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Antibiotic Resistance is a steadily growing problem in today's society. The problem has become so pronounced that many scientists feel public health may soon enter a post-antimicrobial era. Worldwide, many laboratories are addressing this issue by developing novel inhibitors that target essential proteins in the cell. Since the bacterial cell wall is required for survival, enzymes involved in its synthesis are potential targets for the development of novel inhibitors. The enzyme UDP-N-acetyl glucosamine enolpyruvyltransferase (MurA) is a valid target as it is involved in the first committed step of bacterial cell wall synthesis. In this study, we have cloned and overexpressed *murA* from the extremophiles *Oceanobacillus iheyensis* and *Thermatoga maritima,* and the mesophile *Escherichia coli.* Since low guanine plus cytosine Gram-positive bacteria also contain an additional enzyme with UDP-N-acetyl glucosamine enolpyruvyltransferase activity termed MurZ, the gene encoding this protein was also cloned and overexpressed from *O. iheyensis.* Using a whole-cell assay, the overexpression of soluble, enzymatically active MurA (and MurZ) was demonstrated.

Overexpression and Analysis of MurA from Extremophiles

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PREFACE

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Introduction

In 1967 the surgeon general of the United States, William H. Stewart, said it was "time to close the book on infectious diseases and shift all national efforts to chronic diseases." Unfortunately, this same optimism is not present today, as the Director-General of the World Health Organization (WHO) pointed out in 1996, "We are on the brink of a global crisis in infectious diseases. No country is safe from them. No country can any longer afford to ignore this threat."

Bacterial antibiotic resistance has become a societal issue at the local, national and international levels (12). Many factors have lead to this crisis, including the world population growth, urbanization and poverty levels, human behavior changes, and microbial evolution and adaptation. The problem has become so significant that many scientists and researchers feel we are entering a "post-antimicrobial era", suggesting soon our current public health measures will no longer be able to control the spread of infectious diseases (3).

A downturn in antibiotic discovery is also leaving society vulnerable to emerging resistant diseases and helping promote a "post-antimicrobial era" (19). The reasons for this "lack of new discovery" are primarily financially motivated. Drug companies find it easier to spend billions of dollars on the development of drugs for chronic conditions rather than on new antibiotics which a patient may take only for a limited amount of time.

Research is being conducted to address the issue of antibiotic resistant bacteria. Many studies are addressing this problem from the perspective of the host cell. Based on this approach, novel drugs are being developed that would interact with host cells by blocking binding sites utilized by bacterial pathogens (7). Other studies are trying to

determine what molecules are essential for survival in a bacterial cell and to develop "designer drugs" to inhibit these molecules.

The cell wall (peptidoglycan) contains many essential molecules which have been a target for the development of many novel antimicrobials. The advantage of targeting the peptidoglycan is that it allows a bacterial cell to be targeted specifically without causing adverse side effects in the human host. Currently, most antibiotics in existence today that inhibit some aspect of cell wall biosynthesis have been developed as a result of a producing organism's secondary metabolism (19). Common examples include vancomycin, ampicillin, penicillin, and cephalathin. Since the cell wall is critical for survival, any of the enzymes involved in its biosynthesis offer an attractive target for the development of novel drugs.

UDP-N-acetylglucosamine enolpyruvyltransferase (MurA) is involved in the first committed step in bacterial cell wall biosynthesis (2). It acts by catalyzing a reaction involving phosphoenolpyruvate (PEP) that is unique among other PEP utilizing enzymes. Most PEP-dependent enzymes cleave the P-O bond of PEP and transfer the liberated P to another molecule, but enolpyruvyltransferases cleave the C-O bond of PEP and transfer the enolpyruvyl moiety to another substrate (24). Specifically, it catalyzes enolpyruvyl transfer from PEP to the 3-OH of UDP-N-acetylglucosamine (UDPAG), forming UDP-N-acetylglucosamine enolpyruvate (2). Currently, MurA and EPSP synthase (5-enolpyruvyl shikimate-3-phosphate synthase) are the only known examples of enolpyruvyltransferases.

MurA has been extensively studied in *Escherichia coli.* It has a molecular weight of approximately 44,000 Daltons and is encoded by a single gene, *murA* (14).

The crystal structure of MurA has been elucidated in both the absence and presence of ligands. It is composed of two globular domains with a catalytic site located in a deep cleft between the two domains (18, 22). The catalytic site, which contains a Cys-115 residue, is the target of alkylation by the antibiotic fosfomycin, a novel inhibitor of MurA (15, 25).

MurA has also been studied in Gram-positive organisms. Interestingly, it has been shown that Gram-positive bacteria with a low G + C content contain two genes with UDP-N-acetylglucosamine enolpyruvyltransferase activity. While *murA* is common in all bacterial species, *murZ* exists only in low G + C content Gram-positive bacteria. In *Streptococcus pneumoniae,* the genes are only 45% homologous, but they both catalyze a UDP-N-acetylglucosamine enolpyruvyltransferase activity that is inhibited by fosfomycin (5).

Extremophiles are organisms that survive in extreme environments. These diverse environments can have extremes of temperature, pressure, salinity, pH, and radiation (23). Organisms which do live in these extreme environments rely on their ability to redistribute various intramolecular interactions required for both protein stabilization and structural flexibility (6). This reorganization allows the molecule to avoid denaturation and ensures proper ligand binding. By studying molecules from organisms residing in extreme environments and comparing their structure to similar molecules from mesophilic organisms, numerous insights can be obtained into the mechanism of action of a molecule.

The objective of this study was to clone *murA* from *Escherichia coli, Thermatoga maritima,* and *Oceanobacillus iheyensis* (both *murA* and *murZ*) and compare them at the

protein level. Furthermore, an attempt was made to overexpress each gene in *E. coli.* Our objective was to gain information on how a conserved protein functions at different extremes. Our data show that we were able to successfully clone *murA* and *murZ* from all organisms. It also demonstrates that we were able to obtain soluble overexpression of *murA* and *murZ*, but not at a level which would accommodate protein purification.

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. Typical growth mediums used were Tryptic Soy Broth (TSB), Brain Heart Infusion (BHI), and Luria-Bertani (LB). To make agar plates, 20 g/L agar was added to broth media. All cultures were propagated at 37° C unless indicated. Antibiotics used routinely were ampicillin (100 μg/ml) and kanamycin (25 μg/ml). The reagent 5-Bromo-4-Chloro-3-Indoyl-β-D-Galactopyranoside (X-gal) was used at a concentration of 20 μg/ml.

Total DNA Isolation from *Escherichia coli*

Total DNA was isolated from bacterial cultures using a modification of an established procedure (16). Briefly, 25 ml of BHI was inoculated with *E. coli* and incubated overnight at 37° C with shaking in a New Brunswick Series 25 Incubator (Edison, NJ). Cells were harvested using a Sorvall GLC-1 tabletop centrifuge (Newton, CA) at 4000 rpm for 5 min, followed by resuspension in TE buffer (10mM Tris, 1mM EDTA; pH 8). Lysis was accomplished by resuspending cells in 100 μl of 20% sodium dodecyl sulfate (SDS) with subsequent incubation at 60° C for 15 min. Fifty microliter of RNase (1mg/ml) was added and the solution incubated at 37° C for 20 min to degrade contaminating RNA. Subsequently, 75 μl of proteinase K (1mg/ml) was added followed by incubation at 60° C for 1 hour to degrade protein. A phenol extraction was performed by adding 6 ml of phenol (pH 8.0). After gentle mixing by inversion, the organic and aqueous phases were separated by centrifugation at 4000 rpm for 5 min. The

Table 1. Bacterial strains and DNA sources used in this study.

Bacterial Strain	Description	Source
Escherichia coli	Facultative Anaerobe	ESU Culture Collection
Thermatoga maritima[a]	Extremophile (hot vents)	ATCC
Oceanobacillus iheyensis[b]	Extremophile (deep ocean)	Reference 10
Escherichia coli DH5α	Routine Cloning Strain	ESU Culture Collection
Escherichia coli AD494(DE3)	Expression Strain	Novagen
Escherichia coli HMS174(DE3)	Expression Strain	Novagen
Escherichia coli Origami(DE3)	Expression Strain	Novagen

[a] *T. maritima* was not grown in this study. Purified DNA was obtained from the American Type Culture Collection (ATTC).
[b] *O. iheyensis* was not grown in this study. Purified DNA was obtained from T. Hideto listed in reference 10.

Table 2. Plasmids and recombinant plasmids used in this study.

