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Numerous antimicrobial compounds, which once controlled pathogenic organisms, are largely ineffective primarily due to the evolution of antibiotic resistance genes acquired by these pathogenic bacteria. This situation constitutes a serious public health threat that urgently calls for the development of new antibacterial agents to avoid a post-antibiotic era. The search and development of new antimicrobials must be an on-going process in order to maintain a safe and effective drug library. Ideally, the novel agent would target the microbe exclusively by inhibiting a critical cellular process, with little or no toxicity to the host. One possible treatment could be a regime that inhibits enolpyruvyltransferases. These enzymes are involved in enzymatic pathways that are absent from animals and thus, are attractive targets for the development of novel antimicrobials. Currently, MurA (UDP-N-acetylglucosamine enolpyruvyltransferase), encoded by the *murA*1 and *murA*2 genes, and EPSP synthase (5-enolpyruvyl shikimate-3-phosphate synthase), encoded by the *aroA* gene, are the only known examples of enolpyruvyltransferases. In this study we have accomplished 2 specific objectives. First, we have cloned *aroA*, *murA*1, and *murA*2 from *Staphylococcus aureus*. Secondly, we have overexpresed *aroA*, *murA*1, and *murA*2 in *Escherichia coli*. Accomplishing these two objectives will help to better understand the enolpyruvyltransferases, both

structurally and functionally, with the ultimate goal of developing novel antibiotics to combat the almost unabated resurgence of infectious pathogens.

# Cloning and overexpression of the enolpyruvyltransferases from *Staphylococcus aureus*

A Thesis

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## PREFACE

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## Introduction

Pathogenic bacteria have a long history of causing disease in humans. The discovery and clinical use of antibiotics has been of paramount importance in combating these pathogens. In today's world, however, numerous antimicrobial compounds, which once controlled pathogenic organisms, are largely ineffective primarily due to the evolution of antibiotic resistance genes acquired by these pathogenic bacteria (40, 43). While it can be argued that antibiotics have saved millions of lives, they also have provided the selective pressure enabling the production of drug resistant strains (21, 26, 40, 64). Indeed, resistance to all classes of antibiotics has been demonstrated in various bacterial pathogens (23), constituting a serious public health threat (23, 27, 55). This situation urgently calls for the development of new antibacterial agents to avoid a post-antibiotic era.

The genus *Staphylococcus* is composed of Gram-positive cocci that occur in clusters (32). Members of this genus can be isolated from the environment as well as human and animal hosts (32). Currently there are 32 species and 15 subspecies that belong to this genus (32) with the most notorious member being *S. aureus*. It is distinguished from coagulase negative staphylococci by its exclusive ability to clot rabbit plasma (32). The versatility of this organism to survive in various host environments and its adaptability to seemingly unfavorable conditions makes *S. aureus* a formidable pathogen. Of the pathogens we face today, few are more daunting than *S. aureus*, which has been implicated in a host of different diseases, ranging from common skin infections to endocarditis (6, 31).

The discovery of penicillin in the 1920's is considered one of the major medical innovations of the 20th century. Throughout history many *S.aureus* infections were fatal, but the use of penicillin G in the 1940's significantly reduced fatality rates (26). In fact, when penicillin was first introduced, *S. aureus* was almost 94% susceptible to this drug but widespread resistance to penicillin developed in the 1950's (36). Compared to other bacteria, *S. aureus* seemed to have a heightened capacity for the development of antibiotic resistance (46). Indeed, a pandemic of staphylococcal infections occurred in the mid-twentieth century and spanned the years from about 1946 to about 1966 (69). This led to the development of semi-synthetic penicillin's such as amoxicillin and "penicillinase-resistant beta-lactams," such as methicillin, which were introduced in the early 1960's (36). Within a decade the first methicillin-resistant staphylococci were reported in the United Kingdom (9, 54, 63) and in the mid-1970's resistance appeared in the United States (9, 43, 46, 54, 63, 69). Today, the rate of drug resistance has continued to rise (23, 64, 68). In 2000, the first vancomycin-resistant *S. aureus* (VRSA) strain was reported in Japan and in 2002 the Centers for Disease Control and Prevention reported two confirmed cases of high level VRSA in the United States (72, 73). Since vancomycin has long been considered the "drug of last resort", these findings are particularly troublesome.

Due to the emergence of antibiotic resistant bacteria of all genera, especially in common pathogens as *S. aureus*, the search and development of new antimicrobials must be an on-going process in order to maintain a safe and effective drug library. Ideally, the novel antimicrobial agent would target the microbe exclusively by inhibiting a critical cellular process, with little or no toxicity to the host. One possible treatment could be a

regime that inhibits enolpyruvyltransferases. These enzymes are involved in enzymatic pathways that are absent from animals (25) and thus, are attractive targets for the development of novel antimicrobials. Currently, MurA (UDP-N-acetylglucosamine enolpyruvyltransferase) and EPSP synthase (5-enolpyruvyl shikimate-3-phosphate synthase) are the only known examples of enolpyruvyltransferases. These enzymes catalyze a reaction involving phosphoenolpyruvate (PEP). Most PEP-dependent enzymes cleave the P-O bond of PEP and transfer the liberated P to another molecule, but the enolpyruvyltransferases are unique among other PEP utilizing enzymes in that they cleave the C-O bond of PEP and transfer the enolpyruvyl moiety to another substrate (65).

EPSP synthase catalyzes the transfer of enolpyruvate from PEP to the 5-OH of shikimate-3-phosphate (S3P), forming 5-enolpyruvateshikimate-3-phosphate (EPSP) and inorganic phosphate (5). It is the sixth enzyme of the shikimate pathway and is necessary for production of most aromatic compounds, including many important aromatic amino acids. The shikimate pathway is absent in animals (25) but it is essential to the survival of bacteria, plants, fungi, algae, and some parasites (15, 19, 20, 30), thus making enzymes in this pathway a valid and compelling broad-spectrum target for new antibacterial therapies.

EPSP synthase is encoded by the *aroA* gene and has a molecular weight (MW) of 46,000 Daltons. Glyphosate (N-phosphonomethylglycine), a broad-spectrum herbicide, is a competitive inhibitor of EPSP synthase at the shikimate-3-phosphate (S3P) binding site (22, 59). Although EPSP synthase has been studied in depth from a variety of different species, definite evidence on its mode of action beyond its inhibition by

glyphosate, remain questionable (2, 50, 61). It has been demonstrated that univalent cations stimulate EPSP synthase in concentrations comparable to those found in bacterial cells (12). Specifically, they alter the activity of EPSP synthase in *Streptococcus pneumoniae* (12), *Pseudomonas aeruginosa* (17), *Bacillus subtilis* (16), and *Klebsiella pneumoniae* (60) but they have no effect on EPSP synthase from *Escherichia coli*, suggesting a species-specific mechanism of action. Although both S. *pneumoniae* and *B. subtilis* EPSP are activated, glyphosate inhibition of EPSP synthase from *S. pneumoniae* is considerably greater in the presence of the univalent cations $NH_4^+$ and $K^+$ (12). Amino acid sequence comparison of EPSP synthase from *E. coli*, *S. pneumoniae*, and *B. subtilis* reveal low overall sequence identity to each other, but several fully conserved domains implicated in substrate binding and catalysis, are present in each (12). It is hypothesized that species-specific primary structure induces conformational changes in the secondary and tertiary structure, which presumably explains the observed differences in glyphosate inhibition in the presence of univalent cations (12). The elucidation of the crystal structure of EPSP synthase has provided a basis to study the structural and mechanistic similarities with MurA (14, 33, 58, 53, 57) and also its relationship with glyphosate (52, 57).

MurA catalyzes the first committed step in peptidoglycan biosynthesis and has a MW of 44,000 Daltons (8). It is encoded by a single gene, *murA*, which is sometimes referred to as *murZ* in older literature (38). It catalyzes enolpyruvyl transfer from PEP to the 3-OH of UDP-N-acetylglucosamine (UDPAG), forming UDP-N-acetylglucosamine enolpyruvate (7). Although MurA has been extensively studied in *E. coli*, its crystal structure has been elucidated using the enzyme from multiple organisms. In fact, by

studying the structure both in the absence and presence of ligands, investigations have demonstrated two globular domains connected by a double-stranded linker (53, 57). The first domain contains the catalytic site (Cys-115) and the overall protein fold is very similar to that of EPSP synthase. The Cys-115 site is located in a deep cleft between the globular domains and is the target of alkylation by the antibiotic fosfomycin (37, 66), a natural inhibitor of MurA (28). Site-directed mutagenesis of Cys-115 to serine (51) or alanine (56), but not aspartate (29), inactivates the enzyme. In *Mycobacterium tuberculosis* aspartate occurs naturally in position 115 and resistance of this organism to fosfomycin has been suggested to be due to the lack of Cys-115 (10). Replacement of aspartate by a cysteine residue allowed inhibition of MurA by fosfomycin. In *E.coli*, substitution of the active Cys-115 by aspartate made it completely resistant to fosfomycin, while maintaining enzymatic activity (29). The inactivation of MurA by fosfomycin was considerably enhanced in the presence of UDP-N-acetylglucosamine, suggesting it induces confirmational changes in the active site (37). Recently, three new inhibitors of MurA, a cyclic disulfide, a purine analogue, and a pyrazolopyrimidine were identified. Although less potent and structurally dissimilar as compared to fosfomycin, they seem to act in a similar fashion (4). However, their antibacterial action was not specifically due to MurA inhibition, as inhibition of DNA, RNA, and protein synthesis was also observed (4). It has been suggested that these compounds are tightly, but not covalently, associated with MurA at or near a site at which fosfomycin binds.

