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Antibiotic resistance has become an important issue in the treatment of microbial diseases in recent years. One approach by the scientific community is the development of novel inhibitors, which target essential bacterial enzymes needed for cell survival. This project focused on methionine aminopeptidase (MetAP), an essential enzyme due to its function of cleaving the first methionine amino acid in premature proteins. In this study, we have cloned and overexpressed MetAP from *Bacillus anthracis*, *Thermotoga maritima*, *Haemophilus influenzae*, *Staphylococcus aureus*, and *Oceanobacillus iheyensis* as a MetAP-histidine fusion protein. After induction of the gene, MetAP from *T. maritima*, *H. influenzae*, and *B. anthracis* were successfully purified via nickel affinity chromatography. Enzymatic activity of the proteins, however, could not be reproducibly detected.

Cloning, Overexpression, and Purification of Bacterial Methionine Aminopeptidase

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PREFACE

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Introduction

Bacterial infections used to threaten people's lives before the discovery of antibiotics. The average life expectancy during Victorian times was 45 years, and infant mortality (birth to age 5) was 150 per 1000 live births (24). In July 1928, however, the renowned Scottish bacteriologist Alexander Fleming, discovered penicillin accidentally. One of the Petri dishes, which Fleming inoculated with bacteria before leaving on summer vacation, caught his attention when he made an unscheduled return to his laboratory (33). Little did he know the microbial inhibition he observed was due to a substance that would revolutionalize the way infectious diseases were treated. Twelve years later, the first true antibiotic in the world was born when Howard Florey, Ernst Chain, and a team of scientists at Oxford University obtained purified penicillin (33). In fact, it was thought that from this time onward, the end of infectious diseases had begun. Ironically, in 1967 William H. Stewart, the surgeon general of the United States, said it was "time to close the book on infectious diseases and shift all national efforts to chronic diseases (34)."

In today's world, however, people have become aware that antibiotics are no longer a "cure-all" as the number of antibiotic-resistant organisms has increased (24). Although new antibiotics are constantly discovered and marketed for human use, multiple antibiotic resistance is increasingly common following the application of any new antibiotic. In fact, mortality rates from infectious diseases are as high today as before antibiotics arrived in many parts of the world (31).

Researchers constantly seek new antibacterial agents because of rapidly developing antibiotic resistance in both Gram-positive and Gram-negative bacteria. The increasing need for new antibiotics to overcome rapidly developing resistance mechanisms observed in pathogenic bacteria has made researchers investigate alternate approaches to conquer infectious diseases. Thus, the search for new antibiotics with novel mechanisms of action is essential in the war against drug resistant microbial pathogens. Alternatively, searching for new antibacterial agents active against enzyme targets essential for cell survival is one approach being actively pursued by many pharmaceutical companies. One such enzyme that has been the focus of attention by the scientific community in recent years is methionine aminopeptidase (2, 19).

In nascent proteins, protein synthesis begins with methionine in eukaryotes or N-formylmethionine in prokaryotes (31). However, the N-terminal methionine residue needs to be removed for the maturation and proper function of most proteins (32). Methionine aminopeptidase (MetAP) is the enzyme which serves to remove the N-terminal methionine from mature proteins, and the lethality of MetAPs' absence in *Saccharomyces cerevisiae*, *Escherichia coli*, and *Salmonella typhimurium* indicates its physiological importance (16). Methionine aminopeptidase is therefore a valid target for the design of an antibacterial drug. The gene for MetAP is designated as *map*.

Methionine aminopeptidase is differentiated into two major types, designated type I and type II, based on amino acid sequence alignments (16). In type II

enzymes, a helical inserted subdomain, which is approximately 60 residues in length that shares no sequence or structural homology with any other known protein, distinguishes type II from type I enzymes (16). Both type I and type II MetAPs are found in eukaryotic cells, while only either type I or type II is found in bacteria (32, 16). Deletion of either type I or type II MetAP from bacteria is lethal.