Plasmids	Description	Source
PT7Blue3	Cloning Vector	Novagen
pETBlue1	Expression Vector	Novagen
pJLT1	pT7Blue3::*E.coli murA*	This Study
pJLT2	pT7Blue3::*T. maritima murA*	This Study
pJLT3	pT7Blue3::*O. iheyensis murA*	This Study
pJLT4	pT7Blue3::*O. iheyensis murZ*	This Study
pJLT5	PETBlue1::*E. coli murA*	This Study
pJLT6	pETBlue1::*T. maritima murA*	This Study
pJLT7	pETBlue1::*O. iheyensis murA*	This Study
pJLT8	pETBlue1::*O. iheyensis murZ*	This Study

upper aqueous layer was placed into a fresh 15 ml conical tube and the phenol extraction repeated. After the second phenol extraction, a chloroform extraction was performed by adding 6 ml of chloroform/isoamyl alcohol (24:1), followed by gentle mixing and centrifugation at 4000 rpm for 5 min. DNA was precipitated by adding 2.5 volumes of 95% ice-cold ethanol to the aqueous layer. Once DNA was visible, it was removed using a sterile pipette tip and placed in a 1.5 ml microcentrifuge tube. After drying at room temperature for approximately 30 min, the DNA was resuspended in 500 μl of TE buffer. DNA solutions were stored at 4° C until needed.

Plasmid DNA Isolation

Plasmid DNA was isolated from 16 hour cultures using the QIAprep Spin Miniprep kit (Qiagen; Valencia, CA) according to the manufacturer's recommendations.

Quantification and Purity of DNA

DNA purity was determined by measuring the absorbance ratio at 260 nm and 280 nm. For quantity determination, the absorbance at 260 nm was measured. All measurements were performed using a DU Series 50 Spectrophotometer (Beckman; Fullerton, CA). Absorbance values were converted into μg/ml using the equation: (A_{260}) (Dilution Factor) (50μg/ml) = μg/ml.

DNA Sequencing and Analysis

Plasmid DNA was isolated for DNA sequencing using the QIAprep Spin Miniprep Kit described above. Once high quality plasmid DNA was obtained (verified

by absorbance 260 nm/280 nm ratio readings of approximately 1.8), it was sequenced with the assistance of the DNA Sequencing Laboratory at the University of Arkansas for Medical Sciences, Little Rock, Arkansas. DNA sequences were compared to known nucleotide sequences using the Basic Local Alignment Search Tool (BLAST) (1).

Agarose Gel Electrophoresis

DNA was separated via agarose gel electrophoresis according to standard protocols (16). To prepare gels, 0.21g of agarose and 1µl of 10 mg/ml ethidium bromide were added to 30 ml of TAE, prepared from 50X stock (242 g Tris, 57.1 ml acetic acid, and 4 ml 0.5 M EDTA/1L) in a 125 ml flask. After heating in a microwave, the solution was cooled slightly and allowed to solidify in a gel mold. Subsequently, DNA was loaded into preformed wells and electrophoresed using a Bio-Rad model 250/2.5 power supply (Bio-RAD; Hercules, CA). DNA was visualized after electrophoresis using a UV Intensity Transilluminator (Fisher; St. Louis, MO).

Gel Extraction

DNA was extracted from agarose gels using a Qiaex II Agarose Gel Extraction Kit (Qiagen) according to the manufacturer's suggested protocol.

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was used to amplify specific regions of DNA from chromosomal DNA templates using gene specific primers. Primers used in this study are listed in Table 3. Reactions typically consisted of 200 µM of deoxynucleotide triphosphates (dNTP's), 1.5 µM $MgCl_2$, 1µl *Taq* polymerase, 500 ng DNA, and 50 nM of

each primer in buffer containing 50 mM TrisCl, 50 mM KCl and 0.01% Triton-X100 in a final volume of 100 µl. Reactions were run on a Minicycler Thermocycler (MJ Research Inc., Watertown, MA). Thirty cycles were run with each cycle consisting of three steps, a 94° C denaturation step for 1 min, a 55° C annealing step for 1 min and a 72° C extension step for 2 min. Initial denaturation at 94° C was performed for 5 min before the thirty cycles began and an additional extension step at 72° C for 5 min was performed at the end of the thirty cycles.

Phosphorylation of DNA

DNA fragments were phosphorylated at the 5'end using an End Conversion Mix (Novagen, Madison, WI). Briefly, DNA was mixed with the End Conversion Mix which contained adenosine triphosphate (ATP) and T4 DNA kinase. After incubation for 15 min at 22° C, the reaction was terminated by incubation at 75° C for 5 min.

Ligation

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

Table 3. Primers used in PCR.

Primer	Primer sequence 5'-3'
E. coli murA forward	ATG GAT AAA TTT CGT GTT CAG G
E. coli murA reverse	TTA TTC GCC TTT CAC ACG CTC AAT
T. maritima murA forward	ATG GGT AAA CTG GTC CTT CAG GGT
T. maritima murA reverse	TCA ATT TTC TTT TTC AAC ATA CTC GAT
O. iheyensis murA forward	ATG GAA AAA ATC ATC GTA AGT GGC GGA
O. iheyensis murA reverse	TTA ATC CGC TGC TAT TTC ATT TGA
O. iheyensis murZ forward	ATG CAA AAA TTA TTA ATT GAA GGT GGT
O. iheyensis murZ reverse	TTA CAT ATT TTG ATC TTG CAT TAT TTC

Preparation of Competent Cells

Competent *E. coli* cells were prepared according to a modification of a standard protocol (16). Briefly, 0.5 ml of an overnight culture grown in 2X LB at 30° C with shaking was used to inoculate 200 ml of 2X LB. Cultures were propagated at 30° C with shaking until an OD_{600} of 0.3 was reached. Subsequently, 4 ml of 1M $MgCl_2$ was added to the growing cultures and incubation continued until an OD_{600} of 0.45-0.55 was obtained. Cultures were chilled on ice for 2 hours followed by centrifugation at 3000 rpm for 5 min at 4° C in a J2-HS centrifuge (Beckman). Cells were resuspended in 100 mM ice-cold $CaCl_2$ media (0.05 M $CaCl_2$, 0.04 M $MnCl_2$, 0.02 M CH_3COON, pH 7.5) and incubated on ice for an additional 40 min. Centrifugation was repeated as above and cell pellets resuspended in ice-cold $CaCl_2$ media containing 15% glycerol. Cells were stored at –70° C until needed.

Transformation

Transformation of DNA into competent cells was performed under standard conditions (16). Briefly, variable amounts of DNA or ligation mixtures were added to 100 µl of competent *E. coli* cells. Transformation mixtures were incubated on ice for 15 min, heat shocked for 90 seconds at 42° C, and placed back on ice for 2 min. Nine hundred microliter of LB broth was added to the transformed cells and 100 µl of this suspension spread plated on LB agar plates containing ampicillin. When kanamycin was used as a selective agent, transformation mixtures were incubated for 1 hour at 37° C prior to plating on media with kanamycin. Plates were incubated for approximately 16-

20 hours at 37° C. The addition of X-gal to the media allowed blue/white colony screening and aided in the identification of recombinants when appropriate.

PCR-Colony Lysis

PCR-Colony lysis was used to verify the presence of recombinant plasmids. Briefly, a small amount of potentially recombinant *E. coli* cells were aseptically added to 10 µl of sterile water. Cell lysis was accomplished by incubation at 95° C for 5 min. The lysed cell mixture was subsequently used as a DNA source for PCR using gene specific primers under conditions described above. Agarose gel electrophoresis was used to verify the presence of amplified DNA.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Gels (15%) were electrophoresed using a Fisher Biotech Protein Electrophoresis System FE-VE16-1 (Fisher, St. Louis, MO) and a Thermo EC Series 90 Power Supply (Thermo Electron, Holbrook, NY) according to the manufacturer's recommendations. Protein standards were purchased from Sigma (St. Louis, MO).

Overexpression

Cultures of *E. coli* containing an expression plasmid were propagated in LB media containing the appropriate antibiotic at 37° C with shaking until mid log phase (A_{600}~0.5) was obtained. Isopropylthio-β-D-galactoside (IPTG) was added and incubation continued for 2-3 hours at 37° C. Soluble protein was harvested using the

Bugbuster reagent (Novagen). Briefly, 1 ml of culture was transferred to a 1.5 ml microcentrifuge tube and centrifugation performed at high speed for 1 min. The cell pellet was resuspended in 300 µl of Bugbuster reagent and incubated on a rocker platform for 20 min at room temperature. After centrifugation in a microcentrifuge at high speed for 10 min, the resulting supernatant was analyzed by SDS-PAGE.

