Gel Extractions

Gel extractions were performed on the PCR products using a Gel-Extraction kit (Qiagen), according to the specified protocol.

2.4.5. Restriction Digests

All enzymes and buffers for restriction digests were purchased from New England Biolabs (Beverly, MA). Single site restriction digests were performed on the PCR products and pGEX vectors using the following protocol: 4 µl H_2O, 5 µl buffer (EcoR I buffer for *B. subtilis* MurA1 and buffer 3 for *B. subtilis* MurA2), 0.5 µL BSA (1 mg/ml), 1 µl (10 U) Not I, and 40 µl (1 - 5 µg) PCR product or vector. These digests were incubated for 16 h at 37 °C. The DNA was then purified by the QIAquick PCR Purification kit (Qiagen), according to the accompanying literature. DNA was eluted in 40 µl H_2O. A second single site restriction digest for the PCR products and vectors was set up following the same protocol, except that 1 µl (20 U) BamH I or 1 µl (20 U) EcoR I was used in place of Not I. These digests were incubated for 4 h at 37 ^{o}C. The DNA was then purified by the QIAquick PCR Purification kit and eluted in 40 µl H_2O.

2.4.6. Ligations

All enzymes and buffers for ligation were purchased from New England Biolabs. The digested PCR products of *B. subtilis* MurA1 and MurA2, were ligated into the p-GEX-6P-1 vector. Ligations were set up using a 4:1 insert:vector size ratio by adding 4 µl (100 ng) PCR product, 10 µl H_2O, 2 µl 10X ligation buffer, 3 µL (100 ng) digested vector and 2 µl (800 U) ligase to a microcentrifuge tube and incubating the reaction for 16 h at 19 °C. The ligation products were transformed into BL21(DE3) competent cells and several colonies were selected for plasmid preparations. Restriction digests of the new construct were performed using the restriction digest protocol described previously (2.4.5) to verify the size of the inserted gene. The sequence of the new construct was verified by sequencing.

2.4.7. Site-Directed Mutagenesis

Site-directed mutagenesis of *S. aureus* MurA1 and *B. subtilis* MurA2 were performed to correct mutations introduced in the cloning process using the Qwik ChangeII Site-directed Mutagenesis kit (Stratagene). The desired mutation was made to the DNA using PCR and the following protocol: 38.6 µl H_2O, 5 µl 10X reaction buffer, 2 µl (25 - 50 ng) DNA, 1.7 µl (125 ng) of each primer, 1 µl dNTP mix, and 1 µl polymerase was mixed on ice. The reaction was placed in an Eppendorf Mastercycler set for an initial denaturing period at 95 °C for 30 s. A cycle of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min and elongation at 68 °C for 15 min was repeated 30 times. The thermocycler was programmed to hold at 4 °C indefinitely following the completed program. To remove vector that had not been mutated, 1 µl of DpnI was incubated with the reaction mixture for 2 h at 37 ˚C and then stored at -20 ˚C. Following PCR, the products were transformed into DH5α competent cells and several colonies were selected for plasmid preparations. The corrected mutation was verified by DNA sequencing.

2.4.8. Over-expression of MurA

Over-expression of the MurA-GST fusion proteins was carried out using the following protocol: An overnight culture was prepared in which 50 µl of the RIL(DE3) cell glycerol stock was added to 50 ml of LB-Amp-Cm media and incubated overnight at 37 °C while shaking at 250 rpm. A 7 ml aliquot of the overnight culture was added to each of 6 flasks containing 670 ml LB-Amp-Cm media and 1 drop of antifoam. These cultures were incubated at 37 °C while shaking at 200 rpm until reaching an OD_{600} of 0.4 - 0.6. The temperature of the culture was then lowered to 18 ^{o}C and incubation continued for 20 - 30 min before the cells were induced with IPTG to a final concentration of 0.5 mM. After induction, cells were incubated for 25 - 27 h at 18 ˚C while shaking at 200 rpm before being centrifuged for 10 min at 4 ˚C and 6,000 rpm using a Beckman J2-21 centrifuge. The supernatant was discarded, the cell pellets were collected, massed and frozen at -80 °C.

2.4.9. Purification of MurA

The MurA-GST fusion proteins were purified at 4 °C using an ÄKTA FPLC system (GE Healthcare). Cells pellets were suspended in 10 ml extraction buffer per 1 g of cells plus 1 mg lysozyme per 1 g of cells by stirring for 2 h at 4 °C. The solution was sonicated (settings: duty cycle 100%, micro tip 3, pulsed sonication) on ice for 2 x 30 sec and centrifuged for 60 min at 4 ^{o}C and 18,000 rpm using a Beckman J2-21 centrifuge. The supernatant was collected and was loaded onto a 40 ml GSTPrep FF 16/10 column (GE Healthcare) that had been pre-equilibrated with buffer A. The column was washed with 10 CV buffer A to wash out unbound protein and MurA was eluted using a single step-gradient to 100 % buffer A + glutathione. The fractions were analyzed for the MurA-GST fusion protein by SDS-PAGE. Fractions containing the fusion protein were digested with PreScission protease in a 1:25 (mg:mg) ratio of protease to MurA for 4 h at 4 ^{o}C while concentrating in an Amicon device with a 30 kD filter (Millipore, Bedford, MA). This protein solution was subjected to a second purification step using a 20 ml HiLoad 16/10 Q Sepharose FF column (GE Healthcare) or a 6 mL Resourse Q column (GE Healthcare). The HiLoad Q-Seph was used for both MurA1 enzymes due to a high amount of protein (150 – 200 mg) being purified. The Resource Q was used for both MurA2 enzymes due to a limited amount of protein (10 – 15 mg) being purified. The protein was applied to the column which had previously been equilibrated with buffer A. The protein was eluted from the column by increasing the salt concentration in the mobile phase via a gradient from buffer A to buffer A + 1.0 M NaCl from 0% to 40% over 10

CV. The gradient was then increased from 40% to 100% over 2 CV with an added wash of 100% buffer A + 1.0 M NaCl for 3 CV. Fractions were assayed for protein content using SDS-PAGE and were desalted and concentrated to less than 20 ml using an Amicon device and Millipore 30 kD filter. Desalting was accomplished by adding buffer A until the salinity readings on a Corning Checkmate II conductivity meter were less than 10 μS/cm^2. After concentration, the fractions were aliquoted into 2 ml microcentrifuge tubes and stored at -80 ^{o}C.