Investigations of MurA in *Streptococcus pneumoniae* confirmed it contains two distinct *murA* genes (*murA*1 and *murA*2), both of which encode a UDP-N-acetylglucosamine enolpyruvyltransferase that can be inhibited by fosfomycin (11).

Inactivation of each *murA* gene individually using allelic replacement experiments demonstrated that either *murA* gene was sufficient for survival, but deletion of both genes was lethal (11). Structurally and functionally, *murA*1 and *murA*2 appear very similar (45% homology) with each containing the major structural components involved in ligand interaction of *E.coli* MurA (11). Interestingly, *murA*1 is common to all bacteria except *Mycoplasma* species, whereas *murA*2 exists as a second gene copy only in Gram-positive bacteria with a low G+C content (11).

My objectives in this study were to characterize the enolpyruvyltransferases and determine their usefulness as targets for new antimicrobial agents. The specific aims were to clone the EPSP synthase gene (*aroA*) and the MurA genes (*murA*1 & *murA*2) from *S. aureus* followed by overexpression of these genes in *E. coli*.

## Methods and Materials

### Bacterial Strains, Plasmids, and Culture Conditions

Bacterial strains and plasmids used in this study are listed in Table 1 and Table 2, respectively. Recombinant plasmids generated in this study are listed in Table 3. Typically, the following growth media were used to propagate bacterial cultures: Tryptic Soy Broth (TSB), Luria-Bertani (LB), and Brain Heart Infusion (BHI). Agar plates were prepared by adding agar (20 g/L) to liquid media. Antibiotics routinely used were ampicillin (100 μg/ml) and kanamycin (25 μg/ml). The reagent 5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside (X-Gal) was used at a concentration of 20 μg/ml.

### Total DNA Isolation

Total DNA was purified from bacterial cultures using a modification of an established procedure (49). Briefly, 25 ml of BHI was inoculated with the organism in question and incubated overnight at 37°C with shaking at 250 rpm in a New Brunswick Series 25 Incubator (Edison, NJ). Cells were harvested by centrifugation at 4000 rpm for 5 minutes in a Sorvall GLC-1 tabletop centrifuge (Newton, CT). Following resuspension of the cells in TE buffer (10 mM Tris, 1 mM EDTA; pH 8), cell lysis was accomplished by the addition of 100 μl of 20% sodium dodecyl sulfate (SDS) and subsequent incubation at 60°C for 15 minutes. For lysis of *S. aureus*, an initial preincubation with 100 μl of lysostaphin (10mg/ml) (Sigma; St. Louis, MO) at 37°C for 30 min was performed prior to the addition of SDS. Incubation in the presence of 50 μl of RNase (1 mg/ml) at 37°C for 20 minutes followed by the addition of 75 μl of proteinase K (1 mg/ml) and subsequent incubation at 60°C for 1 hour was carried out to degrade contaminating RNA and protein, respectively. Phenol extraction was carried out

**Table 1. Bacterial strains used in this study.**

| Bacterial Strain | Description | Source |
|---|---|---|
| *Staphylococcus aureus* 57-dk | Methicillin resistant clinical isolate | KSU[a] |
| *S. aureus* Dumas | Methicillin resistant clinical isolate | KSU[a] |
| *S. aureus* Japan | Methicillin resistant clinical isolate | KSU[a] |
| *S. aureus* Kasanjian | Methicillin resistant clinical isolate | KSU[a] |
| *S. aureus* 23b | Clinical isolate | Newman Hospital[b] |
| *Escherichia coli* DH5 $\alpha$ | Cloning strain | ESU culture collection |
| *E.coli* Tuner (DE3) | Overexpression strain | Novagen[c] |

[a] Kansas State University College of Veterinary Medicine, Manhattan, KS. Culture collection of John Iandolo. Origin of isolate unknown.
[b] Emporia, KS.
[c] Novagen, Madison, WI.

**Table 2. Plasmids used in this study.**

| Plasmids | Description | Source |
|---|---|---|
| pT7Blue3 | Cloning vector | Novagen |
| pETBlue1 | Expression vector | Novagen |
| pQE30 | Expression vector | Qiagen[a] |
| pREP4 | Contains gene for T7 polymerase | Qiagen[a] |
| pLac1 | Contains repressor T7 polymerase | Novagen |

[a] Qiagen, Valencia, CA

**Table 3. Recombinant plasmids generated in this study.**

| Plasmid | Description |
|---|---|
| pIMS-1 | pT7Blue3::*aroA* PCR product *S.aureus* 23b |
| pIMS-2 | pT7Blue3:: *aroA* PCR product *S.aureus* 57-dk |
| pIMS-3 | pT7Blue3:: *aroA* PCR product *S.aureus* Dumas |
| pIMS-4 | pT7Blue3:: *aroA* PCR product *S.aureus* Japan |
| pIMS-5 | pT7Blue3:: *aroA* PCR product *S.aureus* Kasanjian |
| pIMS-6 | pT7Blue3::*murA*1 PCR product *S.aureus* Japan |
| pIMS-7 | pT7Blue3::*murA*2 PCR product *S.aureus* 57-dk |
| pIMS-8 | pQE30:: *aroA* PCR product *S.aureus* 57-dk |
| pIMS-9 | pETBlue1::*murA*1 PCR product *S.aureus* Japan |
| pIMS-10 | pETBlue1::*murA*2 PCR product *S.aureus* 57-dk |

by adding 6 ml of phenol, gently mixing of the solution by inversion, followed by centrifugation at 4000 rpm for 5 minutes. The upper aqueous layer was placed into a fresh 15 ml conical tube and the phenol extraction repeated as described. Chloroform extraction was performed as described for phenol extraction using 6 ml of chloroform/isoamyl alcohol (24:1). DNA was precipitated from the solution upon the addition of 2.5 volumes of ice-cold 95% ethanol. Once the DNA became visible, it was removed using a sterile pipette tip, placed into a 1.5 ml microcentrifuge tube, and dried at room temperature for 30 minutes. DNA was resuspended in 500 µl of TE buffer and the quantity and purity determined as described below. The DNA sample was stored at 4°C.

**Plasmid DNA Isolation**

Plasmid DNA was isolated from 16 h cultures using a QIAprep Spin Miniprep kit (Qiagen; Valencia, CA) according to the manufacturers recommendations. Quantity and purity was determined as described below, followed by storage at 4°C.

**Quantification and Purity of DNA**

The quantity and purity of DNA was determined by measuring the absorbance at 260 nm and the absorbance ratio at 260 nm and 280 nm, respectively, in a DU Series 50 Spectrophotometer (Beckman; Fullerton, CA). Values obtained were converted into µg/ml using the following equation: $(OD_{260})$ (Dilution factor) (50 µg/ml) = µg/ml.

**DNA Sequencing and Sequence Analysis**

Plasmid DNA was sent to a commercial facility for DNA sequencing. Sequence data obtained was compared to known *Staphylococcus* genes using the Basic Local Alignment Search Tool (BLAST) (1).

**Agarose Gel Electrophoresis**

A 0.7% agarose gel was prepared by dissolving 0.21 g of agarose in 30 ml of 1X TAE, which was prepared from a 50X stock (242 g Tris, 57.1 ml acetic acid, and 4 ml 0.5 M EDTA/1L). Ethidium bromide (1 µl of a 10 mg/ml solution) was added and the solution heated in a microwave to dissolve the agarose. After solidification, gels were placed into a Minicell EC370M electrophoretic chamber (Fisher; St. Louis, MO) powered by a Bio-RAD model 250/2.5 power supply (Bio-RAD; Hercules, CA). DNA containing 1X gel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) was loaded into wells formed in the agarose. After electrophoresis, a UV Intensity Transilluminator (Fisher; St. Louis, MO) was used to visualize the DNA. Documentation was accomplished using a Panasonic CCD Ultra Lum camera and Scion Image software (Ultra Lum; Paramount, CA).

**DNA Extraction from Agarose Gels**

DNA was extracted from agarose gels using a QIAEXII Gel Extraction kit (Qiagen) according to the manufacturers recommendations.

**Polymerase Chain Reaction**

Polymerase chain reaction (PCR) was used to amplify desired regions of DNA. Typical reaction mixtures consisted of 500 ng DNA, 200 µM deoxynucleotide triphosphates (dNTPs), 1.5 µM $MgCl_2$, 1 µl *Taq* polymerase, and 50 nM each of the forward and reverse primers, in buffer containing 50 mM TrisCl, 50 mM KCl and 0.01% Triton-X100 in a final volume of 100 µl. A typical amplification consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of DNA denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and elongation at 72°C for 1 min. Amplification

proceeded using a Minicycler model PT 150 thermocycler (MJ Research; Watertown, Mass). DNA primers used in this study are listed in Table 4.