Whole-Cell Fosfomycin Resistance Assay

Soluble overexpression of MurA was determined using a whole-cell fosfomycin resistance assay. Briefly, cultures to be analyzed were inoculated into 5 ml LB containing the appropriate antibiotic and grown overnight with shaking at 37° C. A fresh 5 ml LB tube containing 5 µl of 100 mM IPTG and 50 µl of the appropriate antibiotic was inoculated with 5 µl of the overnight culture and propagated at 37° C with shaking. After mid-log phase was reached, fresh 5 ml LB tubes containing 50 µl of 100 mM IPTG and variable amounts of fosfomycin were inoculated with 50 µl of the mid-log phase culture. Cultures were allowed to incubate approximately 16 hours at 37° C with shaking. Growth was quantified by measuring the absorbance at 600 nm. The same procedure was also performed at 30° C.

Results

Cloning of *murA* from *E. coli, T. maritima, and O. iheyensis, and murZ* from *O. iheyensis.*

Using gene specific primers, *murA* was amplified from chromosomal DNA templates prepared from each organism via PCR. Additionally, *murZ* was amplified from an *O. iheyensis* DNA template. Each amplicon was cloned into pT7Blue3 and transformed into *E. coli* DH5α. The nucleotide sequence of each gene is illustrated in Figures 1-4. BLAST analysis confirmed the integrity of each cloned gene (data not shown).

Amino Acid Sequence Analysis

Using a web-based analysis program (http://www.ncbi.nih.gov/BLAST/), the nucleotide sequence for each gene was translated into its corresponding amino acid sequence. Examination of each translated protein revealed major structural features (α-helices and β-sheets), as well as areas of ligand interaction are conserved (Figures 5-8).

Each translated MurA and MurZ sequence was aligned with *E. coli* MurA to determine the amount of homology between the two proteins (Figures 9-11). Furthermore, the amino acid sequences of *O. iheyensis* MurA and *O. iheyensis* MurZ were compared (Figure 12). *O. iheyensis* MurA demonstrated the most homology to *E. coli* MurA at 47%, whereas *T. maritima* MurA was 45% homologous. *O. iheyensis* MurA was the least homologous to *E. coli* MurA at 41%. *O. iheyensis* MurA was 43% homologous to *O. iheyensis* MurZ.

Overexpression of *murA* and *murZ*

To assess if the cloned *murA* or *murZ* genes could be expressed as active proteins in *E. coli,* each gene was cloned into pETBlue1, an expression vector which facilitates the overexpression of cloned genes. Nucleotide sequencing of each recombinant plasmid verified each gene was cloned with the proper spacing from the ribosome binding site supplied by the plasmid (data not shown). Each recombinant plasmid was transformed into *E. coli* AD494 (DE3), *E. coli* HMS174 (DE3), and *E. coli* Origami (DE3) cells for subsequent analysis by the whole cell fosfomycin assay (see Methods and Materials). Furthermore, each recombinant plasmid in each *E. coli* host strain was induced at both 30° C and 37° C to determine if temperature affected protein solubility. As shown in Figures 14-19, *E. coli* AD494 was the superior host strain in all cases for protein solubility as determined by increased fosfomycin resistance. Protein solubility was maximal at 37° C for *E. coli* MurA and *O. iheyensis* MurZ, whereas 30° C was optimal for *T. maritima* and *O. iheyensis* MurA. Samples were also examined by SDS-PAGE to visually analyze each overexpressed protein, but in all cases, data obtained were inconclusive (data not shown).

Figure 1. Nucleotide sequence of the *murA* coding sequence from *E. coli*.

1 ATG GAT AAA TTT CGT GTT CAG GGG CCA ACG AAG CTC CAG GGC
43 GAA GTC ACA ATT TCC GGC GCT AAA AAT GCT GCT CTG CCT ATC
85 CTT TTT GCC GCA CTA CTG GCG GAA GAA CCG GTA GAG ATC CAG
127 AAC GTC CCG AAA CTG AAA GAC GTC GAT ACA TCA ATG AAG CTG
169 CTA AGC CAG CTG GGT GCG AAA GTA GAA CGT AAT GGT TCT GTG
211 CAT ATT GAT GCC CGC GAC GTT AAT GTA TTC TGC GCA CCT TAC
253 GAT CTG GTT AAA ACC ATG CGT GCT TCT ATC TGG GCG CTG GGG
294 CCG CTG GTA GCG CGC TTT GGT CAG GGG CAA GTT TCA CTA CCT
337 GGC GGT TGT ACG ATC GGT GCG CGT CCG GTT GAT CTA CAC ATT
379 TCT GGC CTC GAA CAA TTA GGC GCG ACC ATC AAA CTG GAA GAA
421 GGT TAC GTT AAA GCT TCC GTC GAT GGT CGT TTG AAA GGT GCA
462 CAT ATC GTG ATG GAT AAA GTC AGC GTT GGC GCA ACG GTG ACC
505 ATC ATG TGT GCT GCA ACC CTG GCG GAA GGC ACC ACG ATT ATT
547 GAA AAC GCA GCG CGT GAA CCG GAA ATC GTC GAT ACC GCG AAC
589 TTC CTG ATT ACG CTG GGT GCG AAA ATT AGC GGT CAG GGC ACC
631 GAT CGT ATC GTC ATC GAA GGT GTG GAA CGT TTA GGC GGC GGT
673 GTC TAT CGC GTT CTG CCG GAT CGT ATC GAA ACC GGT ACT TTC
715 CTG GTG GCG GCG GCG ATT TCT CGC GGC AAA ATT ATC TGC CGT
757 AAC GCG CAG CCA GAT ACT CTC GAC GCC GTG CTG GCG AAA CTG
798 CGT GAC GCT GGA GCG GAC ATC GAA GTC GGC GAA GAC TGG ATT
841 AGC CTG GAT ATG CAT GGC AAA CGT CCG AAG GCT GTT AAC GTA
883 CGT ACC GCG CCG CAT CCG GCA TTC CCG ACC GAT ATG CAG GCC
925 CAG TTC ACG CTG TTG AAC CTG GTG GCA GAA GGG ACC GGG TTT
967 ATC ACC GAA ACG GTC TTT GAA AAC CGC TTT ATG CAT GTG CCA
1009 GAG CTG AGC CGT ATG GGC GCG CAC GCC GAA ATC GAA AGC
1045 AAT ACC GTT ATT TGT CAC GGT GTT GAA AAA CTT TCT GGC GCA
1087 CAG GTT ATG GCA ACC GAT CTG CGT GCA TCA GCA AGC CTG GTG
1128 CTG GCT GGC TGT ATT GCG GAA GGG ACG ACG GTG GTT GAT CGT
1170 ATT TAT CAC ATC GAT CGT GGC TAC GAA CGC ATT GAA GAC AAA
1213 CTG CGC GCT TTA GGT GCA AAT ATT GAG CGT GTG AAA GGC GAA
1255 TAA

Figure 2. Nucleotide sequence of the *murA* coding sequence from *T. maritima.*

Note that the first nucleotide is a T. When this gene was amplified, it was replaced with an A.