2.4.10. Kinetic Analysis of MurA

Kinetic analysis of MurA using a continuous assay requires coupling to MurB, which utilizes the product EP-UNAG to form UNAG-Mur while consuming NADPH. Control samples with no MurA were used to account for a decrease in absorbance due to oxidation of NADPH. The change in NADPH absorbance was recorded at 340 nm in a 96-well plate using a SpectraMax 340PC plate reader. Enzyme activity is expressed as mmol product / min of reaction / mg of MurA (U/mg). Data evaluation was performed with SigmaPlot (SPSS Science, Chicago, IL, USA).

The kinetic analysis of the MurA enzymes was carried out in 250 μl of 50 mM HEPES (pH 8.0) + 2 mM DTT at 25 ^{o}C using the MurA - MurB coupled assay. All assays were performed with a final concentration of 50 mM KCl, 20 mM glucose, 20 U GOX, 0.3 mM NADPH, 20 μg MurB and enzyme concentrations that were dependent on the activity and availability of each enzyme: 0.01 mg *E. cloacae* MurA, 0.3 mg *S. aureus* and *B. subtilis* MurA1, 0.02 mg *S. aureus* MurA2, and 0.2 mg *B.*

subtilis MurA2. Assays to determine the K_m values were performed with saturating amounts of the first substrate (6 mM UNAG or 4 mM PEP) and increasing concentrations (0.1 – 3.0 mM) of the second substrate. All assays were started by addition of the saturating substrate. The K_m values were determined by fitting the kinetic data to equation 1

$$v = \frac{V_{\max} * [S]}{K_m + [S]}$$ Equation 1

where v is the initial velocity, V_{max} is the maximum velocity, K_m is the Michaelis constant and [S] is the substrate concentration (UNAG or PEP) being varied.

Assays to determine the IC_{50} values were performed for *E. cloacae* MurA, *S. aureus* and *B. subtilis* MurA1 using final concentrations of 1 mM UNAG, 1 mM PEP and increasing concentrations (0.1 – 1000 μM) of fosfomycin. Assays to determine the IC_{50} values for *S. aureus* and *B. subtilis* MurA2 were performed with the K_m value for UNAG (43 μM), 1 mM PEP and increasing concentrations (0.1 – 1000 μM) of fosfomycin. All assays were started with the addition of PEP following a 15 min incubation period. IC_{50} values were determined by fitting the data to equation 2

$$v = V_{\min} + \frac{V_{\max} - V_{\min}}{1 + \left(\frac{[I]}{IC_{50}} \right)^n}$$ Equation 2

where v is the initial velocity, V_{max} is the maximum velocity, V_{min} is the minimum velocity, [I] is the concentration of inhibitor and n is the hill slope.

The pH profile of the *S. aureus* and *B. subtilis* MurA enzymes was examined and compared to *E. cloacae* MurA. Assays were performed in 250 µl of 50 mM MES (pH 6.0) + 2 mM DTT, 50 mM MOPS (pH 7.0) + 2 mM DTT, 50 mM HEPES (pH 8.0) + 2 mM DTT and 50 mM TAPS (pH 9.0) + 2 mM DTT at 25 ^{o}C using the MurA - MurB coupled assay. All assays were performed with a final concentration of 50 mM KCl, 20 mM glucose, 20 U GOX, 0.3 mM NADPH, 20 µg MurB, 1 mM UNAG, 1mM PEP and the same enzyme concentrations that were used for the kinetic analysis. All pH profile assays were started by addition of PEP.

The effect of specific cations and anions on the activity of *S. aureus* and *B. subtilis* MurA enzymes was examined and compared to *E. cloacae* MurA. Assays were performed in 250 µl of 50 mM HEPES (pH 8.0) + 2 mM DTT at 25 ^{o}C using the MurA - MurB coupled assay. All assays were performed with a final concentration of 20 mM glucose, 20 U GOX, 0.3 mM NADPH, 20 µg MurB, 1 mM UNAG, 1 mM PEP and the same enzyme concentrations that were used for the kinetic analysis. Three concentrations (50 mM, 100 mM, 250 mM) of the following salts were used to test each enzyme: CaCl, KCl, NaCl, NH_4Cl, $Ca(CH_3CO_2)_2$, KCH_3CO_2, $NaCH_3CO_2$, $NH_4CH_3CO_2$, Na_2SO_4 and $(NH_4)_2SO_4$. All ionic-dependence assays were started by addition of PEP.

The effect of detergents and organic solvents on the activity of *S. aureus* and *B. subtilis* MurA enzymes was examined and compared to *E. cloacae* MurA. Buffer solutions were prepared containing 50 mM HEPES (pH 8.0) + 2 mM DTT and 5 % and 10 % (v/v) of the following solutions: DMSO, EtOH, Triton X-100, glycerol, and n-Octyl-β-D-glucoside. Assays were performed in 250 µl of each buffer at 25 ^{o}C using the MurA - MurB coupled assay. All assays were performed with a final concentration of 50 mM KCl, 20 mM glucose, 20 U GOX, 0.3 mM NADPH, 20 µg MurB, 1 mM UNAG, 1 mM PEP and the same enzyme concentrations that were used for the kinetic analysis. All detergent and solvent assays were started by addition of PEP.

2.5. PreScission Protease

2.5.1. DNA

PreScission protease was provided by Dr. Ernst Schönbrunn.

2.5.2. Over-expression of PreScission

Over-expression of the PreScission-GST fusion protein was carried out using the following protocol: an overnight culture was prepared in which 50 µl of the glycerol stock in pLysS(DE3) cells was added to 50 ml of LB-Cm media and incubated overnight at 37 °C while shaking at 250 rpm. A 7 ml aliquot of the overnight culture was added to each of 6 flasks containing 670 ml LB-Cm media and 1 drop of antifoam. These cultures were incubated at 37 °C while shaking at 200 rpm until an OD_{600} of 0.9 – 1.1 was reached. The cells were induced with IPTG to a final concentration of 0.3 mM, and were allowed to grow for 4 - 5 h at 37 ˚C while shaking at 200 rpm before being centrifuged for 10 min at 4 °C and 6,000 rpm using a

Beckman J2-21 centrifuge. The supernatant was discarded, the cell pellets were collected, massed and frozen at -80 °C.

2.5.3. Purification of PreScission

The PreScission-GST fusion protein was purified at 4 °C using an ÄKTA FPLC system. Cells pellets were suspended in 10 ml PreScission buffer A + 0.1 % Tween 20 per 1 g of cells plus 1 mg lysozyme per 1 g of cells by stirring for 2 h at 4 °C. The solution was sonicated (settings: duty cycle 100%, micro tip 3, pulsed sonication) on ice for 2 x 30 sec and centrifuged for 60 min at 4 ^{o}C and 18,000 rpm using a Beckman J2-21 centrifuge. The supernatant was collected and was loaded onto a 40 ml GSTPrep FF 16/10 column that had been pre-equilibrated with PreScission buffer A. The column was washed with 10 CV PreScission buffer A to wash out unbound protein and PreScission was eluted using a single step-gradient to 100 % PreScission buffer A + glutathione. The fractions were analyzed for the PreScission-GST fusion protein by SDS-PAGE. Fractions containing the fusion protein were concentrated to less than 20 ml using an Amicon device with a 30kD filter. After concentration, 20 % (v/v) glycerol was added and the fractions were aliquoted into microcentrifuge tubes and stored at -80 ^{o}C.