**Ligation Reaction**

DNA fragments to be cloned were ligated into the appropriate plasmid using DNA ligase. Briefly, variable amounts of the DNA to be cloned were phosphorylated, when necessary, and were mixed with approximately 50 ng of plasmid DNA, and 1 µl of DNA ligase (3 U/µl) in a buffer consisting of 66 mM Tris, 6.6 mM $MgCl_2$, and 10 mM DTT (pH 7.6). Reaction mixtures were incubated for 2 h at 22°C.

**Phosphorylation of DNA**

Phosphorylation of DNA involved the transfer of the gamma-phosphate of adenosine triphosphate (ATP) to the 5' terminus of DNA. Briefly, gel purified PCR products (concentration undetermined) were incubated with an end conversion mix (Novagen; Madison, WI) containing T4 DNA kinase and ATP at 22°C for 15 min. Following inactivation of the DNA kinase at 75°C for 5 min, the reaction was incubated on ice for 2 min. Ligation into an appropriate plasmid was performed as described above.

**Preparation of Competent Cells**

Competent cells were prepared using a modification of an established procedure (49). Briefly, 2 ml of 2X LB liquid media was inoculated with the appropriate *E.coli* strain and incubated overnight at 30°C with shaking at 250 rpm in a New Brunswick Series 25 Incubator (Edison, NJ). A fresh 200 ml 2X LB liquid media was inoculated using 0.5 ml of the overnight culture and allowed to grow at 30°C with shaking until an $OD_{600}$ of 0.3 was reached. After adding 4 ml of 1 M $MgCl_2$, the cells were allowed to

**Table 4. Primers used in PCR.**

| Primer | Primer sequence 5'-3' |
|---|---|
| *aroA* | Forward: GATATCGTTGTACAACATGTTGAT C<br>Reverse: GCT TCA TTA ACA CGA GAG TCT AAA |
| *aroA*-BamH1 construct | Forward: CGACGGATCCATGGTAAATGAACAAACCATTGA<br>Reverse: GTTTTCCCAGTCACGACGT |
| *murA*1 | Forward: TGT TAC AAT ACT CGA CGC AGG TAA AAT<br>Reverse: GCC ACA CAA CGT TGA CCT TCT TCA |
| *murA*2 | Forward: GGT CAA GTG GGG AAG AAC AGC ATA<br>Reverse: CCA TCA TCT TCA CCT CTC ATC AAT AAA |
| *murA*1 expression | Forward: ATG GAT AAA ATA GTA ATC AAA GGT GGA<br>Reverse: GCC ACA CAA CGT TGA CCT TCT TCA |
| *murA*2 expression | Forward: ATG GCT CAA GAG GTA ATA AAA ATA AGA<br>Reverse: CCA TCA TCT TCA CCT CTC ATC AAT AAA |
| *aroA* gap scan | AACTTATGGATGCGAATATTGAAGGT |
| *aroA* inframe[a] | ACA CCT TCA GCT AGC GAC GCG AAC |

[a] Primer used to determine if *aroA* was placed in frame with the histidine codons supplied by pQE30.

grow until an $OD_{600}$ of 0.4 to 0.55 was attained. Following incubation of the cells on ice for 2 h, cells were harvested by centrifugation at 3000 rpm for 5 min at 4°C in a J2-HS centrifuge (Beckman). Following resuspension in 100 mM ice-cold $CaCl_2$ media (0.05 M $CaCl_2$, 0.04 M $MnCl_2$, 0.02 M $CH_3COONa$, pH 7.5) the cells were incubated on ice for 40 min, and then reharvested by centrifugation as described above. Precipitated cells were resuspensed in ice-cold $CaCl_2$ media containing 15% glycerol and stored at -70°C.

**Transformation of Competent Cells**

Competent *E.coli* cells were transformed according to a standard protocol (49). Briefly, 100 µl of competent cells were incubated with either plasmid DNA (variable amounts) or ligation reactions on ice for 15 min followed by a heat shock at 42°C for 90 seconds. After incubation on ice for two minutes, 900 µl of TSB was added. Transformed cells were allowed to incubate at 37°C for 1 h prior to spread plating 100 µl on LB plates containing the appropriate antibiotic and subsequent overnight incubation at 37°C. When ampicillin was used as the selective agent, the 1 h incubation prior to spread plating could be omitted.

**Restriction Enzyme Digestions**

Restriction enzyme digestions were performed to digest DNA at specific nucleotides. Typical reactions consisted of DNA (variable amounts), 1X reaction buffer (supplied with restriction endonuclease) and 1 µl of the appropriate restriction enzyme in a variable total volume. Reactions were allowed to proceed at the temperature specific for the restriction enzyme for at least 1 h. Digested DNA was analyzed by agarose gel electrophoresis.

**Alkaline Phosphatase Treatment of Plasmids**

Restriction enzyme digested DNA was treated with alkaline phosphatase according to a standard protocol (49). Briefly, 1 μg of DNA was mixed with alkaline phosphatase (0.01 U/μl) in a buffer supplied with the enzyme. After incubation at 37°C for 30 min, an equal amount of alkaline phophatase (0.01 U/μl) was added again followed by a second incubation at 37°C for 30 min. The reaction was stopped by adding 300 μl of stop buffer (10 mM Tris, 1 mM EDTA, 200 mM NaCl, and 0.5% SDS). Following extraction by phenol and chloroform/isoamyl alcohol (24:1), the DNA was precipitated using ethanol. The sample was taken to dryness in a Savant Integrated Speed Vac System ISS110 (Holbrook, NY) and resuspended in water or TE.

**PCR Screening of Recombinants**

Bacterial colonies containing potential recombinant plasmids were screened using a modification of a PCR based procedure. Using a sterile toothpick, potentially recombinant colonies were inoculated onto the appropriate media with antibiotic selection and placed into 5 μl of sterile water. The cell solution was heated at 95°C for 5 min to lyse the cells. The crude lysate was used in a PCR to amplify the cloned gene using gene specific primers. Amplicons were visualized by agarose gel electrophoresis.

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

Discontinuous polyacrylamide gels were prepared and subjected to electrophoresis according to the Laemmli system (34). Polyacrylamide gels were electrophoresed using a Fisher Biotech Protein Electrophoresis System FE-VE16-1 (Fisher) and a Thermo EC Series 90 power supply (Thermo Electron, Holbrook, NY)

according to the manufacturer's recommendations. Protein standards used for comparison were purchased from Sigma (St. Louis, MO).

**Overexpression of Cloned Genes**

Overexpression of cloned genes was accomplished by growing the appropriate *E. coli* strain containing the recombinant expression plasmid to an $OD_{600}$ of approximately 0.6 in BHI containing the appropriate antibiotic. Control samples (1 ml) were taken at this point which was considered time zero. The gene was expressed by adding isopropylthio-β-D-galactoside (IPTG) to a final concentration of 2 mM and samples (1 ml) were taken at 1, 2, 3, and 4 hours. Cells were harvested by centrifugation and lysed using 5X SDS-PAGE loading buffer (60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, and 0.1% bromophenol blue). Cell lysates were examined by SDS-PAGE as described above.

**Purification of Histidine Fusion Protein**

Proteins over-expressed as a histidine fusion protein were purified using a Ni-NTA agarose affinity column (Qiagen; Valencia, CA) according to the manufacturer's recommendations. Briefly, total cell lysates were added to the Ni-NTA column and the column was washed with 0.1 M phosphate buffered saline containing 20 mM imidazole, pH 7. Proteins bound by the column were eluted with 0.1 M phosphate buffered saline containing 200 mM imidazole, pH 7. The purified product was analyzed using SDS-PAGE as described above.

## Results

### Cloning of *aroA* from *Staphylococcus aureus*

Using gene-specific primers, *aroA* was amplified via PCR from 5 *S. aureus* isolates obtained from distinct geographic locations. Using the cloning scheme depicted in Figure 1, amplicons were ligated into pT7Blue3. Following transformation, recombinants were verified by digestion with the restriction enzyme *Eco*R1 (Fig. 2).

### Cloning of *murA*1 and *murA*2 from *Staphylococcus aureus*

Using gene-specific primers, *murA*1 and *murA*2 were each amplified from *S. aureus*. The same general cloning scheme was followed as performed for *aroA* (Fig. 1). Recombinant plasmids containing *murA*1 and *murA*2 were verified using a colony lysis PCR based protocol as shown in Figure 3 and Figure 4, respectively.

### DNA sequencing of *aroA*, *murA*1, and *murA*2 from *Staphylococcus aureus*

Recombinant plasmids were purified and sent for nucleotide sequencing at a commercial DNA sequencing facility. *aroA* was sequenced in completion and the nucleotide sequence from each of the five *S. aureus* isolates is depicted in Appendices 1-5. Recombinant plasmids containing *murA*1 and *murA*2 were confirmed by DNA sequencing of the 5' and 3' ends (data not shown).