1 TTG GGT AAA CTG GTC GTT CAG GGT GGC GCC GTT CTC GAA GGA
43 GAA GTG GAG ATA TCG GGT TCA AAA AAC GCT GCT CTT CCA ATA
85 ATG GCA GCA GCG ATT TTG TGT GAT GAG GAG GTA ATT CTC AAA
127 AAC GTA CCG AGA CTC CAA GAT GTC TTC GTC ATG ATA GAC ATC
169 CTG AGA TCC ATT GGA TTC AGA GTG GAA TTC GAG GAG AAC GAA
211 CTG AAG ATC AAA AGA GAA AAC GAT ATC TCA CAG GAA GTG CCT
253 TAC GAA CTT GTC AGG AAG ATG AGG GCG TCC TTC AAC GTG CTC GGT
298 CCG ATC GCT GTG AGA ACT GGA AGA GCG AAG GTT GCT CTT CCA
340 GGT GGG TGT TCC ATA GGA GTC AGA CCT GTG GAC TTT CAT CTT GAA
385 GGC CTC AAA AAA ATG GGA TTC TCG ATA AAA GTG GAA CAT GGC
427 TTT GTT GAA GCC TGC TTT GAA AGA AGA ATC GAT TAT GTG ACG ATT
469 ACC CTT CCT TTT CCA AGC GTT GGT GCC ACG GAG CAC CTG ATG ACC
514 ACA GCG GCT CTC TTA AAA GGT GCC CGT GTA GTG ATT GAA AAC
556 GCC GCA ATG GAA CCT GAA ATC GTA GAC CTT CAA AAT TTC ATA
598 AAC AGA ATG GGT GGA CAT ATT GAA GGA GCC GGA ACC AGC CGG
640 ATA GTG ATT GAA GGC GTG GAG AAA ATG CAG GGA GTT GAA TAC
682 AGC ATC ATT CCC GAT CGA ATA GAA GCT GGA ACG TAC CTG GTA
724 GCC ATC GCA GCA AGT CGT GGA AAA GGT CTG GTG AAG AAT GTA
766 AAC CCG GAT CAC CTC ACA AAC TTT TTT GAG AAA CTG GAA GAA
808 ACA GGG GCG AAA CTT AAA GTT CTT GGA AAC GAA GTA GAG ATC
850 GAA ATG AGA GAA AGA CCA AAA GCG GTG GAT GTT ACA ACG AAT
892 CCG TAC CCT GGT TTT CCC ACG GAT CTT CAG CCT CAG ATG ATG GCG
937 TAT CTA TCG ACA GCG TCG GGA GTC TCG GTT ATA ACC GAA AAC GTC
952 TTC AAA ACG AGG TTC TTA CAC GTG GAC GAG TTG AAA AGA ATG
994 GGA GCG GAC ATA GAA GTT TCT GGA AAC GTC GCC ATA GTG AAA
1036 GGC GTT GAA AAA CTC AGC GGT GCC CCC GTT GAG GGA ACG GAT
1078 CTC AGG GCA ACC GCT GCC CTT CTC ATA GCG GGA ATC ATA GCA
1120 GAT GGA GTC ACT GAG ATA AGC AAC GTC GAA CAC ATA TTC AGA
1162 GGT TAC GAA GAT GTC ATA GAC AAA TTC AGT GAA CTG GGA GCA
1204 AAA ATC GAG TAT GTT GAA AAA GAA AAT TGA

Figure 3. Nucleotide sequence of the *murA* coding sequence from *O. iheyensis.*

1 ATG GAA AAA ATC GTA AGT GGC GGA CAC CAA TTG AAT GGC ACC
43 GTA CGG CTT GAA GGT GCT AAA AAC GCT GTA CTA CCT GTT TTA
86 GCT GCA AGT TTA ATT GCG AGT GAA GGG GAA AGC GTT ATT AAA
128 GAA GTA CCA GTT TTA GCA GAC GTG TAC ACA ATT AAT GAA GTA
170 TTA CGT AAT CTA AAC GCA GAA GTT GAA TTT GAT TCA ACA ACA
212 AAG ACA GTG AAT ATT AAT GCA TCA CAA CAA TTA GAG ACA GAA
254 GCC CCA TTT GAA TAT GTA AGA AAA ATG CGT GCA TCT GTA CTT
295 GTT TTG GGA CCA CTT TTG GCC CGT TAT GGA CAC GCG AAA GTT
338 GCT ATG CCA GGA GGA TGT GCG ATT GGT TCT CGA CCA ATT GAT
380 TTA CAC CTT AAA GGT TTC GAA GCA ATG GGA GCA GAA ATC CAT
422 GTA GGT AAC GGC TAT GTG GAA GCA AAT GTG AAT GGC AGA TTA
463 CAA GGT GCG AAG ATT TAT CTT GAT ATG CCA AGT GTA GGT GCG
506 ACG GAA AAC ATT ATG ATG GCT GCA GCA CTT GCA GAG GGT AAG
548 ACA GTA ATT GAA AAT GCA GCA AAA GAA CCA GAA ATA GTT GAT
590 TTA GCA AAT TAT CTT AAT AAG ATG GGT GAG AAT ATC GTT GGA
632 GCA GGT ACG GAA ACA ATC CGT ATT ATT GGT GTA GAA AAA CTT
674 CGC GGT ACG GAG CAC ATG ATT ATA CCT GAT CGT ATT GAA GCT
716 GGT ACC TTT ATG GTT GCC TCT GCA ATT ACT GGA GGT AAT GTA
758 TTC ATT GAG AAT GCA ATG CGT GAA CAT TTA CGC TCT GTA ATT
799 TCA AAG TTA GAA GAA ATG AAT GTT GAT GTA ATT GAT GAG AAT
842 GGC GGT CTA CGA ATC ATA GGG CCA GAG AAA TTA AAA TCA ACA
884 GAT ATT AAA ACA TTA CCG CAT CCT FFT TTT CCT ACA GAT ATG
926 CAA TCA CAA ATG ATG TCA TTA ATG CTT CGT GCA GAA GGC ACA
968 GGT GTG ATT ACG GAG ACT GTT TTC GAA AAT CGT TTT ATG CAT
1010 GTA GAA GAA TTT CGA CGC ATG AAT GCG AAT ATC AAA ATT GAA
1052 GGA CGC AGT GTG ATT ATT GAA GGT ATT TCA GAG TTG CAA GGC
1094 GCT GAA GTA GCA GCA ACA GAT CTT CGT GCG GCA GCG GCA CTT
1136 ATT TTA GCA GGT CTT GTA AGC GAT GGA TAC ACA CGT GTG ACG
1178 GAG CTT AAG CAT CTA GAC CGT GGC TAT GTT GAT ATT GTT GAT
1220 AAG TTA GCG GCA CTT GGT GCA GAT ATC AAG AGA GTG GAT GAG
1262 AAC GGA GTA GTT GTA CAA CCA CTC TAC GTA ACT GCT GCA AAA
1304 GAA TCA AAT GAA ATA GCA GCG GAT TAA

Figure 4. Nucleotide sequence of the *murZ* coding sequence from *O. iheyensis*.

1 ATG CAA AAA TTA TTA ATT GAA GGT GGT CAT GAT CTA ACT GGC
43 CAA GTT CGA ATT AGT GGA GCC AAG AAC AGT GCG GTT GCT TTA
86 CTT CCT GCT GCA ATA TTG GCG GAT TCA GCC GTG ACG ATT GAA
128 GGA TTA CCA GAA ATT TCT GAT GTA GAT ACA TTA GGG GAT TTG
170 CTT GAA GAA ATT GGA GGT AGT GTA TCT AGA GAT GGA CAA GAT
212 ATC ACT ATT CAT CCT GAG AAG ATG ATG GCA ATG CCT TTA CCA
254 AAC GGC AAG GTT AAA AAA CTT CGT GCA TCC TAT TAT TTT ATG
295 GGG GCA ATG CTT GGT AAA TTT AAC AAA GCA GTA ATT GGT TTA
338 CCT GGA GGA TGT TTT TTA GGA CCA CGC CCA ATA GAT CAA CAT
380 ATC AAG GGT TTT GAA GCG CTG GGT GCA GAG GTT ACTAAT GAA
422 CAA GGT GCA ATT TAC TTG CGA GCA AAC GAA TTG CGT GGA GCG
463 CGT ATC TAT CTT GAC GTT GTT AGT GTT GTT GCA ACG ATA AAT
506 ATT ATG TTG GCA GCA GTA AAA GCA AAG GGA AGA ACT AAC ATT
548 GAG ATT GCT GCT AAA GAG CCA GAA ATT ATC GAT GTA GCA ACA
590 CTA CTG ACG AAT ATG GGA GCA AAA ATT AAA GGT GTA GGT ACC
632 GAC GTG ATA CGT ATT GAT GGA GTT CCT TCT TTA CAT GGT TGC
674 CGT CAT ACG ATT ATT CCT GAT CGA ATA GAA GCT GGT ACG TAT
716 GCA ATT GCT GCA GCA GCG AAA GGG AAG GAA GTA ATT ATT GAT
758 AAT GTG ATT CCA CAG CAT TTA GAA TCA CTA ATT GCA AAA CTT
799 CGT GAA ATG GAT GTA ACC ATT GAA GAA AGT GAT GAA CAA TTA
842 TAT ATT GCA CGA AAC CGA CCA CTA AAG AGT GTG GAT ATT AAA
884 ACG TTA GTA TAC CCT GGG TTT CCA ACA GAT TTA CAA CAA CCA
926 TTT ACT TCT TTA CTA ACA CAA GAG ACC CAT TCT GGT GTA ATT
968 ACA GAT ACA ATT TAT TCT GCT AGA CTA AAA CAT ATT GAT GAA
1010 TTA CGT AGG ATG AAT GCC GTA ATT AAA GTT GAA GGC GGG TCG
1052 GTG ATC GTT TCC GGT CCT GTT CAA TTA GAA GGT GCA CGT GTA
1094 AAA GCA AGT GAT CTT CGT GCA GGA GCA TCT CTG ATT ATT GCT
1136 GGT CTG TTA GCT GAT GGT ATT ACT GAA ATA ACT GGA CTG GAT
1178 CAT ATA GAT AGA GGA TAT GAA AGA TTA ACA GAG AAA TTA TCT
1220 TCA TTA GGC GCG AAT ATT TGG CGT GAA GAA ATG ACA GAT ATA
1262 GAA ATA ATG CAA GAT CAA AAT ATG TAA

Figure 5. Translated Protein sequence of *E. coli* MurA. Conserved residues of ligand interaction are underlined as identified in *E. coli* (5, 21).