3. Results and Discussion

3.1. Molecular Alignments of Pathogenic Bacteria

Table 1: Identity and similarity of MurA from pathogenic bacteria to *E. Cloacae* MurA.

Organism	Identity/Similarity to *E. cloacae* MurA	
Staphylococcus aureus	MurA1	49% / 72%
	MurA2	42% / 68%
Bacillus subtilis	MurA1	48% / 71%
	MurA2	45% / 69%
Streptococcus pneumoniae	MurA1	50% / 69%
	MurA2	44% / 67%
Enterococcus faecalis	MurA1	45% / 70%
	MurA2	46% / 68%
Clostridium tetani	MurA1	46% / 70%
	MurA2	48% / 70%
Helicobacter pylori	MurA	47% / 69%
Campylobacter jejuni	MurA	49% / 73%
Vibrio vulnificus	MurA	78% / 91%
Mycobacterium tuberculosis	MurA	43% / 66%
Escherichia coli	MurA	93% / 97%

Sequence alignment is a tool that can be used to identify regions of similarity in genetic material. In protein sequences, the degree of similarity between amino acids occupying a particular position can be interpreted as a rough measure of how conserved a region is among lineages. The absence of substitutions in a particular region, or the presence of only very conservative substitutions, suggests that this region has structural or functional importance. Global alignments (similarity matrix PAM250) of MurA from several pathogenic bacteria reveal approximately 50 % identity and 70 % similarity (Table 1) to *E. cloacae* MurA. Similar values are also found when aligning the two MurA genes from the same species. The sequence alignments of *E. cloacae* MurA versus *S. aureus* and *B. subtilis* MurA1 and MurA2 are shown in Table 2. Since fosfomycin inhibits *E. cloacae* MurA by forming a covalent bond with the active site residue Cys115, we can speculate whether the *S. aureus* or *B. subtilis* MurA will be inhibited by fosfomycin. Indeed, the sequence alignments do show that these particular gram-positive MurA enzymes possess the corresponding Cys115 residue. The outcome of the sequence alignments led us to pursue the biochemical characterization of these enzymes. The MurA1 and MurA2 genes were amplified by PCR followed by restriction digestion and ligation into pGEX-6p-1 vector as described in section 2.4.

Table 2: Sequence alignments of *E. cloacae* MurA, *S. aureus* and *B. subtilis* MurA1 and MurA2. Residues that are identical in all sequences are highlighted and correspond to the loop region of E. *cloacae* MurA (residues 111-122).

```
                  ....|....|....|....|....|....|....|....|....|....|
                      5         15        25        35        45
E.cloacae MurA    --MDKFRVQGPTRLQGEVTISGAKNAALPILFAALLA-EEPVEIQNVPKL
S.aureus MurA1    --MDKIVIKGGNKLTGEVKVEGAKNAVLPILTASLLASDKPSKLVNVPAL
S.aureus MurA2    MAQEVIKIRGGRTLNGEVNISGAKNSAVAIIPATLLA-QGHVKLEGLPQI
B.subtilis MurA1  --MEKIIVRGGQKLNGTVKVEGAKNAVLPVIAASLLASEEKSVICDVPTL
B.subtilis MurA2  --MEKLNIAGGDSLNGTVHISGAKNSAVALIPATILA-NSEVTIEGLPEI

                  ....|....|....|....|....|....|....|....|....|....|
                      55        65        75        85        95
E.cloacae MurA    KDIDTTMKLLTQLGTKVE-RNGS--VWIDASNVNNFSAPYDLVKTMRASI
S.aureus MurA1    SDVETINNVLTTLNADVTYKKDENAVVVDATKTLNEEAPYEYVSKMRASI
S.aureus MurA2    SDVKTLVSLLEDLNIKASLNGTE--LEVDTTEIQNAALPNNKVESLRASY
B.subtilis MurA1  SDVYTINEVLRHLGADVHFENNE--VTVNASYALQTEAPFEYVRKMRASV
B.subtilis MurA2  SDIETLRDLLKEIGGNVHFENGE--MVVDPTSMISMPLPNGKVKKLRASY

                  ....|....|....|....|....|....|....|....|....|....|
                      105       115       125       135       145
E.cloacae MurA    WALGPLVARFGQGQVSLPGGCAIGARPVDLHIFGLEKLGAEIKLEEGYVK
S.aureus MurA1    LVMGPLLARLGHAIVALPGGCAIGSRPIEQHIKGFEALGAEIHLENGNIY
S.aureus MurA2    YMMGAMLGRFKKCVIGLPGGCPLGPRPIDQHIKGFKALGAEIDESSTTSM
B.subtilis MurA1  LVMGPLLARTGHARVALPGGCAIGSRPIDQHLKGFEAMGAEIKVGNGFIE
B.subtilis MurA2  YLMGAMLGRFKQAVIGLPGGCHLGPRPIDQHIKGFEALGA EVTNEQGAI

                  ....|....|....|....|....|....|....|....|....|....|
                      155       165       175       185       195
E.cloacae MurA    ASVNGRLKGAHIVMDKVSVGATVTIMSAATLAEGTTIIENAAREPEIVDT
S.aureus MurA1    ANAKDGLKGTSIHLDFPSVGATQNIIMAASLAKGKTLIENAAKEPEIVDL
S.aureus MurA2    KIEAKELKGAHIFLDMVSVGATINIMLAAVYATGQTVIENAAKEPEVVDV
B.subtilis MurA1  AEVKGRLQGAKIYLDFPSVGATENLIMAAALAEGTTTLENVAKEPEIVDL
B.subtilis MurA2  YLRAERLRGARIYLDVVSVGATINIMLAAVLAEGKTIIENAAKEPEIIDV

                  ....|....|....|....|....|....|....|....|....|....|
                      205       215       225       235       245
E.cloacae MurA    ANFLVALGAKISGQGTDRITIEGVERLGGGVYRVLPDRIETGTFLVAAAI
S.aureus MurA1    ANYINEMGGRITGAGTDTITINGVESLHGVEHAIIPDRIEAGTLLIAGAI
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S.aureus MurA2    ANFLTSMGANIKGAGTSTIKINGVKELHGSEYQVIPDRIEAGTYMCIAAA
B.subtilis MurA1  ANYINGMGGKIRGAGTGTIKIEGVEKLHGVKHHIIPDRIEAGTFMVAAAI
B.subtilis MurA2  ATLLTSMGAKIKGAGTNVIRIDGVKELHGCKHTIIPDRIEAGTFMIAGAA

                  ....|....|....|....|....|....|....|....|....|....|
                      255       265       275       285       295
E.cloacae MurA    SGGKIVCRNAQPDTLDAVLAKLREAGADIETGEDWISLDMHGKRPKAVTV
S.aureus MurA1    TRGDIFVRGAIKEHMASLVYKLEEMGVELDYQEDGIRVRA EGELQPVDI
S.aureus MurA2    CGENVILNNIVPKHVETLTAKFSELGVNVDVRDERIRINN NAPYQFVDI
B.subtilis MurA1  TEGNVLVKGAVPEHLTSLIAKMEEMGVTIKDEGEGLRVIG PKELKPIDI
B.subtilis MurA2  MGKEVIIDNVIPTHLESLTAKLREMGYHIETSDDQLLIVGGQKNLKPVDV

                 ....|....|....|....|....|....|....|....|....|....|
                      305       315       325       335       345
E.cloacae MurA    RTAPHPAFPTDMQAQFTLLNLVAEGTGVITETIFENRFMHVPELIRMGAH
S.aureus MurA1    KTLPHPGFPTDMQSQMMALLLTANGHKVVTETVFENRFMHVAEFKRMNAN
S.aureus MurA2    KTLVYPGFATDLQQPITPLLFMANGPSFVTDTIYPERFKHVEELKRMGAN
B.subtilis MurA1  KTMPHPGFPTDMQSQMMALLLRASGTSMITETVFENRFMHAEEFRRMNGD
B.subtilis MurA2  KTLVYPGFPTDLQQPMTALLTRAKGTSVVTDTIYSARFKHIDELRRMGAN

                  ....|....|....|....|....|....|....|....|....|....|
                      355       365       375       385       395
E.cloacae MurA    AEIESNTVICHGVEKLSGAQVMATDLRASASLVLAGCIAEGTTVVDRIYH
S.aureus MurA1    INVEGRSAKLEGKSQLQGAQVKATDLRAAAALILAGLVADGKTSVTELTH
S.aureus MurA2    IEVDEGTATIK-PSTLHGAEVYASDLRAGACLIIAGLIAEGVTTIYNVKH
B.subtilis MurA1  IKIEGRSVIINGPVQLQGAEVAATDLRAGAALILAGLVAEGHTRVTELKH
B.subtilis MurA2  MKVEGRSAIITGPVELQGAKVKASDLRAGSCLVVAGLMADGVTEITGLEH

                  ....|....|....|....|....|....|....|....|.
                      405       415       425       435
E.cloacae MurA    IDRGYERIEDKLRALGANIERVKGE----------------
S.aureus MurA1    LDRGYVDLHGKLKQLGADIERIND-----------------
S.aureus MurA2    IYRGYTDIVEHLKALGADIWTETV-----------------
B.subtilis MurA1  LDRGYVDFHQKLAALGADIERVNDESASEQENKEVVSDLNA
B.subtilis MurA2  IDRGYSSLEKKLEGLGATIWRERMTDEEIEQLQNS------
```