### Construction of a Histidine-*aroA* Fusion

*aroA* was amplified from pIMS-2 using a 5' gene specific primer containing a *Bam*H1 site and a 3' primer targeting outside the multiple cloning site of pT7Blue3 (Table 4). The PCR product was gel purified and digested using the restriction enzymes *Bam*H1 and *Hind* III followed by ligation into alkaline phosphate treated pQE30 digested using the same restriction enzymes. The recombinant plasmid (pIMS-8) was subjected to

**Figure 1**. General cloning scheme using the cloning vector pT7Blue3 and gel purified PCR products (*aroA*, *murA*1, and *murA*2).

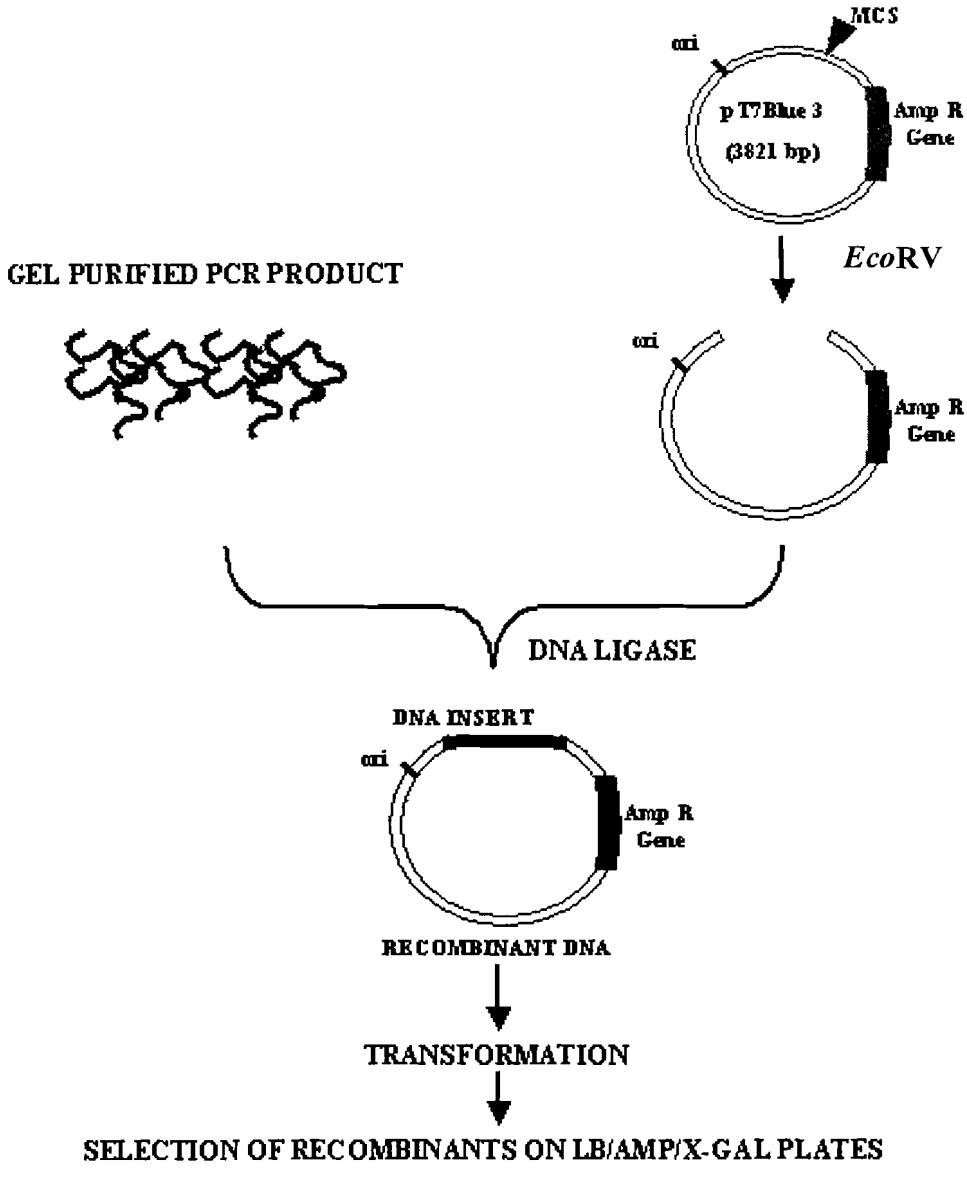

MCS
ori
pT7Blue 3
(3821 bp)
Amp R
Gene
EcoRV
GEL PURIFIED PCR PRODUCT
ori
Amp R
Gene
DNA LIGASE
DNA INSERT
ori
Amp R
Gene
RECOMBINANT DNA
TRANSFORMATION
SELECTION OF RECOMBINANTS ON LB/AMP/X-GAL PLATES

**Figure 2**. Agarose gel electrophoresis of *Eco*R1 digested pIMS-2. Lane 1, molecular weight markers; lane 2, *Eco*R1 digested pIMS-2 showing both the cloning vector (3821 bp) and *aroA* from *S. aureus*. This gel is representative of all 5 isolates from whom this gene was sequenced.

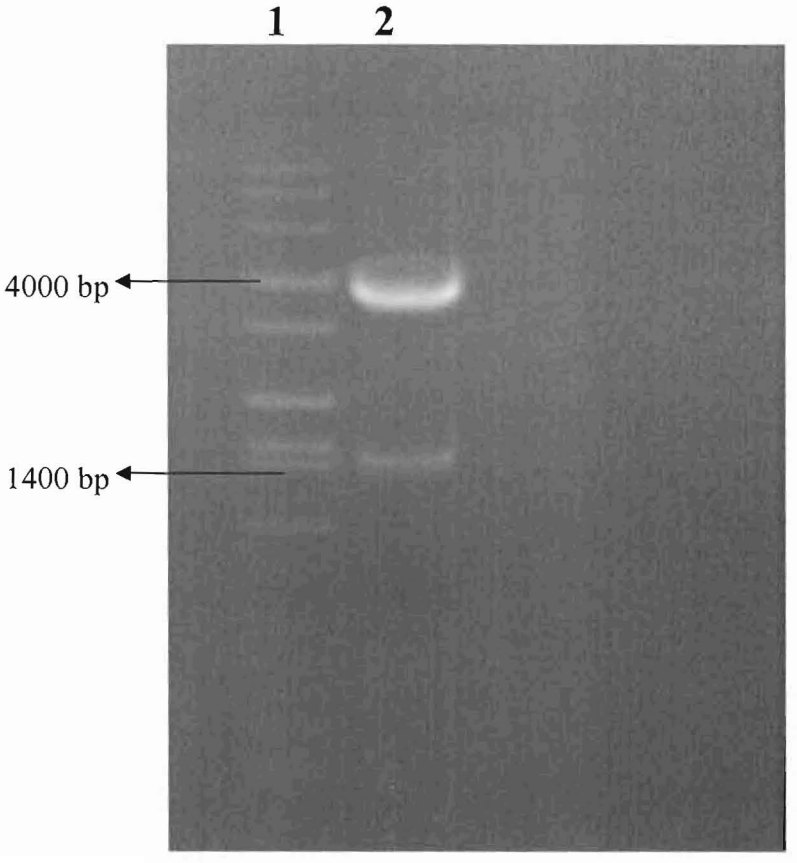

1
2
4000 bp
1400 bp

**Figure 3**. Agarose gel electrophoresis of *murA*1 amplicons from pIMS-6. Lane 1, molecular weight markers; lane 3 to 7, *S. aureus murA*1 amplicons from pIMS-6.

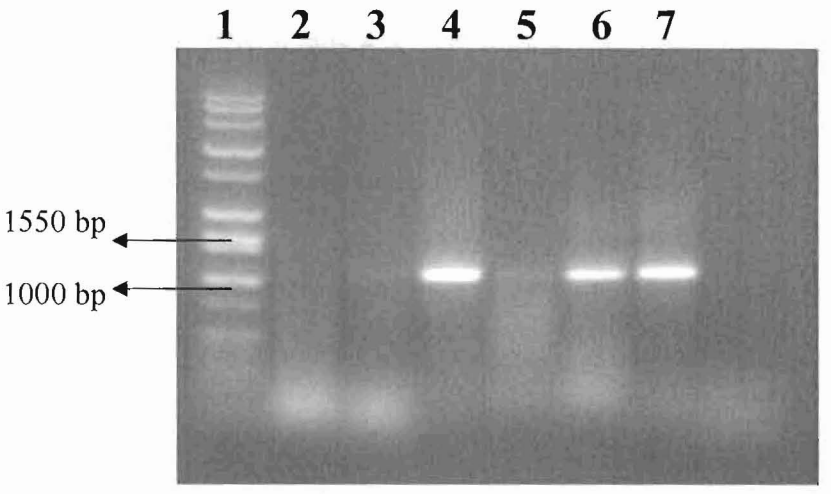
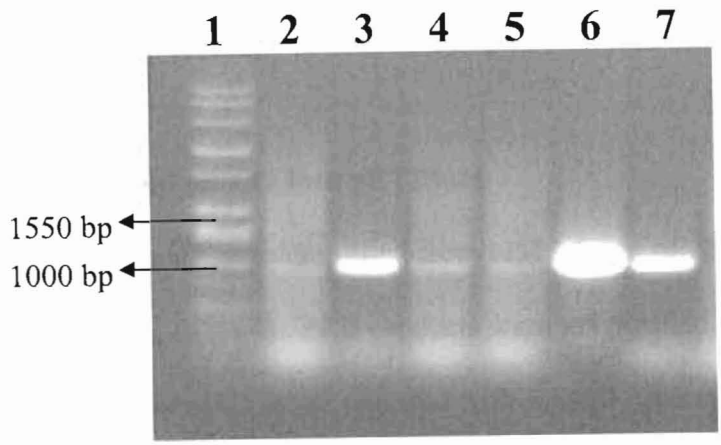

1 2 3 4 5 6 7
1550 bp
1000 bp

**Figure 4**. Agarose gel electrophoresis of *murA*2 amplicons from pIMS-7. Lane 1, molecular weight markers; lane 2 to 7, *S. aureus murA*2 amplicons from pIMS-7.