1 MDKFRVQGPT KLQGEVTISG AKNAALPILF AALLAEEPVE
41 IQNVPKLKDV DTSMKLLSQL GAKVERNGSV HIDARDVNVF
81 CAPYDLVKTM RASIWALGPL VARFGQGQVS LPGGCTIGAR
121 PVDLHISGLE QLGATIKLEE GYVKASVDGR LKGAHIVMDK
161 VSVGATVTIM CAATLAEGTT IIENAAREPE IVDTANFLIT
201 LGAKISGQGT DRIVIEGVER LGGGVYRVLP DRIETGTFLV
241 AAAISRGKII CRNAQPDTLD AVLAKLRDAG ADIEVGEDWI
281 SLDMHGKRPK AVNVRTAPHP AFPTDMQAQF TLLNLVAEGT
321 GFITETVFEN RFMHVPELSR MGAHAEIESN TVICHGVEKL
361 SGAQVMATDL RASASLVLAG CIAEGTTVVD RIYHIDRGYE
401 RIEDKLRALG ANIERVKGE

Figure 6. Translated protein sequence of *T. maritima* MurA. Conserved residues of ligand interaction are underlined as identified in *E. coli* (5, 21).

1 MAAAILCDEE VILKNVPRLQ DVFVMIDILR SIGFRVEFEE
41 NELKIKREND ISQEVPYELV RKMRASFNVL GPIAVRTGRA
81 KVALPGGCSI GVRPVDFHLE GLKKMGFSIK VEHGFVEACF
121 ERRIDYVTIT LPFPSVGATE HLMTTAALLK GARVVIENAA
161 MEPEIVDLQN FINRMGGHIE GAGTSRIVIE GVEKMQGVEY
201 SIIPDRIEAG TYLVAIAASR GKGLVKNVNP DHLTNFFEKL
241 EETGAKLKVL GNEVEIEMRE RPKAVDVTTN PYPGFPTDLQ
281 PQMMAYLSTA SGVSVITENV FKTRFLHVDE LKRMGADIEV
321 SGNVAIVKGV EKLSGAPVEG TDLRATAALL IAGIIADGVT
361 EISNVEHIFR GYEDVIDKFS ELGAKIEYVE KEN

Figure 7. Translated protein sequence of *O. iheyensis* MurA. Conserved residues of ligand interaction are underlined as identified in *E. coli* (5, 21).

1 MEKIIVSGGH QLNGTVRLEG AKNAVLPVLA ASLIASEGES
41 VIKEVPVLAD VYTINEVLRN LNAEVEFDST TKTVNINASQ
81 QLETEAPFEY VRKMRASVLV LGPLLARYGH AKVAMPGGCA
121 IGSRPIDLHL KGFEAMGAEI HVGNGYVEAN VNGRLQGAKI
161 YLDMPSVGAT ENIMMAAALA EGKTVIENAA KEPEIVDLAN
201 YLNKMGANIV GAGTETIRII GVEKLRGTEH MIIPDRIEAG
241 TFMVASAITG GNVFIENAMR EHLRSVISKL EEMNVDVID
281 NGGLRIIGPE KLKSTDIKTL PHPGFPTDMQ SQMMSLMLRA
321 EGTGVITETV GENRFMHVEE FRRMNANIKI EGRSVIIEGI
361 SELQGAEVAA TDLRAAAALI LAGLVSDGYT RVTELKHLDR
401 GYVDIVDKLA ALGADIKRVD ENGVVVQPLY VTAAKESNEI
441 AAD

Figure 8. Translated protein sequence of *O. iheyensis* MurZ. Conserved residues of ligand interaction are underlined as identified in *E. coli* (5, 21).

1 MQKLLIEGGH DLTGQVRISG AKNSAVALLP AAILADSAVT
41 IEGLPEISDV DTLGDLLEEI GGSVSRDGQD ITIHPEKMMA
81 MPLPNKVKK LRASYYFMGA MLGKFNKAVI GLPGGCFLGP
121 RPIDQHIKGF EALGAEVTNE QGAIYLRANE LRGARIYLDV
161 VSVGATINIM LAAVKAKGRT TIENAAKEPE IIDVATLLTN
201 MGAKIKGVGT DVIRIDGVPS LHGCRHTIIP DRIEAGTYAI
241 AAAAKGKEVI IDNVIPQHLE SLIAKLREMD VTIEESDEQL
281 YIARNRPLKS VDIKTLVYPG FPTDLQQPFT SLLTQATHSG
321 VITDTIYSAR LKHIDELRRM NAVIKVEGGS VIVSGPVQLE
361 GARVKASDLR AGASLIIAGL LADGITEITG LDHIDRGYER
401 LTEKLSSLGA NIWREEMTDI EIMQDQNM

Figure 9. Amino acid comparison of *T. maritima* and *E. coli* MurA. Identical amino acids: 178/391 (45%); Positive amino acids: 239/391 (61%); Groups created for proper alignment: 3/391 (0%).

```
T. mar    AAILCDEEVILKNVPRLQDVFVMIDILRSIGFRVEFEENELKIKRENDISQEVPYELVRK
          AA+L +E V ++NVP+L+DV   + +L  +G +VE     + I   +       PY+LV+
E. coli   AALLAEEPVEIQNVPKLKDVDTSMKLLSQLGAKVE-RNGSVHIDARDVNVFCAPYDLVKT

T. mar    MRASFNVLGPIAVRTGRAKVALPGGCSIGVRPVDFHLEGLKKMGFSIKVEHGFVEACFER
          MRAS   LGP+  R G+ +V+LPGGC+IG RPVD H+ GL+++G +IK+E G+V+A  +
E. coli   MRASIWALGPLVARFGQGQVSLPGGCTIGARPVDLHISGLEQLGATIKLEEGYVKASVDG

T. mar    RIDYVTITLPFPSVGATEHLMTTAALLKGARVVIENAAMEPEIVDLQNFINRMGGHIEGA
          R+    I +   SVGAT  +M  A L +G   +IENAA EPEIVD  NF+  +G  I G
E. coli   RLKGAHIVMDKVSVGATVTIMCAATLAEGT-TIIENAAREPEIVDTANFLITLGAKISGQ

T. mar    GTSRIVIEGVEKMQGVEYSIIPDRIEAGTYLVAIAASRGKGLVKNVNPDHLTNFFEKLEE
          GT RIVIEGVE++ G  Y ++PDRIE GT+LVA A SRGK + +N  PD L     KL +
E. coli   GTDRIVIEGVERLGGGVYRVLPDRIETGTFLVAAAISRGKIICRNTQPDTLDAVLAKLRD

T. mar    TGAKLKVLGNEVEIEMR-ERPKAVDVTTNPYPGFPTDLQPQMMAYLSTASGVSVITENVF
           GA ++V  + + ++M  +RPKAV+V T P+P FPTD+Q Q       A G   ITE VF
E. coli   AGADIEVGEDWISLDMHGKRPKAVNVRTAPHPAFPTDMQAQFTLLNLVAEGTGFITETVF

T. mar    KTRFLHVDELKRMGADIEVSGNVAIVKGVEKLSGAPVEGTDXXXXXXXXXXXXDGVTE
          + RF+HV EL RMGA  E+  N  I  GVEKLSGA V  TD            +G T
E. coli   ENRFMHVPELSRMGAHAEIESNTVICHGVEKLSGAQVMATDLRASASLVLAGCIAEGTTV

T. mar    ISNVEHIFRGYEDVIDKFSELGAKIEYVEKE
          +  + HI RGYE + DK   LGA IE V+ E
E. coli   VDRIYHIDRGYERIEDKLRALGANIERVKGE
```