3.2. Over-expression of *S. aureus* and *B. subtilis* MurA

Induction studies to determine suitable conditions for protein over-expression were performed as described in section 2.2.6. The MurA constructs were used to transform BL21(DE3), ArcticExpress(DE3), pLysS(DE3) and RIL(DE3) competent cell lines. All cell lines except ArcticExpress(DE3) displayed reasonable levels of over-expression at 37 ˚C, but the over-expression corresponded only to insoluble protein. RIL(DE3) and pLysS(DE3) cell lines displayed the highest levels of over-expression and solubility in all cases when induction studies were repeated with incubation temperatures ranging from 13 ˚C to 20 ˚C post-induction. In an attempt to increase the amount of soluble protein, induction studies were repeated at 18 ˚C post-induction with final concentrations of IPTG at 0.5 mM and 1.0 mM and total incubation time at 5 h and 24 h. The highest levels of over-expression and solubility for all enzymes were induction with IPTG to a final concentration of 0.5 mM in the RIL(DE3) cell line with a 24 h incubation at 18 ˚C post-induction. Using these conditions, *S. aureus* and *B. subtilis* MurA1 had approximately 60% soluble over-expression and *S. aureus* and *B. subtilis* MurA2 had approximately 10% soluble over-expression.

3.3. Purification of *S. aureus* and *B. subtilis* MurA and PreScission

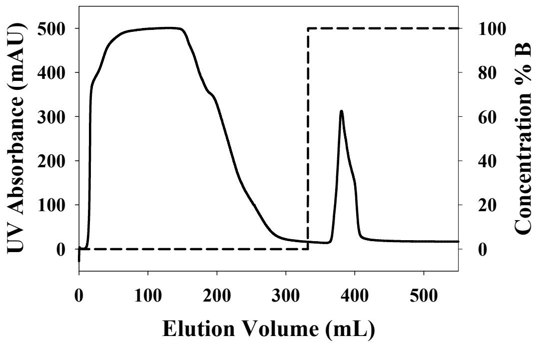


Figure 5: GST chromatographic profile for PreScission protease.

Protein purification of *E. cloacae* MurA was done by Huijong Han and purification of *E. Coli* MurB was done by Martha Healy-Fried. Purification of *S. aureus* and *B. subtilis* enzymes was done as described in section 2.4.9 and PreScission protease was purified as described in section 2.5.3. Using these conditions, all enzymes were purified to greater than 95% homogeneity. Purification from a 4 L culture of each enzyme yielded 50 mg of PreScission, 50 mg of *S. aureus* and *B. subtilis* MurA1 and less than 5 mg of *S. aureus* and *B. subtilis* MurA2. Representative SDS-PAGE gels for the MurA purification are shown in Figure 6. Representative chromatographic profiles for PreScission are shown in Figure 5, *S. aureus* and *B. subtilis* MurA1 in Figure 7 and *S. aureus* and *B. subtilis* MurA2 in Figure 8.