Divalent metals determine the activity of the MetAP enzymes, which is indicated by the loss of enzymatic activity upon treatment with EDTA (1, 16). Co(II) has been studied and confirmed to reproducibly activate MetAP *in vitro* (16). However, questions remain as to if this metal activates *in vivo* since there are other possible activating divalent metals.

MetAP type I and type II from bacteria are different from MetAP type I and type II in humans based on the metal used for activation. Thus, enough variation exists between prokaryotic and eukaryotic type I and II MetAP's to make them potential targets for the development of broad-spectrum antibacterial compounds. The development of inhibitors with specificity toward the physiologically relevant metalloform of MetAP is the underlying goal of researchers trying to develop new inhibitors toward this enzyme. However, since Co(II) is a very potent *in vitro* activator of MetAP, most studies focus on looking for possible drug candidates to inhibit this metalloform first.

To better understand MetAP and to determine if different metalloforms predominate in MetAP's from different organisms, the objective of this study was to clone, overexpress, and purify recombinant MetAP from *Staphylococcus aureus,*

Bacillus anthracis, Oceanobacillus iheyensis, Thermotoga maritima, Haemophilus influenzae, and *Escherichia coli*. Once purified enzyme is obtained, investigation into the enzymatic properties of each MetAP can be initiated.

Methods and Materials

Bacterial Strains, Culture Conditions, and DNA Used In this Study

Bacterial strains used in this study are listed in Table 1. Recombinant plasmids generated in this study are listed in Table 2. Organisms were typically propagated in Luria-Bertani (LB) media unless otherwise noted. Agar plates were prepared by adding agar (20 g/L) to liquid media. Antibiotics routinely used were ampicillin (100 μg/ml), kanamycin (30 μg/ml), and chloramphenicol (34 μg/ml).

Isolation of Chromosomal DNA

Chromosomal DNA was purchased or obtained from collaborators for *Bacillus anthracis, Oceanobacillus iheyensis,* and *Thermotoga maritima.* Chromosomal DNA from *Staphylococcus aureus, Haemophilus influenzae,* and *Escherichia coli O157:H7* was isolated using a DNeasy Tissue Kit (Qiagen; Valencia, CA) according to the manufacturer's recommendations. Since *H. influenzae* would not grow in LB broth, organisms were obtained by scraping the surface of a chocolate agar plate.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) was performed using gene specific primers as indicated in Table 3. Briefly, reaction mixes consisted of 50-100 ng of chromosomal DNA, 100 pMol of each primer, 1.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates, and 1 unit of Taq polymerase in a final volume of

Table 1. Bacterial strains used in this study.

Bacterial Strain	Description	Source
Staphylococcus aureus	Clinical Isolate	ESU Culture Collection
Bacillus anthracis[b]	Environmental Isolate	Kansas State University
Oceanobacillus iheyensis[b]	Environmental Isolate	Reference 31
Thermotoga maritima[b]	Environmental Isolate	ATCC[a]
Hamophilus influenzae	Clinical Isolate	ATCC
Escherichia coli O157:H7	Clinical Isolate	ESU Culture Collection
E. coli NovaBlue	Cloning Strain	Novagen
E. coli BL21(DE3)	Expression Strain	Novagen

[a]ATCC --- American type culture collection.

[b]DNA was obtained from the source indicated.

Table 2. Recombinant plasmids generated in this study.

Description
pET30EK/LIC :: *Staphylococcus aureus* MetAP
pET30EK/LIC :: *Bacillus anthracis* MetAP
pET30EK/LIC :: *Oceanobacillus iheyensis* MetAP
pET30EK/LIC :: *Thermotoga maritima* MetAP
pET30EK/LIC :: *Haemophilus influenzae* MetAP
pET30EK/LIC :: *Escherichia coli O157:H7* MetAP

Table 3. Nucleotide sequence (5' to 3') of primers used to amplify MetAP from various organisms.