Figure 10. Amino acid comparison of *O. iheyensis* and *E. coli* MurA. Identical amino acids: 201/421 (47%); Positive amino acids: 274/421 (65%); Groups created for proper alignment: 9/421 (2%).

```
O. ihey   MEKIIVSGGHQLNGTVRLEGAKNAVLPVLAASLIASEGESVIKEVPVLADVYTINEVLRN
          M+K  V G  +L G V + GAKNA LP+L A+L+A E    I+ VP L DV T  ++L
E. coli   MDKFRVQGPTKLQGEVTISGAKNAALPILFAALLAEEPVEIQNVPKLKDVDTSMKLLSQ

O. ihey   LNAEVEFDSTTKTVNINASQQLETEAPFEYVRKMRASVLVLGPLLARYGHAKVAMPGGCA
          L A+VE + +   V+I+A       AP++ V+ MRAS+  LGPL+AR+G  +V++PGGC
E. coli   LGAKVERNGS---VHIDARDVNVFCAPYDLVKTMRASIWALGPLVARFGQGQVSLPGGCT

O. ihey   IGSRPIDLHLKGFEAMGAEIHVGNGYVEANVNGRLQGAKIYLDMPSVGATENIMMAAALA
          IG+RP+DLH+ G E +GA I +  GYV+A+V+GRL+GA I +D  SVGAT  IM AA LA
E. coli   IGARPVDLHISGLEQLGATIKLEEGYVKASVDGRLKGAHIVMDKVSVGATVTIMCAATLA

O. ihey   EGKTVIENAAKEPEIVDLANYLNKMGANIVGAGTETIRIIGVEKLRGTEHMIIPDRIEAG
          EG T+IENAA+EPEIVD AN+L  +GA I G GT+ I I GVE+L G  + ++PDRIE G
E. coli   EGTTIIENAAREPEIVDTANFLITLGAKISGQGTDRIVIEGVERLGGGVYRVLPDRIETG

O. ihey   TFMVASAITGGNVFIENAMREHLRSVISKLEEMNVDV---IDNGGLRIIGPEKLKSTDIK
          TF+VA+AI+ G +   NA  + L +V++KL +   D+    D   L + G ++ K+ +++
E. coli   TFLVAAAISRGKIICRNAQPDTLDAVLAKLRDAGADIEVGEDWISLDMHG-KRPKAVNVR

O. ihey   TLPHPGFPTDMQSQMMSL-MRAEGTGVITETVGENRFMHVEEFRRMNANIKIEGRSVIIE
          T PHP FPTDMQ+Q   L + AEGTG ITETV ENRFMHV E  RM A+ +IE  +VI
E. coli   TAPHPAFPTDMQAQFTLLNLVAEGTGFITETVFENRFMHVPELSRMGAHAEIESNTVICH

O. ihey   GISELQGXXXXXXXXXXXXXXXXXXVSDGYTRVTELKHLDRGYVDIVDKLAALGADIKR
          G+ +L G                    +++G T V  + H+DRGY  I DKL ALGA+I+R
E. coli   GVEKLSGAQVMATDLRASASLVLAGCIAEGTTVVDRIYHIDRGYERIEDKLRALGANIER

O. ihey   V
          V
E. coli   V
```

Figure 11. Amino acid comparison of *O. iheyensis* MurZ and *E. coli* MurA. Identical amino acids: 172/419 (41%); Positive amino acids 247/419(58%); Groups created for proper alignment: 10/419 (2%).

```
O. ihey  MQKLLIEGGHDLTGQVRISGAKNSAVALLPAAILADSAVTIEGLPEISDVDTLGDLLEEI
         M K  ++G  +L G+V ISGAKN+A+ +L AA+LA+  V I+ +P++ DVDT   LL ++
E. coli  MDKFRVQGPTELQGEVTISGAKNAALPILFAALLAEEPVEIQNVPKLKDVDTSMKLLSQL

O. ihey  GGSVSRSGQDITIHPEKMMAMPLP-NKVKKLRASYYFMGAMLGKFNKAVIGLPGGCFLCP
         G  V R+G  + I    +     P + VK +RAS + +G ++ +F +  + LPGGC +
E. coli  GAKVERNGS-VHIDARDVNVFCAPYDLVKTMRASIWALGPLVARFGQGQVSLPGGCTIGA

O. ihey  RPIDQHIKGFEALGAEVTNEQGAIYLRAN---ELRGARIYLDVVSVGATINIMLAAVKAK
         RP+D HI G E LGA +  E+G  Y++A+    L+GA I +D VSVGAT+ IM AA  A+
E. coli  RPVDLHISGLEQLGATIKLEEG--YVKASVDGRLKGAHIVMDKVSVGATVTIMCAATLAE

O. ihey  GRTTIENAAKEPEIIDVATLLTNMGAKIKGVGTDVIRIDGVPSLHGCRHTIPPDRXXXXX
         G T IENAA+EPEI+D A  L  +GAKI G GTD I I+GV  L G  + + PDR
E. coli  GTTIIENAAREPEIVDTANFLITLGAKISGQGTDRIVIEGVERLGGGVYRVLPDRIETGT

O. ihey  XXXXXXXXXXXVIIDNVIPQHLESLIAKLREMDVTIEESDE--QLYIARNRPLKSVDIKT
                    +I  N  P  L++++AKLR+    IE  ++   L +   RP K+V+++T
E. coli  FLVAAAISRGKIICRNAQPDTLDAVLAKLRDAGADIEVGEDWISLDMHGKRP-KAVNVRT

O. ihey  LVYPGFPTDLQQPFTSLLTQATHSGVITDTIYSARLKHIDELRRMNAVIKVEGGSVIVSG
           +P FPTD+Q  FT L   A  +G IT+T++  R  H+ EL RM A  ++E  +VI  G
E. coli  APHPAFPTDMQAQFTLLNLVAEGTGFITETVFENRFMHVPELSRMGAHAEIESNTVICHG

O. ihey  PVQLEGARVKASDLRXXXXXXXXXXXXXXXXTEITGLDHIDRGYERLTEKLSSLGANIWR
           +L GA+V A+DLR                T +  + HIDRGYER+ +KL +LGANI R
E. coli  VEKLSGAQVMATDLRASASLVLAGCIAEGTTVVDRIYHIDRGYERIEDKLRALGANIER
```

Figure 12. Amino acid comparison of *O. iheyensis* MurA and *O. iheyensis* MurZ. Identical amino acids: 187/426 (43%); Positive amino acids: 272/426 (62%); Groups created for proper alignment: 4/426 (0%).

```
MurA    MEKIIVSGGHQLNGTVRLEGAKNAVLPVLAASLIASEGESVIKEVPVLADVYTINEVLRN
        M+K+++ GGH L G VR+ GAKN+ + +L A+++A    + I+ +P ++DV T+ ++L
MurZ    MQKLLIEGGHDLTGQVRISGAKNSAVALLPAAILADSAVT-IEGLPEISDVDTLGDLLEE

MurA    LNAEVEFDSTTKTVNINASQQLETEAPFEYVRKMRASVLVLGPLLARYGHAKVAMPGGCA
            V  D   + + I+  + +    P   V+K+RAS   +G +L ++  A + +PGGC
MurZ    IGGSVSRDG-QDITIHPEKMMAMPLPNGKVKKLRASYYFMGAMLGKFNKAVIGLPGGCF

MurA    IGSRPIDLHLKGFEAMGAEIHVGNGYVEANVNGRLQGAKIYLDMPSVGATENIMMAAALA
        +G RPID H+KGFEA+GAE+    G +    N  L+GA+IYLD+ SVGAT NIM+AA  A
MurZ    LGPRPIDQHIKGFEALGAEVTNEQGAIYLRAN-ELRGARIYLDVVSVGATINIMLAAVKA

MurA    EGKTVIENAAKEPEIVDLANYLNKMGANIVGAGTETIRIIGVEKLRGTEHMIIPDRIEAG
        +G+T IENAAKEPEI+D+A  L  MGA I G GT+ IRI GV  L G  H IIPDRIEAG
MurZ    KGRTTIENAAKEPEIIDVATLLTNMGAKIKGVGTDVIRIDGVPSLHGCRHTIIPDRIEAG

MurA    TFMVASAITGGNVFIENAMREHLRSVISKLEEMNVDVIDENGGLRIIGPEKLKSTDIKTL
        T+ +A+A  G  V I+N + +HL S+I+KL EM+V + + +  L I     LKS DIKTL
MurZ    TYAIAAAAKGKEVIIDNVIPQHLESLIAKLREMDVTIEESDEQLYIARNRPLKSVDIKTL

MurA    PHPGFPTDMQSQMMSLMLRAEGTGVITETVFENRFMHVEEFRRMNANIKIEGRSVIIEGI
         +PGFPTD+Q    SL+ +A  +GVIT+T++  R  H++E RRMNA IK+EG SVI+ G
MurZ    VYPGFPTDLQQPFTSLLTQATHSGVITDTIYSARLKHIDELRRMNAVIKVEGGSVIVSGP

MurA    SELQGXXXXXXXXXXXXXXXXXXVSDGYTRVTELKHLDRGYVDIVDKLAALGADIKRVD
         +L+G                    ++DG T +T L H+DRGY  + +KL++LGA+I R +
MurZ    VQLEGARVKASDLRAGASLIIAGLLADGITEITGLDHIDRGYERLTEKLSSLGANIWREE

MurA    ENGVVV
           + +
MurZ    MTDIEI
```

Figure 13. Fosfomycin resistance of *E. coli murA*. Data points are the result of compiled data from three different experimental trials for each cell type and temperature.