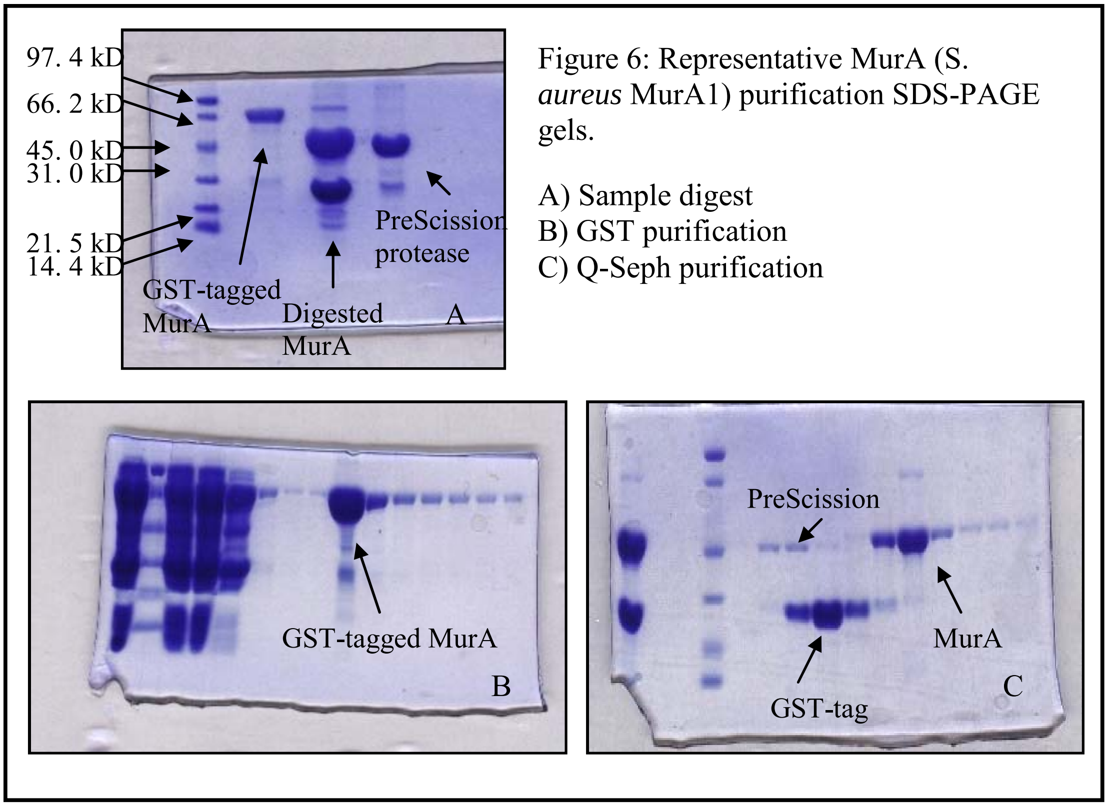


Figure 6: Representative MurA (S. *aureus* MurA1) purification SDS-PAGE gels.

A) Sample digest
B) GST purification
C) Q-Seph purification

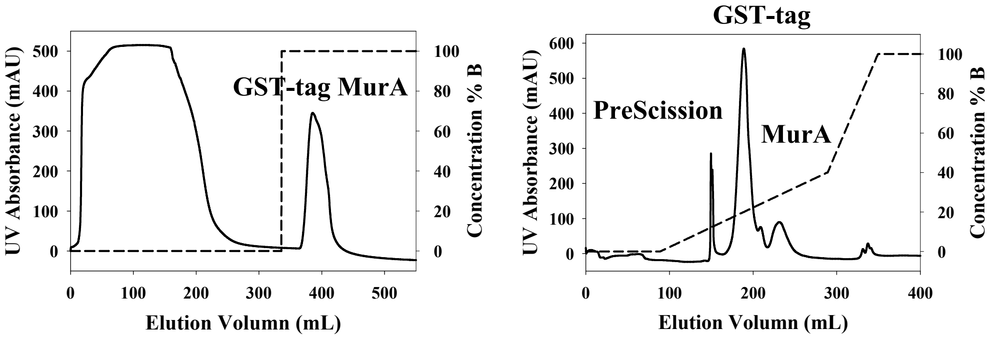


Figure 7: Representative GST (left) and Q-Seph (right) chromatographic profiles for *S. aureus* and *B. subtilis* MurA1.

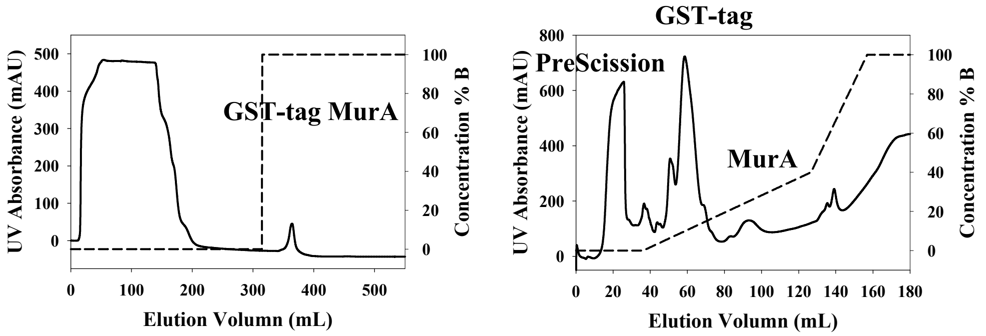


Figure 8: Representative GST (left) and Resource Q (right) chromatographic profiles for *S. aureus* and *B. subtilis* MurA2.

3.4. Kinetic Analysis of *S. aureus* and *B. subtilis* MurA

Steady-state kinetic characterization of *S. aureus* and *B. subtilis* MurA1 and MurA2 was performed in parallel experiments with the MurA enzyme from *E. cloacae*.

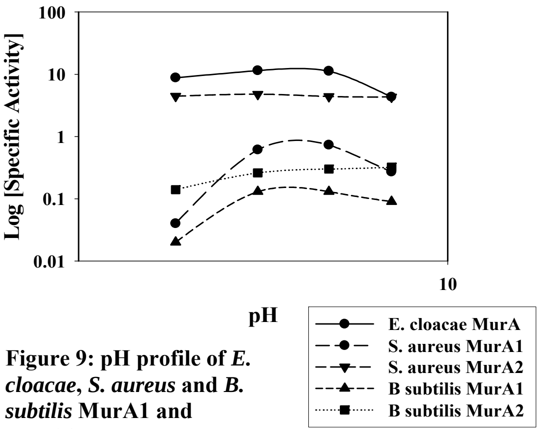


Figure 9: pH profile of *E. cloacae*, *S. aureus* and *B. subtilis* MurA1 and MurA2.

Table 3: Specific activities (U/mg) of *E. cloacae* MurA, *S. aureus* and *B. subtilis* MurA1 and MurA2 with change in pH.