Organism		
Bacillus anthracis	F	ATGATTATTCGTAATGAACAAGATTTA
	R	TTATATTTCTGTTAAAATAATCGG
Thermotoga maritima	F	ATGATAAGAATAAAGACACCCTCT
	R	TCATCCCTCCTTGGTCAATATCTC
Oceanobacillus iheyensis	F	ATGATTGCAAAGACAGAAGCGGAT
	R	TTACAATGTCGTAATAATTGGGCC
Haemophilus influenzae	F	ATGGCTATTCCAATTAGAACTGAA
	R	TTACACATTTACCATAATCCGCGA
Escherichia coli O157 : H7	F	ATGGCTATCTCAATCAAGACCCCA
	R	TTATTCGTCGTGCGAGATTATCGC
Staphylococcus aureus	F	ATGATTGTAAAAACAGAAGAAGAATT
	R	CTATTCTTCTTCAATTTTTGTCGT
EK/LIC specific sequences[a]	F	GACGACGACAAGAT
	R	GAGGAGAAGCCCGGT

[a]EK/LIC specific sequences were added to each primer used to amplify MetAP in order to facilitate cloning into pET30EK/LIC.

F: Forward; R: Reverse.

100µl. In general, thirty cycles were performed consisting of a 94°C denaturation step for 1 min, a 52°C annealing step for 1 min, and finally a 45-sec primer extension step at 72°C. The annealing step temperature was varied depending on the chromosomal DNA used. For *H. influenzae,* the annealing temperature was 52°C. Amplification proceeded using a Minicycler model PT 150 thermocycler (MJ Research; Watertown, Mass).

Agarose Gel Electrophoresis

A 0.7% agarose gel was prepared by dissolving 0.21g 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 approximately 1 min to dissolve the agarose. After solidification, gels were placed into a Minicell EC370 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 Kodak DC290 Zoom Digital Camera system (Kodak; Rochester, NY).

Purification of PCR Products

Amplified DNA was purified from PCR mixes using a Montage^{TM} PCR

Centrifugal Filter Devices Spin Column (Millipore; Bedford, MA) according to the manufacturer's recommendations. Alternatively, a gel extraction procedure was performed to purify the amplicon when more than one amplification product was present. In such cases, DNA was extracted from agarose gels using a Qiaex Gel Extraction Kit (Qiagen) according to the manufacturer's suggested protocol.

T4 DNA Polymerase Treatment DNA

Insert DNA was treated with T4 DNA polymerase to generate a vector with compatible overhangs according to the manufacturer's instructions. Briefly, reaction mixes consisted of approximately 0.2 pmol purified PCR product, 2.5 mM dATP, 5 mM DTT, and 0.05 U of T4 DNA polymerase in a T4 DNA polymerase buffer (Novagen). Reactions were initiated upon addition of the enzyme followed by incubation at 22°C for 30 min. Termination was accomplished by inactivating the enzyme with a 75°C incubation for 20 min.

Annealing Reactions

T4 DNA polymerase treated inserts were annealed into a pET30EK/LIC (Novagen). Reactions consisted of 50 ng of pET30EK/LIC and 0.01 pmol of T4 DNA polymerase treated insert DNA. After incubation at 22°C for 5 min, 1 µl of 25 mM EDTA was added followed by incubation at 22°C for 5 min to terminate the reaction.

Transformation

pET30EK/LIC – T4 DNA Polymerase treated inserts were transformed into *E. coli* NovaBlue competent cells using a modification of a standard condition (29). Briefly, 1 µl of the annealing reaction was added to 50 µl of competent *E. coli* NovaBlue cells. Transformation mixtures were incubated on ice for 5 min, heat shocked for 30 sec at 42°C, and placed back on ice for 2 min. 250 µl of room temperature SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM $MgSO_4$, and 20 mM glucose) was added to the transformed cells. The final mixture was incubated at 37°C while shaking at 250 rpm for 60 min. 20 µl of the suspension was combined with 100 µl of LB and spread plated on LB agar plates containing kanamycin (30 mg/ml). Plates were incubated for approximately 16-20 h at 37°C.