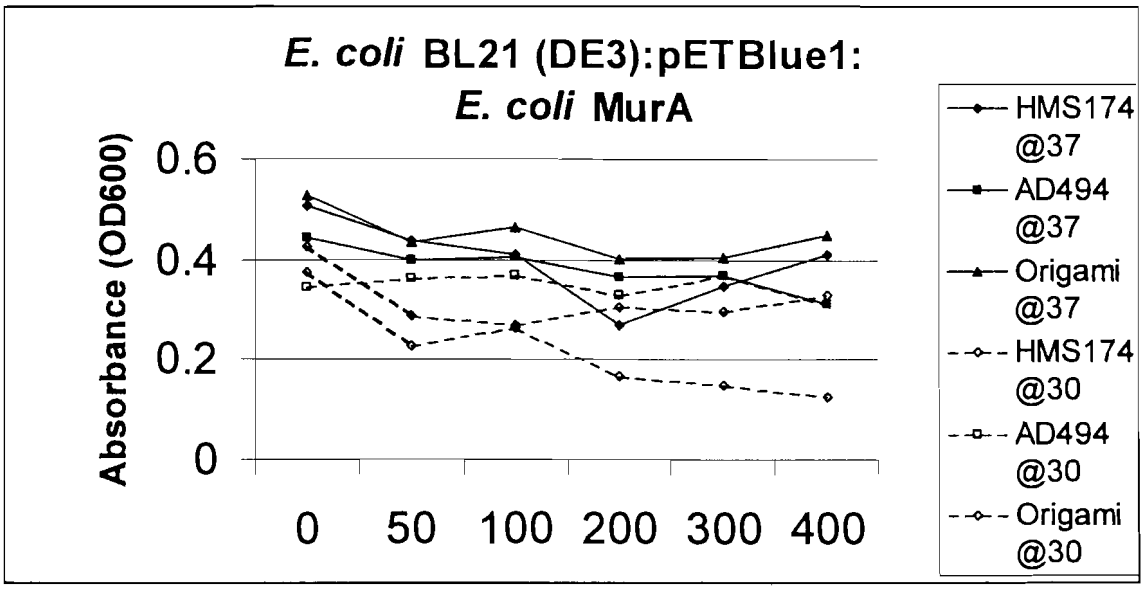

E. coli BL21 (DE3):pETBlue1:
E. coli MurA
Absorbance (OD600)
0.6
0.4
0.2
0
0
50
100
200
300
400
HMS174 @37
AD494 @37
Origami @37
HMS174 @30
AD494 @30
Origami @30

Figure 14. Fosfomycin resistance in *T. maritima murA*. Data points are the result of compiled data from three different experimental trials with all cell types and temperatures.

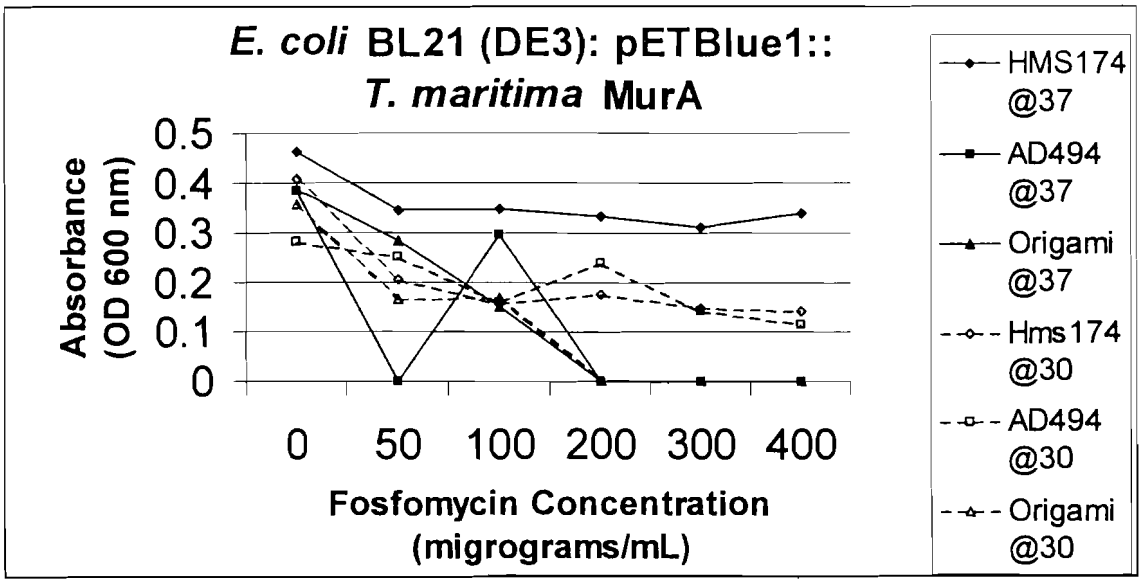

E. coli BL21 (DE3): pETBlue1::
T. maritima MurA
Absorbance (OD 600 nm)
0.5
0.4
0.3
0.2
0.1
0
0
50
100
200
300
400
Fosfomycin Concentration (migrograms/mL)
HMS174 @37
AD494 @37
Origami @37
Hms174 @30
AD494 @30
Origami @30

Figure 15. Fosfomycin resistance in *O. iheyensis murA*. Data points are the result of compiled data from three different experimental trials with all cell types and temperatures.

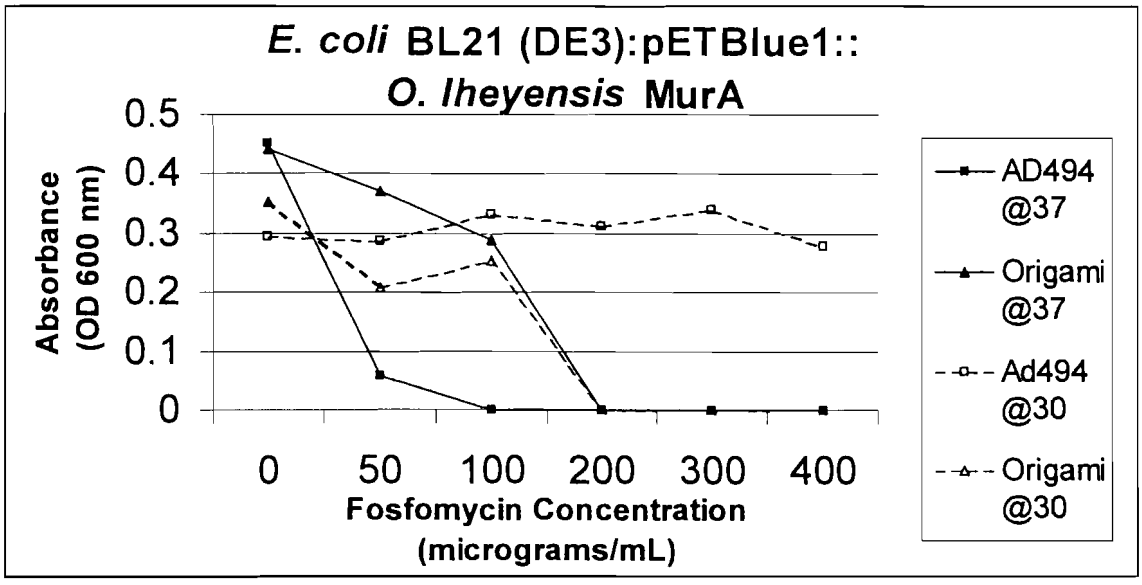
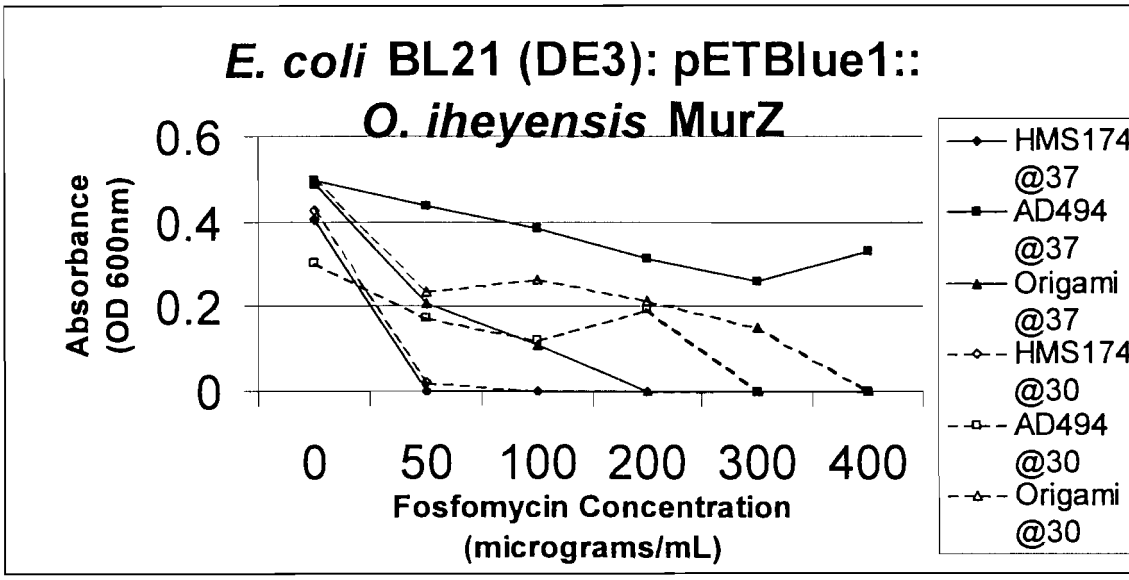