1 2 3 4 5 6 7
1550 bp
1000 bp

DNA sequencing to verify that *aroA* was in frame with the 6 histidine codons (data not shown).

**Cloning of *murA*1 and *murA*2 into pETBlue1**

*murA*1 and *murA*2 were amplified from pIMS-6 and pIMS-7, respectively, using gene specific primers (Table 4). PCR products were gel purified and phosphorylated followed by ligation into pETBlue1 digested with *Eco*RV. Recombinant plasmids (pIMS-9 and pIMS-10) were subjected to DNA sequencing to verify the start codon of each gene was at the appropriate distance from the ribosomal binding site supplied by pETBlue1.

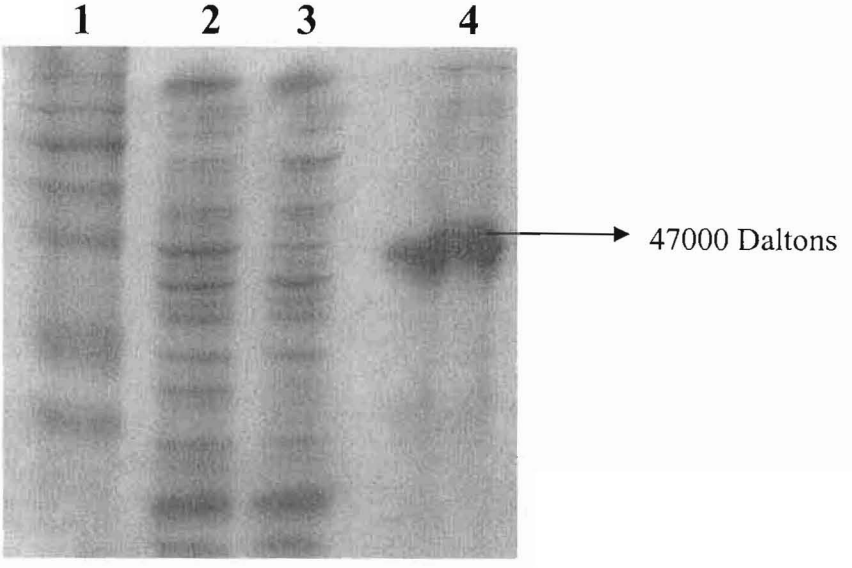
**Overexpression of *aroA***

Overexpression of *aroA* was accomplished by growing *E.coli* DH5α (pRep 4) (pIMS-8) to an $OD_{600}$ of approximately 0.6 in BHI broth containing 100 μg/ml of ampicillin and 25 μg/ml of kanamycin. Overexpression and purification was preformed as described in the Methods and Materials. Both the crude lysate and the purified product were analyzed by SDS-PAGE (15%) (Fig. 5). The overexpressed protein constituted approximately 50% of the total protein in the sample (visual estimation). The elutant from the nickel affinity column contained a distinct protein band corresponding to a size of 47000 Daltons as determined by SDS-PAGE (Fig. 5).

**Overexpression of *murA*1 and *murA*2**

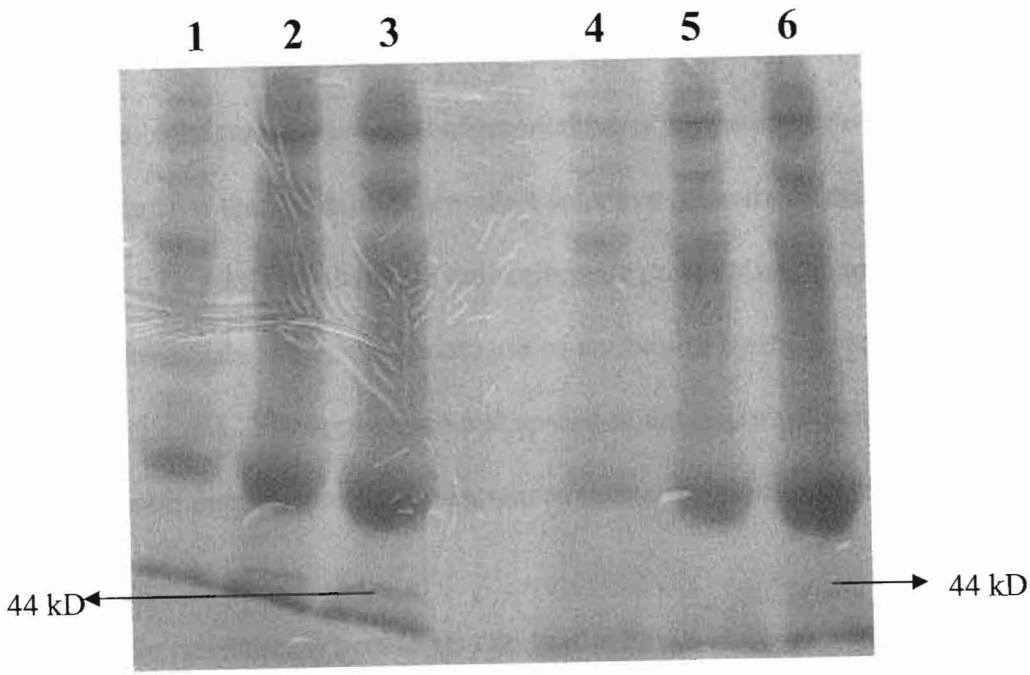
Overexpression of *murA*1 and *murA*2 was accomplished by growing *E.coli* Tuner (DE3) (pLac1) (pIMS-9) and *E.coli* Tuner (DE3) (pLac1) (pIMS-10) to an $OD_{600}$ of approximately 0.6 in BHI broth containing 100 μg/ml of ampicillin. Overexpression was preformed as described in the Methods and Materials section, and the protein product was

**Figure 5.** Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis of Histidine-EPSP synthase fusion protein. Lane 1, molecular weight markers; lane 2, total cellular lysate from the uninduced sample; lane 3, total cellular lysate from the induced sample at 2 hours; lane 4, overexpressed protein after Nickel chromatography.


1
2
3
4
47000 Daltons

analyzed by SDS-PAGE (7.5%). Although overexpression of each protein (44000 Daltons) was achieved (Fig. 6), no attempt was made to purify the protein.

**Figure 6.** Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis of MurA1 and MurA2. Lane 1, total cellular lysate from the uninduced MurA1 sample; lane 2 and 3, total cellular lysate from the induced sample with overexpressed MurA1; lane 4, total cellular lysate from the uninduced MurA2 sample; lane 5 and 6, total cellular lysate from the induced sample with overexpressed MurA2.


1
2
3
4
5
6
44 kD
44 kD

## Discussion

Pathogenic organisms that were once controlled with antibiotics are resurging back into the forefront and this has translated into increased antimicrobial resistance among common pathogens (23). The fundamental basis for the appearance and spread of antibiotic resistance is that antibiotics provide a selective pressure on microorganisms, which in turn can be partly attributed to indiscriminate prescription of antibiotics by health care professionals and inappropriate use of antibiotics by the general population (21, 23, 26, 40, 55, 64). The appearance and spread of antimicrobial resistance has had a major public health impact, resulting in increased mortality, morbidity, and costs of health care (23, 27, 55).

Antibiotic resistance is here to stay no matter how wisely we use our antibiotics. The synthesis of numerous antibiotics over past decades has created a false sense of complacency about the threat of antibiotic resistance. Now, however, there is an urgent and on-going need to control this surge in antimicrobial resistance by searching for new treatment possibilities (43, 68). The scientific community needs to find novel bacterial targets in order to specifically direct antibiotics with little or no toxicity to the host. One such possible target is a group of enzymes called enolpyruvyltransferases. These enzymes are involved in critical enzymatic pathways that are absent from animals (25) and are therefore, attractive targets for the development of novel antimicrobials. The logic of using a specific cellular target for the development of novel treatment strategies has been demonstrated by numerous previous studies.