Colony Lysis PCR

Colony lysis PCR was performed on transformants to determine the presence of an insert DNA. 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. Amplification products were subsequently visualized by agarose gel electrophoresis.

Plasmid DNA Isolation

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

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/280nm ratio reading of approximately 1.8), it was sequenced either with the assistance of the DNA Sequencing Laboratory at the University of Arkansas for Medical Sciences, Little Rock, Arkansas, or in the Department of Biological Sciences, Emporia State University using a LI-COR 4300 DNA Analyzer. DNA sequences were compared to known nucleotide sequences using the Basic Local Alignment Search Tool (BLAST) on the NCBI web site (http://www.ncbi.nlm.nih.gov/BLAST/) to confirm the identity of the insert.

Overexpression of the MetAP gene and Preparation of Soluble Extracts

Recombinant plasmids containing *map* were transformed into the expression strain *E. coli* BL21 (DE3). Strains 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-ß-D-galactoside (IPTG) was added to a final concentration of 1 mM for induction purposes and incubation continued for 2-3 h at 37°C. Soluble protein was harvested using the Bugbuster reagent (Novagen; Madison, WI). Briefly, cell pellets from the overexpressed culture were incubated with the Bugbuster reagent for 20 min on a rocker platform at room temperature. After centrifugation in a microcentrifuge at high speed for 10 min, the resulting supernatant contained the soluble extract.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Pre-cast gels (15%) obtained from Cambrex Bio Science Rockland, inc. (Cambrex; Rockland, ME) were electrophoresed using a Fisher Biotech Protein Electrophoresis System FE-VE 16-1 (Fisher, St. Louis, MO) powered by 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).

Western Blot Analysis

Western Blot analysis was performed on electrophoresed protein gels according to standard conditions (21). Briefly, a "gel sandwich" was assembled on a Bio-Rad Mini Trans-Blot Electrophoresis Transfer Cell, which consisted of filter paper, a nitrocellulose membrane, and a polyacrylamide gel. The transferring of electrophoresed proteins from the polyacrylamide gel to the nitrocellulose membrane was accomplished in an hour under standard conditions (29).

Following transfer, the membrane was blocked using BSA in TBS buffer consisting of 150 mM NaCl and 10 mM Tris-HCl (pH 7.5). After washing twice in TBS, the membrane was washed in TBSTT buffer (500 mM NaCl, 20 mM Tris-HCl, 0.2% v/v Triton X-100, and 0.05% v/v Tween -20, pH 7.5) two times. After equilibration in TBS, the primary antibody (Novagen), which recognized histidine-tagged proteins, was incubated with the membrane for one h. Subsequently, the membrane was incubated with the sec antibody, a goat anti-Mouse IgG-alkaline phosphatase conjugate. After several washing in TBSTT buffer, the membrane was developed using a developing solution consisting of p-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in a buffer supplied by the manufacturer.

Nickel Affinity Chromatography

Nickel affinity chromatography was employed to purify recombinant proteins containing a histidine tag. Briefly, soluble extracts obtained using the BugBuster

reagent were applied to an equilibrated nickel column. The column was equilibrated using a binding buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9) followed by application of the soluble extract. Proteins binding nonspecifically to the column were removed using a wash buffer consisting of 0.5 M NaCl, 60 mM imidazole and 20 mM Tris-HCl. Elution of bound proteins was accomplished using a buffer consisting of 1 M imidazole, 0.5 M NaCl and 20 mM Tris-HCl. Purification was monitored via SDS-PAGE.