Enzyme	MES pH 6	MOPS pH 7	HEPES pH 8	TAPS pH 9
E. cloacae MurA	8.78	11.44	11.20	4.31
S. aureus MurA1	0.04	0.61	0.73	0.27
S. aureus MurA2	4.43	4.77	4.38	4.31
B. subtilis MurA1	0.02	0.13	0.13	0.09
B. subtilis MurA2	0.14	0.26	0.30	0.32

The pH profiles of *S. aureus* MurA1 and *B. subtilis* MurA1 are similar to that of *E. cloacae* MurA (Figure 9, Table 3). These enzymes display higher activities between pH 7 and pH 8 with an average decrease in activity of 1- to 2-fold at pH 6 or pH 9, respectively. *S. aureus* MurA1 appears particularly effected at lower pH, displaying a 15-fold decrease in activity from pH 7 to pH 6. The *S. aureus* and *B. subtilis* MurA2 enzymes are less affected by changes in the pH. *B. subtilis* MurA2 does mimic the behavior of *E. cloacae* MurA at pH 6, but displayed no loss of activity over pH ranging from 7 to 9. *S. aureus* MurA2 displayed no loss of activity over the entire pH range of 6 to 9.

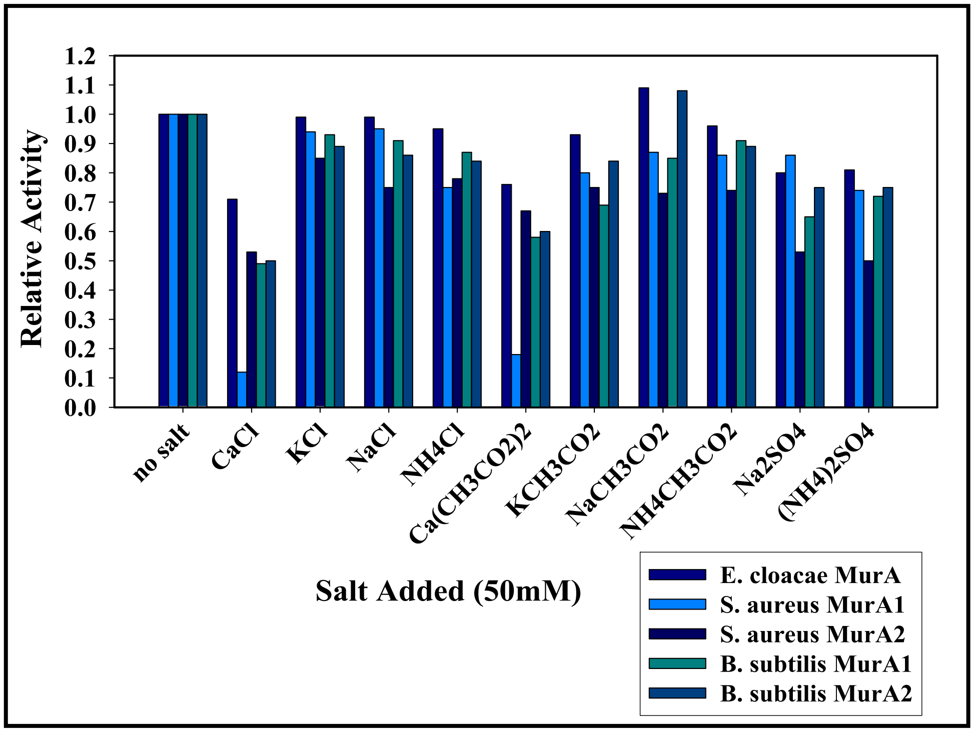
The effect of anions and cations on the enzymatic activity of *S. aureus* and *B. subtilis* MurA is comparable to their effect on *E. cloacae* MurA (Table 4 and Figure 10). Potassium, sodium and ammonium ions displayed little change in activity compared to that observed with no cations present. Calcium ion, however, provided

Table 4: Relative activities of *E. cloacae* MurA, *S. aureus and B. subtilis* MurA1 and MurA2 in the presence of salt.

	Salt Concentration	CaCl	KCl	NaCl	NH_4Cl	$Ca(CH_3CO_2)_2$	KCH_3CO_2	Na CH_3CO_2	NH_4 CH_3CO_2	Na_2 SO_4	$(NH_4)_2$ SO_4
E. cloacae MurA	50mM	0.71	0.99	0.99	0.95	0.76	0.93	1.09	0.96	0.80	0.81
	100mM	0.45	0.94	0.91	0.81	0.53	0.95	0.98	0.93	0.68	0.67
	250mM	0.06	0.79	0.77	0.72	0.37	0.90	0.86	0.85	0.48	0.43
S. aureus MurA1	50mM	0.12	0.94	0.95	0.75	0.18	0.80	0.87	0.86	0.86	0.74
	100mM	0.04	0.91	0.92	0.80	0.14	0.88	0.86	0.78	0.74	0.66
	250mM	0.02	0.82	0.82	0.57	0.11	0.89	0.91	0.80	0.49	0.42
S. aureus MurA2	50mM	0.53	0.85	0.75	0.78	0.67	0.75	0.73	0.74	0.53	0.50
	100mM	0.23	0.82	0.78	0.78	0.49	0.80	0.79	0.84	0.46	0.50
	250mM	0.05	0.55	0.55	0.50	0.19	0.65	0.67	0.68	0.25	0.24
B. subtilis MurA1	50mM	0.49	0.93	0.91	0.87	0.58	0.69	0.85	0.91	0.65	0.72
	100mM	0.22	0.88	0.86	0.81	0.45	0.91	0.75	0.75	0.65	0.60
	250mM	0.17	0.65	0.65	0.59	0.25	0.74	0.72	0.74	0.43	0.42
B. subtilis MurA2	50mM	0.50	0.89	0.86	0.84	0.60	0.84	1.08	0.89	0.75	0.75
	100mM	0.25	0.84	0.80	0.76	0.53	0.84	0.89	0.92	0.65	0.65
	250mM	ND	ND	ND	ND	0.31	0.93	0.95	0.82	0.50	0.48

Table 5: Relative activities of *E. cloacae* MurA, *S. aureus* and *B. subtilis* MurA1and MurA2 in the presence of detergents and organic solvents.