The decision to study the enolpyruvyltransferases in *Staphylococcus aureus* was made for several reasons. It is one of the most common, yet one of the most pathogenic

of all bacteria. Infact, this bacterium epitomizes bacterial antimicrobial resistance (43, 69). Since little information exists on these enzymes in bacteria other than *E. coli*, a detailed analysis of these enzymes in *S. aureus* seems long overdue. A detailed examination of these enzymes from *S. aureus* will require the cloning and overexpression of the respective genes, which was the objective of the study presented herein.

The gene encoding EPSP synthase (*aroA*) was cloned and the nucleotide sequence determined from 5 discrete *S.aureus* isolates obtained from diverse geographical locations. Results obtained by a BLAST analysis search indicated 97-99% homology among *aroA* from the 5 isolates (Appendices 1-5). These data suggest *aroA* is a valid target for the development of novel drugs. What also makes it appealing is its wide distribution among bacteria, both Gram-negative and Gram-positive (12, 13, 15, 18, 24, 39), and in fungi, yeasts, and higher plants (19, 20, 30).

Several studies have shown that glyphosate, the active ingredient in the widely used herbicide, Roundup, inhibits EPSP synthase (5, 15, 48, 59). The fact that this broad-spectrum herbicide inhibits EPSP synthase almost universally makes this enzyme of the shikimate pathway a well-suited target for the development of broad-spectrum antimicrobials. Clustal sequence alignment revealed that the *S. aureus aroA* gene product has 43% and 29% amino acid sequence identity with the *B. subtilis* and *E. coli* EPSP synthase enzyme, respectively, with several amino acids being highly conserved (44). Data presented herein confirm these findings. Along similar lines, it has been shown that EPSP synthase from *Streptococcus pneumoniae* is 53% identical to that of *B. subtilis* and 25% identical to that of *E.coli* (12). Indeed, alignment of the amino acid sequence of EPSP synthase from multiple organisms led to the identification of a highly conserved

region, which has been validated by site-directed mutagenesis and kinetic analysis of the mutant enzymes (35, 45).

In this study, EPSP synthase has been overexpressed and purified in an *E. coli* expression system. By using the histidine expression vector, the *aroA* gene was expressed as a histidine fusion and purified by nickel chromatography (Fig. 5). The solubility of the expressed protein was confirmed by a functional assay of enzymatic activity developed by collaborators at the University of Kansas, Lawrence, KS.

The *murA* genes, whose gene products are also a member of the enolpyruvyltransferase family, were cloned from *S. aureus*. Both, *murA*1 and *murA*2 were cloned and confirmed by DNA sequencing. Previous studies have confirmed the presence of these genes in multiple bacterial organisms (10, 11, 38, 41, 67) with the important distinction that Gram-negative bacteria have one copy of *murA* (38) and Gram-positive bacteria have 2 copies (11). Of these genes, *murA*1 of Gram-positive bacteria is more closely related to *murA* of Gram-negative bacteria on the amino acid level (11). In the study presented herein, only the 5' and 3' ends of the cloned genes were sequenced. Based on these data, the integrity of the cloned insert was verified by a BLAST analysis (data not shown).

Comparison of *S. pneumoniae* MurA1 and MurA2 revealed they are similar-sized polypeptides (419 to 436 amino acids in length) and are 45% identical at the amino acid level (11). The essentiality of the genes was further confirmed by experiments with mutants in which *murA*1 or *murA*2, or both *murA*1 and *murA*2 were deleted. Although one gene can substitute for the other, deletion of both genes in *S. pneumoniae* or the single *murA* gene in Gram-negative bacteria is lethal (8, 11).

Fosfomycin, a naturally occurring broad-spectrum antibiotic, specifically inhibits MurA (28, 37) and has been very useful in structure/function studies. Unfortunately its toxic side effects limit its usefulness clinically. Intriguingly, most clinical antibiotics that act on the cell wall, β-lactams and glycopeptides, do so by inhibiting later steps in peptidoglycan biosynthesis. The earlier steps of the biosynthesis of cytoplasmic peptidoglycan precursors, such as MurA, remain poorly exploited as antimicrobial targets. Of the 11 enzymes involved in peptidoglycan synthesis in *E. coli* (41), 6 are essential in bacteria and their Mur protein products are highly conserved among various bacterial species (71). For this reason, not just MurA but the other Mur proteins also make attractive choices for the development of novel inhibitors that are bactericidal with a broad-spectrum (3, 42, 47, 62, 70). These facts make MurA1 and MurA2 attractive targets in *S. aureus*.

Although both MurA1 and MurA2 were overexpressed as native protein in an *E. coli* expression system (Fig. 6), no attempt was made to purify them. Furthermore, the proteins were likely expressed as insoluble inclusion bodies as determined by collaborators at the University of Kansas. A previous study used lower temperatures to induce expression of *murA*1 (18°C for 24 h) and *murA*2 (30°C for 3.5 h) in order to obtain soluble protein (11). Future studies will examine induction temperatures and the possibility of creating a histidine fusion protein as was done for EPSP synthase. However, the histidine tag may affect protein morphology and functionality, although this was not the case for EPSP synthase. While expression of the native protein is better suited to structure and function studies, it makes purification more challenging.

Looking at the enolpyruvylrtransferases as a group, many striking similarities are apparent. When examined at the amino acid level, clustal alignment showed EPSP synthase is 18.3% identical to MurA over 420 amino acids in *E. coli* (38) and the 2 enzymes have a very similar structure (33). However, despite similarities in structure and function between EPSP synthase and MurA, their mode of action is considerably different (33). Infact, EPSP synthase is not inactivated by fosfomycin and MurA is not inhibited by glyphosate (61).

In summary, the *aroA*, *murA*1, and *murA*2 genes were cloned from *S. aureus*. Overexpression and purification of EPSP synthase was accomplished, which will allow future studies to examine the mode of action of this enzyme. Collaborators at the University of Kansas are currently attempting to crystallize this enzyme. Although *murA*1 and *murA*2 have been cloned and overexpressed, experiments are currently under way to obtain functional protein in order to study their mechanism of action and look for novel inhibitors using High Throughput Screening. This will allow the development of an enhanced library of inhibitors that can be approached for the control of clinical infections.

## Appendix 1

*Staphylococcus aureus* # 23b (Lab strain). GenBank: BH861100.

**1** TGT ACA ACA TGT TGA TCA ACT AAC ATT ACA ACA TGC ATG TGA ACA
**46** ATT AAA AAC ATA TTT TAA GTA GGT GAA TGA A**AT G**GT AAA TGA ACA
**91** AAT CAT TGA TAT TTC AGG TCC GTT AAA GGG CGA AAT AGA AGT GCC
**136** GGG CGA TAA GTC AAT GAC ACA CCA GTG CAA TCA TGT TGG CGT
**178** CGC TAG CTG AAG GTG TAT CTA CTA TAT ATA AGC CAC TAC TTG GCG
**223** AAG ATT GTC GTC GTA CGA TGG ACA TTT TCC GAC TGT TAG GTG TAG
**268** AAA TCA AAG AAG ATG ATG AAA AAT TAG TTG TGA CTT CCC CAG
**310** GAT ATC AAT CTT TTA ACA CGC CAC ATC AAG TAT TGN ATA CAG GTA
**355** ATT NTG GTA CGA CAA CAC GAT TAT TGG CAG GTT TGT TAA GTG GTT
**400** TAN GTA TTG AAA GTG TTT TGT CCT GGC GAT GTT TCA ATT GGT AAA
**445** AGG CCA ATG GAT CGT GTC TTG AGA CCA TTG AAA CTT ATG GAT GCC
**490** AAN ATT GAA GGT ATT GAA GAT AAT TAT ACA CCA TTA ATT ATT AAG
**535** CCA TCT GTC ATA AAA GGT ATA AAT TAT CAA ATG GAA GTT GCA AGT
**580** GCA CAA GTA AAA AGT GCC ATT TTA TTT GCA AGT TTG TTT TCT AAG
**625** GAA CCG ACC ATC ATT AAA GAA TTA GAT GTA AGT CGA AAT CAT
**667** ACT GAG ACG ATG TTC AAA CAT TTT AAT ATT CCA ATT GAA GCA GAA
**712** GGG TTA TCA ATT AAT ACA ACC CCT GAA GCA ATT CGA TAC ATT AAA
**757** CCT GCA GAT TTT CAT GTT CCT GGC GAT ATT TCA TCT GCA GCG TTC
**802** TTT ATT GTT GCA GCA CTT ATC ACA CCA GGA AGT GAT GTA ACA ATT
**847** CAT AAT GTT GGA ATC AAT CCA ACA CGT TCA GGT ATT ATT GAT ATT
**892** GTT GAA AAA ATG GGC GGT AAT ATC CAA CTT TTC AAT CAA ACA ACT
**937** GGT GCT GAA CCT ACT GCT TCT ATT CGT ATT CAA TAC ACA CCA ATG
**982** CTT CAA CCA ATA ACA ATC GAA GGA GAA TTA GTT CCA AAA GCA
**1024** ATT GAT GAA CTG CCT GTA ATA GCA TTA CTT TGT ACA CAA GCA
**1066** GTT GGC ACG AGT ACA ATT AAA GAT GCC GAG GAA TTA AAA GTA
**1108** AAA GAA ACA AAT AGA ATT GAT ACA ACG GCT GAT ATG TTA AAC
**1150** TTG TTA GGG TTT GAA TTA CAA CCA ACT AAT GAT GGA TTG ATT
**1192** ATT TAT CCG TCA GAA TTT AAA ACA AAT GCA ACA GTT GAT AGT
**1234** TTA ACT GAT CAT CGA ATA GGA ATG ATG CTT GCA GTT GCT TCT
**1276** CTA CTT TCA AGC GAG CCT GTC AAA ATC AAA CAA TTT GAT GCT
**1318** GTA AAT GTA TCA TTT CCA GGA TTT TTA CCA AAA CTA AAG CTT TTA
**1363** GAA AAT GAG GGA **TAA** TAT AAA ATG GAA GAT ATC TAT AAA TTA
**1405** ATA GAC GAT ATC AAT CTA CAA AAA CTA GAA AAT TTA GAC TCT
**1447** CGG TTA ATG AAG C

The start codon (ATG) and the stop codon (TAA) are highlighted with bold font.