E. coli BL21 (DE3):pETBlue1::
O. Iheyensis MurA
Absorbance
(OD 600 nm)
0.5
0.4
0.3
0.2
0.1
0
0
50
100
200
300
400
Fosfomycin Concentration
(micrograms/mL)
AD494
@37
Origami
@37
Ad494
@30
Origami
@30

Figure 16. Fosfomycin resistance in *O. iheyensis murZ*. Data points are the result of compiled data from three different experimental trials of all cell types and temperatures.


E. coli BL21 (DE3): pETBlue1::
O. iheyensis MurZ
Absorbance
(OD 600nm)
0.6
0.4
0.2
0
0
50
100
200
300
400
Fosfomycin Concentration
(micrograms/mL)
HMS174 @37
AD494 @37
Origami @37
HMS174 @30
AD494 @30
Origami @30

Discussion

Antibiotic resistance is a growing concern among today's medical professionals. While multiple causes have led to this problem, little has been accomplished in preventing or reversing this growing trend. The longer the scientific community waits to address this increasing problem, the greater the impact will be on public health. The result will be increased mortality and morbidity rates as well as a steadily increasing cost of health care (8, 9, 20). Without basic research into this emerging threat, the human race will begin to succumb to the most primitive of diseases.

Research is being done to help alleviate the problem of bacterial antibiotic resistance by focusing on the organism itself. Much of the current research focus is on identifying essential systems in the bacterial organism whose inhibition is lethal to the cell. In this study, I have focused specifically on the enzyme UDP-N-acetylglucosamine enolpyruvyltransferase (MurA) due to its essentiality in the bacterial cell. This enzyme, which catalyzes the first committed step in bacterial cell wall biosynthesis, is conserved in all bacterial species with a cell wall, but is absent in humans. We also chose to investigate MurA from multiple organisms to see if there was a difference in the protein as a function of the habitat of the organism.

Oceanobacillus iheyensis THE381 is a Gram-positive, strictly aerobic, rod-shaped, spore forming, extremely halotolerant, alkaliphilic bacterium. It was first isolated from deep-sea sediment on the Iheya Ridge at a depth of 1050 m (10). Since this organism was only recently isolated, there have been no in depth investigations with regard to particular enzymes from this organism. MurA (and MurZ) from *O. iheyensis* provides an enzyme for investigation that has adapted to extreme pressure and cold.

Thermatoga maritima is a Gram-negative bacterium found in the vents of volcanoes. It is very tolerant to high sulfur levels, but can grow at a pH of 9.5. *T. maritima* has been categorized as an evolutionary ancient hyperthermophilic bacterium (13). MurA from this organism allows insights into enzyme action at high temperatures. *Escherichia coli* is a Gram-negative facultative anaerobe. It was chosen in part due to the large amount of previous research already performed on it, in addition to inhabiting more physiological conditions. MurA has been well characterized from *E. coli* and it provides an excellent system for comparison to the enzyme from *T. maritima* and *O. iheyensis*.

MurA (and MurZ from *O. iheyensis*) from each organism were analyzed at the protein level. The Mur family of proteins are highly conserved among all bacterial species with a cell wall (21, 5, 26), and data acquired in this study support this finding. Conserved regions previously identified (21, 5) were present in all enzymes; however, slight amino acid variation existed. The most important amino acid, a cystine residue present at amino acid 115 in *E. coli* was present in MurZ and all MurA examined. This residue has been shown to be essential to catalytic activity in multiple studies. Schonbrunn et al. (2000) showed that site-directed mutagenesis of this cystine to serine inactivates the enzyme. Skarzynski et al. (1998) also showed inactivation if cystine was replaced with an alanine. *Mycobacterium tuberculosis,* which is naturally resistant to fosfomycin, has aspartate in position 115; when this aspartate is replaced with a cystine, the organism becomes sensitive to fosfomycin (4). Less data are available on the remaining conserved areas of ligand interaction, as they are only predicted from computer generated models. They appear to be conserved in the proteins examined in this study although slight amino acid variation exists. Comparisons of this nature are

important in determining the likelihood of being able to use a single antibiotic to inhibit MurA from a wide spectrum of bacteria or if a unique antibiotic would be required for each different organism.

A whole cell fosfomycin assay was used to determine if *murA* or *murZ* from each of the different organisms was overproduced as soluble, active protein in *E. coli*. The mechanism of action of fosfomycin resides in its ability to inhibit MurA (25, 15). Recombinant *E. coli* grown in the presence of fosfomycin that overexpress *murA* or (*murZ)* as insoluble protein will be sensitive to fosfomycin. However, if the recombinant cells are overproducing soluble MurA (or MurZ), they will exhibit increased resistance to fosfomycin. Since soluble protein overexpression is dependent upon the growth conditions of the host cell, we examined induction temperature as a possible variable to the production of maximal levels of soluble MurA and MurZ. Temperature was examined due to the findings of a previous study which determined a reduced temperature of gene induction for *Streptococcus pneumoniae murA* (18° C at 24 h) and *murZ* (30° C at 3.5 h) in *E. coli* resulted in the ability to obtain soluble protein (5). Our results indicated *murA* from *E. coli* was overexpressed at maximal levels in recombinant *E. coli* Origami cells grown at 37° C. All cell types, with the exception of HMS174 grown at 37° C, showed overexpression of *murA* at both temperatures tested. *T. maritima murA* was best overexpressed in recombinant *E. coli* AD494 cells grown at 30° C. Overexpression of *T. maritima murA* was also accomplished in HMS174 cells, but at lower levels. *O. iheyensis murA* was overexpressed in *E. coli* AD494 cells grown at 30° C. No soluble overexpression was achieved at 37° C. For *O. iheyensis murZ,* soluble expression was maximal in recombinant *E. coli* AD494 cells grown at 37° C. These

experiments demonstrate that successful overexpression of foreign genes in an *E. coli* background is dependent upon not only the unique characteristics of the protein itself but the host strain as well. Even though proteins may be similar in composition and structures, all conditions for overexpression must be determined empirically.

Although we were able to show overexpression of *murA* or *murZ*, we were unable to express the proteins at a level that could be identified by SDS-PAGE. Zoeiby et al. (2001) engineered recombinant MurA to contain a six amino acid histidine tag. Once purified by Nickel chromatography, proteins were sufficiently concentrated in order to allow identification by SDS-PAGE. This approach would be a viable alternative to the overexpression attempted in this study to obtain large amounts of protein for mechanism of action studies.

In conclusion, *murA* and *murZ* were successfully cloned from organisms residing in extreme environments. Conserved proteins, such as MurA and MurZ, demonstrate that although there may be little similarity between organisms, individual enzymes can perform similar functions. It has also served as a building block for further studies to maximize overexpressed protein yields for subsequent purification. Although overexpression was accomplished, it was not to a level sufficient for purification. Other parameters, such as media composition, concentration of IPTG, etc. will need to be examined.

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