Enzyme	5% DMSO	10% DMSO	5% EtOH	10% EtOH	5% Triton	10% Triton	5% Glycerol	10% Glycerol	5% glucoside	10% glucoside
E. cloacae MurA	1.07	1.15	1.05	0.78	1.12	0.98	1.06	0.96	0.95	0.82
S. aureus MurA1	0.90	0.90	0.60	0.23	0.11	0.08	0.75	0.89	0.21	0.02
S. aureus MurA2	0.71	0.65	0.69	0.65	0.67	0.49	0.50	0.51	0.72	0.53
B. subtilis MurA1	0.92	0.69	0.80	0.54	0.43	0.50	0.90	0.92	0.70	0.29
B. subtilis MurA2	0.97	0.95	0.87	0.64	0.66	0.63	0.80	0.83	0.73	0.55



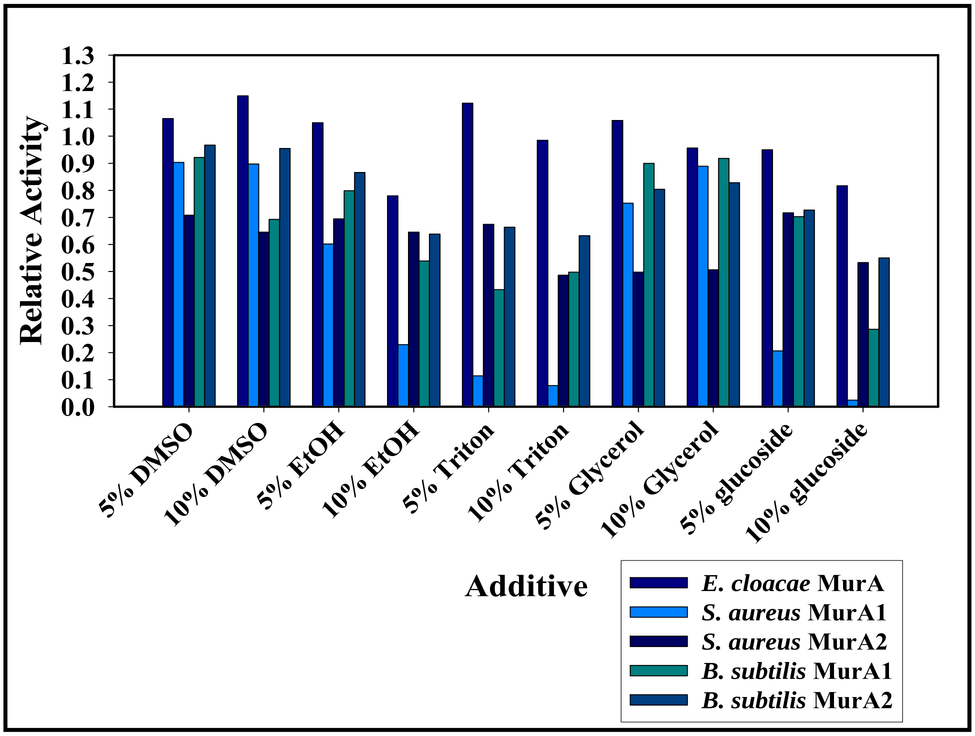


Figure 10: Relative activities of *E. cloacae* MurA, *S. aureus* and *B. subtilis* MurA1 and MurA2 in the presence of 50mM salt (top) and detergent or solvents (bottom).

a significant decrease in the activity of all enzymes, in particular *S. aureus* MurA1 which displayed a 90 % loss of activity at the lowest concentration tested. In comparison, the activity of *S. aureus* MurA2 and both *B. subtilis* enzymes decreased by 50 % and *E. cloacae* activity decreased by 30 %. Chloride, acetate and sulfate ions displayed little change in activity of all enzymes compared to that observed with no anions present. Changes in the ionic concentration of the assay solution, determined that there was little change in activity of the enzymes at the lower concentrations (50 mM and 100 mM), but a significant loss of activity at higher concentrations (250 mM).

The addition of detergents and organic solvents to the assay solutions for *S. aureus* and *B. subtilis* enzymes resulted in a loss of activity in all cases unlike that of *E. cloacae* MurA which remained mostly unchanged (Table 5 and Figure 10). In the presence of 5 and 10% DMSO or glycerol, both MurA1 enzymes and *B. subtilis* MurA2 displayed a slight loss of activity, while *S. aureus* MurA2 activity decreased 30%. Enzymatic activity of MurA1 and MurA2 for *S. aureus* and *B. subtilis* assayed with 5 % EtOH, decreased 35% and 15%, respectively, with higher activity loss in the presence of 10 % EtOH. Triton decreased enzymatic activity by over 40% in all enzymes, with *S. aureus* MurA1 noticeably having the largest loss. Enzymatic activity of MurA2 for *S. aureus* and *B. subtilis* MurA1 and MurA2 assayed with 5 % n-Octyl-β-D-glucoside, decreased 80% and 30%, respectively, with higher activity loss in the presence of 10 % glucoside.

S. aureus and *B. subtilis* MurA enzymes display normal saturation behavior when the specific activities (U/mg) are plotted as a function of substrate concentration (Figure 11). The kinetic constants derived from these graphs (Table 6) demonstrate that the affinity for both substrates (UNAG and PEP) is generally lower than that of *E. cloacae*. *S. aureus* MurA2 and *B. subtilis* MurA2, however, did display K_m constants for UNAG which were 2-fold higher than that of *E. cloacae*. The catalytic efficiency (k_{cat}/K_m) for all of the enzymes is lower in comparison to *E. cloacae* MurA, however, *S. aureus* MurA2 is only slightly lower than *E. cloacae* MurA where the remaining enzymes were more than 100-fold lower. All enzymes were inhibited by the antibiotic fosfomycin but none were as sensitive as *E. cloacae* MurA (Figure 12).

Table 6: Kinetic parameters for *E. cloacae* MurA, *S. aureus and B. subtilis* MurA1 and MurA2.

Enzyme	K_m UNAG (mM)	V_{max} UNAG (U/mg)	k_{cat}/K_m UNAG ($M^{-1}s^{-1}$)	K_m PEP (mM)	V_{max} PEP (U/mg)	k_{cat}/K_m PEP ($M^{-1}s^{-1}$)	IC_{50} FOS (µM)
E. cloacae MurA	0.081 ±0.02	5.3 ±0.2	6.0E+03	0.016 ±0.004	6.8 ±0.3	3.9E+04	1.04 ±0.05
S. aureus MurA1	0.38 ±0.03	1.3 ±0.04	1.2E+01	0.063 ±0.01	1.3 ±0.04	7.0E+01	62 ±10
S. aureus MurA2	0.042 ±0.01	4.3 ±0.2	5.2E+03	0.033 ±0.004	4.1 ±0.1	6.2E+03	12 ±0.8
B. subtilis MurA1	0.23 ±0.02	0.48 ±0.01	6.9E+00	0.066 ±0.01	0.49 ±0.02	2.5E+01	3.8 ±0.9
B. subtilis MurA2	0.043 ±0.01	0.17 ±0.01	2.0E+01	0.017 ±0.002	0.21 ±0.01	6.3E+01	220 ±200