## Appendix 2

*Staphylococcus aureus* # 67 (57-dk) MRSA. GenBank: BH861101.

**1** GAT ATC GTT GTA CAT NAT GTT GAT CAA CTA ACA TTA CAA CAT GCA
**46** TGT GAA CAA TTA AAA ACA TAT TTT AAG TAG GTG AAT GAA **ATG** GTA
**91** AAT GAA CAA ATC ATT GAT ATT TCA GGT CCG TTA AAG GGC GAA ATA
**136** GAA GTG CCG GGC GAT AAG TCA ATG ACA CAC CGT GCA ATC ATG
**178** TTG GCG TCG CTA GCT GAA GGT GTA TCT ACT ATA TAT AAG CCA CTA
**223** CTT GGC GAA GAT TGT CGT CGT ACG ATG GAC ATT TTC CGA CTG TTA
**268** GGT GTA GAA ATC AAA GAA GAT GAT GAA AAA TTA GTT GTG ACT
**310** TCC CCA GGA TAT CAA TCT TTT AAC ACG CCA CAT CAA GTA TTG TAT
**355** ACA GGT AAT TCT GGT ACG ACA ACA CGA TTA TTG GCA GGT TTG TTA
**400** AGT GGT TTA GGT ATT GAA AGT GTT TTG TCT GGC GAT GTT TCA ATT
**445** GGT AAA AGG CCA ATG GAT CGT GTC TTG AGA CCA TTG AAA CTT
**487** ATG GAT GCG AAT ATT GAA GGT ATT GAA GAT AAT TAT ACA CCA TTA
**532** ATT ATT AAG CCA TCT GTC ATA AAA AGT ATA AAT TAT CAA ATG GAA
**577** GTT GCA AGT GCA CAA GTA AAA AGT GCC ATT TTA TTT GCA AGT TTG
**622** TTT TCT AAG GAA CCG ACC ATC ATT AAA GAA TTA GAT GTA AGT CGA
**667** AAT CAT ACT GAG ACG ATG TTC AAA CAT TTT AAT ATT CCA ATT GAA
**712** GCA GAA GGG TTA TCA ATT AAT ACA ACC CCT GAA GCA ATT CGA
**754** TAC ATT AAA CCT GCA GAT TTT CAT GTT CCT GGC GAT ATT TCA TCT
**799** GCA GCG TTC TTT ATT GTT GCA GCA CTT ATC ACA CCA GGA AGT GAT
**844** GTA ACA ATT CAT AAT GTT GGA ATC AAT CCA ACA CGT TCA GGT ATT
**889** ATT GAT ATT GTT GAA AAA ATG GGC GGT AAT ATC CAA CTT TTC AAT
**934** CAA ACA ACT GGT GCT GAA CCT ACT GCG TCT ATT CGT ATT CAA TAC
**979** ACA CCA ATG CTT CAA CCA ATA ACA ATC GAA GGA GAA TTA GTT
**1021** CCA AAA GCA ATT GAT GAA CTG CCT GTA ATA GCA TTA CTT TGT
**1063** ACA CAA GCA GTT GGC ACG AGT ACA ATT AAA GAT GCC GAG GAA
**1105** TTA AAA GTA AAA GAA ACA AAT AGA ATT GAT ACA ACG GCT GAT
**1147** GTG TTA AAC TTG TTA GGG TTT GAA TTA CAA CCA ACT AAT GAT
**1189** GGA TTG ATT ATT TAT CCG TCA GAA TTT AAA ACA AAT GCA ACA
**1231** GTT GAT AGT TTA ACT GAT CAT CGA ATA GGA ATG ATG CTT GCA
**1273** NGT TGC TTC TCT ACT TTC AAG CGA GCC TGT CAA AAT CAA ACA
**1315** ATT TGA TGC TGT AAA TGT ATC ATT TCC AGG ATT TTT ACC AAA ACT
**1360** AAA GCT TTT AGA AAA TGA GGG A**TA** ATA TAA AAT GGA AGA TAT
**1402** CTA TAA ATT AAT AGA CGA TAT CAA TCT ACA AAA ACT AGA AAA
**1444** TTT AGA CTC TCG

The start codon (ATG) and the stop codon (TAA) are highlighted with bold font.

## Appendix 3

*Staphylococcus aureus* # 69 (Kasanjian) MRSA. GenBank: BH861102.

**1** TAC AAC ATG TTG ATC AAC TAA CAT TAC AAC ATG CAT GTG AAC AAT
**46** TAA AAA CAT ATT TTA AGT AGG TGA ATG AA**A TG**G TAA ATG AAC AAA
**91** TCA TTG ATA TTT CAG GTC CGT TAA AGG GCG AAA TAG AAG TGC CGG
**136** GCG ATA AGT CAA TGA CAC ACC GTG CAA TCA TGT TGG CGT CGC
**178** TAG CTG AAG GTG TAT CTA CTA TAT ATA AGC CAC TAC TTG GCG AAG
**223** ATT GTC GTC GTA CGA TGG ACA TTT TCC GAC TGT TAG GTG TAG AAA
**268** TCA AAG AAG ATG ATG AAA AAT TAG TTG TGA CTT CCC CAG GAT
**310** ATC AAT CTT TTA ACA CGC CAC ATC AAG TAT TGT ATA CAG GTA ATT
**355** CTG GTA CGA CAA CAC NAT TAT TGG CAG GTT TGT TAA GTG GTT TAG
**400** GTA TTG AAA GTG TTT TGT CTG GCG ATG TTT CAA TTG GTA AAA GGC
**445** CAA TGG ATC GTG TCT NGA GAC CAT TGA AAC TTA TGG ATG CNA
**487** ATA TTG AAG GTA TTG AAG ATA ATT ATA CAC CAT TNA TTA CTA AGC
**532** CAT CTG NCA TAA AAG GTA TAA ATT ATC AAA TGG AAG TTG CAA
**574** GTG CAC AAG TAA AAA GTG CCA TTT TAT TTG CAA GTT TGT TTT CTA
**619** AGG AAC CGA CCA TCA TTA AAG AAT TAG ATG TAA GTC GAA ATC
**661** ATA CTG AGA CGA TGT TCA AAC ATT TTA ATA TTC CAA TTG AAG CAG
**706** AAG GGT TAT CAA TTA ATA CAA CCC CTG AAG CAA TTC GAT ACA TTA
**751** AAC CTG CAG ATT TTC ATG TTC CTG GCG ATA TTT CAT CTG CAG CGT
**796** TCT TTA TTG TTG CAG CAC TTA TCA CAC CAG GAA GTG ATG TAA CAA
**841** TTC ATA ATG TTG GAA TCA ATC CAA CAC GTT CAG GTA TTA TTG ATA
**886** TTG TTG AAA AAA TGG GCG GTA ATA TCC AAC TTT TCA ATC AAA CAA
**931** CTG GTG CTG AAC CTA CTG CTT CTA TTC GTA TTC AAT ACA CAC CAA
**976** TGC TTC AAC CAA TAA CAA TCG AAG GAG AAT TAG TTC CAA AAG
**1018** CAA TTG ATG AAC TGC CTG TAA TAG CAT TAC TTT GTA CAC AAG
**1060** CAG TTG GCA CGA GTA CAA TTA AAG ATG CCG AGG AAT TAA AAG
**1102** TAA AAG AAA CAA ATA GAA TTG ATA CAA CGG CTG ATA TGT TAA
**1144** ACT TGT TAG GGT TTG AAT TAC AAC CAA CTA ATG ATG GAT TGA
**1186** TTA TTT ATC CGT CAG AAT TTA AAA CAA ATG CAA CAG TTG ATA
**1228** GTT TAA CTG ATC ATC GAA TAG GAA TGA TGC TTG CAG TTG CTT
**1270** CTC TAC TTT CAA GCG AGC CTG TCA AAA TCA AAC AAT TTG ATG
**1312** CTG TAA ATG TAT CAT TTC CAG GAT TTT TAC CAA AAC TAA AGC TTT
**1357** TAG AAA ATG AGG GA**T AA**T ATA AAA TGG AAG ATA TCT ATA AAT
**1399** TAA TAG ACG ATA TCA ATC TAC AAN AAC TAG AAA ATT TAG ACT
**1441** CTC GGT TAA TGA AGC

The start codon (ATG) and the stop codon (TAA) are highlighted with bold font.