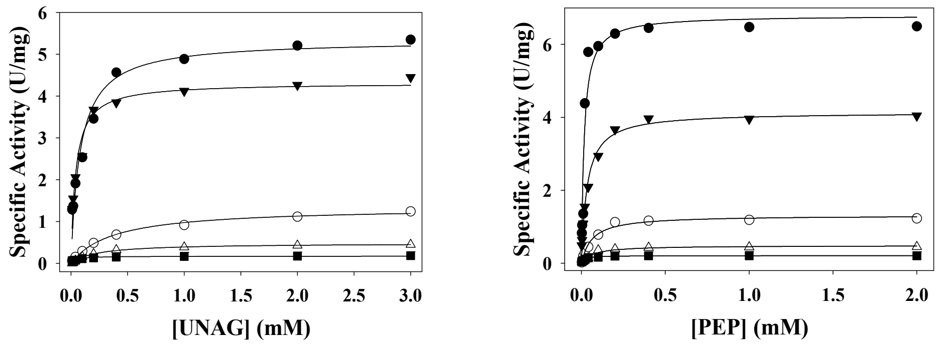


Figure 11: Comparison of the steady-state kinetics of *E. cloacae* MurA, *S. aureus* and *B. subtilis* MurA1 and MurA2. Enzymatic assays with 6 mM UNAG (top) or 4 mM PEP (bottom) and increasing concentrations of the second substrate. All data were fit to equation 1 yielding the kinetic parameters shown in Table 6.

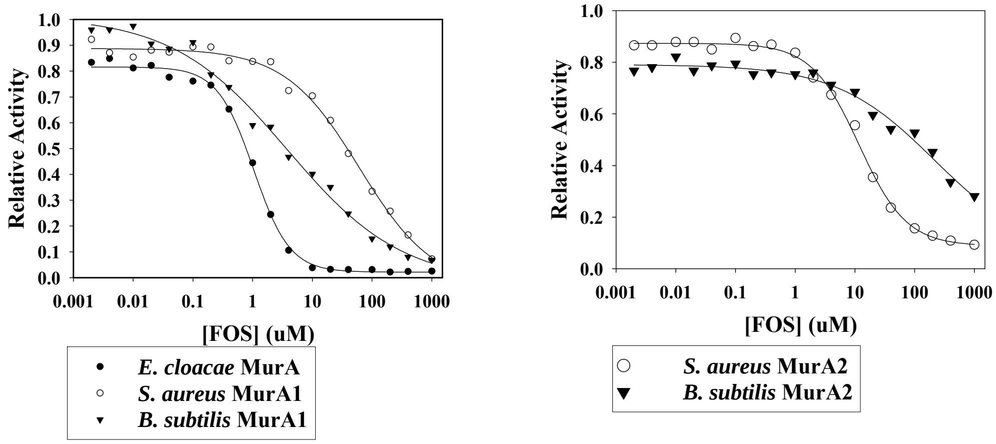


Figure 12: Inhibition of *E. cloacae* MurA, *S. aureus* and *B. subtilis* MurA1 and MurA2 by fosfomycin. IC_{50} values of fosfomycin inhibition were determined using 1 mM UNAG (left) and 43μM UNAG (right), 1 mM PEP and increasing concentrations of fosfomycin. All data were fit to equation 2 yielding the kinetic parameters shown in Table 6.

While none of the enzymes were catalytically as efficient as *E. cloacae* MurA, the MurA2 enzymes in both organisms are clearly more efficient than the MurA1 enzymes, which supports what was found for the *S. pneumoniae* MurA1 and MurA2 enzymes previously studied [34]. Structural analysis would be beneficial in determining why the kinetic data for *S. aureus* and *B. subtilis* MurA1 and MurA2 enzymes are substantially different than for *E. cloacae* MurA. Of more interest may be what structural differences cause the MurA2 enzyme to be more active than the MurA1 enzyme as well as determining fosfomycin potency and substrate binding capabilities.

3.5. Future Analysis

To continue to obtain a better understanding of the *S. aureus* and *B. subtilis* MurA enzymes, further work needs to be completed. Experiments should be continued with the MurA2 enzymes in an attempt to increase the amount of soluble protein. Utilization of an alternate tagged vector system or refolding experiments could enhance solubility. The addition of the charged amino acids, L-Arg and L-Glu, at a final concentration of 50 mM in the extraction buffer, has been shown to increase the maximum achievable concentration of soluble protein while preventing protein aggregation and precipitation [38] which happens to be another problem to be investigated. These enzymes do not concentrate well, which leads to difficulty in trying to obtain a high enough enzyme concentration for kinetic assays and crystallization trials. Attempts were made to crystallize *S. aureus* MurA1 using numerous crystallization buffers, however, no crystals were formed. Aggregation or the formation of oligomers in these proteins is suspected as being a concern based on preliminary gel filtration experiments using FPLC. Trials were done on several sizes of gel filtration column, however, the enzyme consistently eluted in the void volume of the column. In addition, determination of protein size using native protein gel electrophoresis was not effective.

Following the development of a more refined protocol to produce these enzymes, a structural study should be performed. To date, no crystal structures for any gram-positive MurA enzyme have been solved. Doing so will reveal the variations of the gram-positive MurA active sites from those that are known for other species. Based on this structural information and the kinetic data shown in this thesis, the discovery of novel antibiotics targeting MurA may not be far away.

4. Conclusions

Sequence analysis of *S. aureus* and *B. subtilis* MurA1 and MurA2 indicates that all genes are complete and that the enzymes contain the important catalytic residues previously identified in *E. cloacae* MurA. To demonstrate that these genes encode active enzymes, they were each over-expressed and purified. Kinetic characterization revealed that the enzymes from both organisms are active and can catalyze the reaction between UNAG and PEP to give EP-UNAG and P_i. For *B. subtilis* MurA1 and MurA2, the enzymes have higher K_ms for their substrates and lower k_{cat}s than *E. cloacae* MurA suggesting that, at a kinetic level, MurA1 more closely resembles MurA2 than its gram-negative counterpart. That does not seem to be the case for the *S. aureus* MurA enzymes, since MurA1 in this organism is kinetically more similar to *E. cloacae* MurA. In any case, inhibitor design would have to possess activity against both MurA1 and MurA2 to ensure antibacterial activity against gram-positive pathogens.

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The Thesis Committee for Jennifer J. Biery certifies

That this is the approved version of the following thesis:

Probing the Antibiotic Target MurA

from *S. aureus* and *B. subtilis*

Thesis Committee:

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Chairperson

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Date approved: __December 7, 2007___