## Appendix 4

*Staphylococcus aureus* # 70 (Japan) MRSA. GenBank: BH861098.

**1** GAT ATC GTT GTA CAA CAT GTT GAT CAA CTA ACA TTA CAA CAT GCA
**46** TGT GAA CAA TTA AAA ACA TAT TTT AAG TAG GTG AAT GAA **ATG** GTA
**91** AAT GAA CAA ATC ATT GAT ATT TCA GGT CCG TTA AAG GGC GAA ATA
**136** GAA GTG CCG GGC AGA TAA GTC AAT GAC ACA CCG TGC AAT CAT
**178** GTT GGC GTC GCT AGC TGA AGG TGT ATC TAC TAT ATA TAA GCC ACT
**223** ACT TGG CGA AGA TTG TCG TCG TAC GAT GGA CAT TTT CCG ACT GTT
**268** AGG TGT AGA AAT CAA AGA AGA TGA TGA AAA ATT AGT TGT GAC
**310** TTC CCC AGG ATA TCA ATC TTT TAA CAC GCC ACA TCA AGT ATT GTA
**355** TAC AGG TAA TTC TGG TAC GAC AAC ACG ATT ATT GGC AGG TTT GTT
**400** AAG TGG TTT AGG TAT TGA AAG TGT TTT GTC TGG CGA TGT TTC AAT
**445** TGG TAA AAG GCC AAT GGA TCG TGT CTT GAG ACC ATT GAA ACT TAT
**490** GGA TGC GAA TAT TGA AGG TAT TGA AGA TAA TTA TAC ACC ATT AAT
**535** TAT TAA GCC ATC TGT CAT AAA AGG TAT AAA TTA TCA AAT GGA AGT
**580** TGC AAG TGC ACA AGT AAA AAG TGC CAT TTT ATT TGC AAG TTT GTT
**625** TTC TAA GGA ACC GAC CAT CAT TAA AGA ATT AGA TGT AAG TCG
**667** AAA TCA TAC TGA GAC GAT GTT CAA ACA TTT TAA TAT TCC AAT TGA
**712** AGC AGA AGG GTT ATC AAT TAA TAC AAC CCC TGA AGC AAT TCG
**754** ATA CAT TAA ACC TGC AGA TTT TCA TGT TCC TGG CGA TAT TTC ATC
**799** TGC AGC GTT CTT TAT TGT TGC AGC ACT TAT CAC ACC AGG AAG TGA
**844** TGT AAC AAT TCA TAA TGT TGG AAT CAA TCC AAC ACG TTC AGG TAT
**889** TAT TGA TAT TGT TGA AAA AAT GGG CGG TAA TAT CCA ACT TTT CAA
**934** TCA AAC AAC TGG TGC TGA ACC TAC TGC TTC TAT TCG TAT TCA ATA
**979** CAC ACC AAT GCT TCA ACC AAT AAC AAT CGA AGG AGA ATT AGT
**1021** TCC AAA AGC AAT TGA TGA ACT GCC TGT AAT AGC ATT ACT TTG
**1063** TAC ACA AGC AGT TGG CAC GAG TAC AAT TAA AGA TGC CGA GGA
**1105** ATT AAA AGT AAA AGA AAC AAA TAG AAT TGA TAC AAC GGC TGA
**1147** TAT GTT AAA CTT GTT AGG GTT TGA ATT ACA ACC AAC TAA TGA
**1189** TGG ATT GAT TAT TTA TCC GTC AGA ATT TAA AAC AAA TGC AAC
**1231** AGT TGA TAG TTT AAC TGA TCA TCG AAT AGG AAT GAT GCT TGC
**1273** AGT TGC TTC TCT ACT TTC AAG CGA GCC TGT CAA AAT CAA ACA
**1315** ATT TGA TGC TGT AAA TGT ATC ATT TCC AGG ATT TTT ACC AAA ACT
**1360** AAA GCT TTT AGA AAA TGA GGG A**TA** ATA TAA AAT GGA AGA TAT
**1402** CTA TAA ATT AAT AGA CGA TAT CAA TCT ACA AAA ACT AGA AAA
**1444** TTT AGA CTC TCG GTT AAT GAA GC

The start codon (ATG) and the stop codon (TAA) are highlighted with bold font.

## Appendix 5

*Staphylococcus aureus* # 71 (Dumas) MRSA. GenBank: BH861099.

**1** GAT ATC GGN GTA CAA CAT GTT GAT CAA CTA ACA TTA CAA CAT GCA
**46** TGT GAA CAA TTA AAA ACA TAT TTT AAG TAG GTG AAT GAA **ATG** GTA
**91** AAT GAA CAA ATC ATT GAT ATT TCA GGT CCT GTT AAA GGG CGA AAT
**136** AGA AGT GCC GGG CGA TAA GTC AAT GAC ACA CCG TGC AAT CAT
**178** GTT GGC GTC GCT AGC TGA AGG TGT ATC TAC TAT ATA TAA GCC ACT
**223** ACT TGG CGA AGA TTG TCG TCG TAC GAT GGA CAT TTT CCG ACT GTT
**268** AGG TGT AGA AAT CAA AGA AGA TGA TGA AAA ATT ANT TGT GAC
**310** TTC CCC AGG ATA TCA ATC TTT TAA CAC GCC ACA TCA AGT ATT GTA
**355** TAC AGG TAA TTC TGG TAC GAC AAC ACG ATT ATT GGC AGG TTT GTT
**400** AAG TGG TTN AGG TAT TGA AAG TGT TTT GTC TGG CGA TGT TTC AAT
**445** TGG TAA AAG GCC AAT GGA TCG TGT CTT GAG ACC ATT GAA ACT TAT
**490** GGA TGC GAA TAT TGA AGG TAT TGA AGA TAA TTA TAC ACC ATT AAT
**535** TAT TAA GCC ATC TGT CAT AAA AGG TAT AAA TTA TCA AAT GGA AGT
**580** TGC AAG TGC ACA AGT AAA AAG TGC CAT TTT ATT TGC AAG TTT GTT
**625** TTC TAA GGA ACC GAC CAT CAT TAA AGA ATT AGA TGT AAG TCG
**667** AAA TCA TAC TGA GAC GAT GTT CAA ACA TTT TAA TAT TCC AAT TGA
**712** AGC AGA AGG GTT ATC AAT TAA TAC AAC CCC TGA AGC AAT TCG
**754** ATA CAT TAA ACC TGC AGA TTT TCA TGT TCC TGG CGA TAT TTC ATC
**799** TGC AGC GTT CTT TAT TGT TGC AGC ACT TAT CAC ACC AGG AAG TGA
**844** TGT AAC AAT TCA TAA TGT TGG AAT CAA TCC AAC ACG TTC AGG TAT
**889** TAT TGA TAT TGT TGA AAA AAT GGG CGG TAA TAT CCA ACT TTT CAA
**934** TCA AAC AAC TGG TGC TGA ACC TAC TGC TTC TAT TNG TAT TCA ATA
**979** CAC ACC AAT GCT TCA ACC AAT AAC AAT CGA AGG AGA ATT AGT
**1021** TCC AAA AGC AAT TGA TGA ACT GCC TGT AAT AGC ATT ACT TTG
**1063** TAC ACA AGC AGT TGG CAC GAG TAC AAT TAA AGA TGC CGA GGA
**1105** ATT AAA AGT AAA AGA AAC AAA TAG AAT TGA TAC AAC GGC TGA
**1147** TAT GTT AAA CTT GTT AGG GTT TGA ATT ACA ACC AAC TAA TGA
**1189** TGG ATT GAT TAT TTA TCC GTC AGA ATT TAA AAC AAA TGC AAC
**1231** ANG TTG ATA GTT TAA CTG ATC ATC GAA TAG GAA TGA TGC TTG
**1273** CAG TTG CTT CTC TAC TTT CAA GCG AGC CTG TCA AAA TCA AAC
**1315** AAT TTG ATG CTG TAA ATG TAT CAT TTC CAG GAT TTT NAC CAA
**1357** AAC TAA AGC TTT TAG AAA ATG AGG GA**T AA**T ACA AAA TGG AAG
**1399** ATA TCT ATA AAT TAA TAG ACA GAT ATC AAT CTA CAA AAA CTA
**1441** GAA AAT TTA GAC TCT CGG TTA ATG AAG

The start codon (ATG) and the stop codon (TAA) are highlighted with bold font.

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CLONING AND OVEREXPRESSION OF THE ENOLPYRUVYLTRANSFERASES FROM STAPHYLOCOCCUS AUREUS

Title of Thesis

Doug Cooper

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