Dialysis

Dialysis was performed to replace the buffer contained in the MetAP preparations obtained post-nickel chromatography. Briefly, samples were placed in 6,000-8,000 molecular weight cut off dialysis tubing (Fisher) and sealed. The dialysis bad with the sample was placed in a flask containing 1 L of 50 mM MOPS (pH 7.0). After continuous stirring overnight at 4°C, samples were stored at -20°C.

MetAP Assay

Activity determination of MetAP was performed in Dr. Qizhuang Ye's laboratory at the University of Kansas according to established methods, or in the Department of Biological Sciences, Emporia State University. A buffer consisting of 50 mM MOPS (pH 7.0), 400 μM Met-AMC (7-amino-4-methylcoumarin; Bacham Bioscience; King of Prussia, PA), and various concentrations (10^{-1}-10^{5} μM) of metals ($CoCl_2$, $MnCl_2$, $ZnCl_2$, $NiCl_2$, $CdCl_2$, $CaCl_2$, and $CuCl_2$) was incubated with

1.0 μM MetAP in a final volume of 100 μl contained in a 96-well clear polystyrene plate. Plates were incubated 30 min at room temperature while they were continuously monitored in plate reader at λex= 360 nm and λem= 460 nm for development of fluorescence.

Results

Cloning of *map*

Using gene specific primers (Table 3), *map* was amplified from chromosomal DNA templates prepared from each organism via PCR. After amplification, each reaction was examined by agarose gel electrophoresis for the presence of an approximate 750 bp band (Figure 1). Each amplicon was subsequently cloned into pET30EK/LIC and transformed into *E. coli* NovaBlue.

Nucleotide Sequence Analysis

Recombinant plasmids were purified and the nucleotide sequence of each cloned gene determined by DNA sequencing (Figures 2-7). BLAST analysis confirmed the integrity of each cloned gene. Furthermore, each nucleotide sequence was aligned with the original *map* sequence from the source organism to determine if errors were introduced. Each cloned *map* was 100% identical at the nucleotide level (data not shown). Using the same analysis tool, the nucleotide sequence for each gene was translated into its corresponding amino acid sequence and aligned with *E. coli* MetAP (Figures 8-12). As shown in Table 4, the protein similarity varied from 34-42% as compared to *E. coli* MetAP. Since MetAP from *E. coli* has been extensively studied and the Ye laboratory at the University of Kansas could supply us with *E. coli* MetAP, we made the decision to not overexpress MetAP from this organism.

Overexpression of MetAP

Each recombinant plasmid was transformed into *E. coli* BL21 (DE3) to facilitate overexpression. Furthermore, each recombinant plasmid was induced at both room temperature and 37°C to determine if temperature affected protein solubility. As shown in Figure 13, protein solubility was maximal at 37°C for all of the organisms except *O. iheyensis* MetAP. For these organisms, induction at 30°C was better than at 37°C. Western blot analysis using anti-histidine antibodies confirmed the overexpressed protein was MetAP (Figure 14). Since MetAPs from *B. anthracis*, *T. maritima*, and *H. influenzae* were overexpressed at the highest levels in *E. coli*, these three proteins were chosen for further study.

Purification of MetAP

After *map* induction from *E. coli* cultures containing either *B. anthracis*, *T. maritima*, or *H. influenzae* genes, soluble extracts were added to a nickel affinity column. Recombinant histidine fusion proteins were eluted from the Ni column and subjected to dialysis. A SDS-PAGE gel showing pre- and post-Ni column chromatography and dialysis is shown in Figure 15.

Enzymatic Activity of *E. coli* MetAP

E. coli MetAP obtained from the Ye laboratory at the University of Kansas was used to demonstrate the activity of MetAP could be effectively assayed at ESU. As seen in Figure 16, *E. coli* MetAP was activated to different levels by the metals Co,

Mn, and Ni. No activation was accomplished using Ca, Cd, Cu, and Zn. Using the same protocol to assay our recombinant MetAPs, we could not demonstrate activity. However, some enzymatic activity from *T. maritima* MetAP was observed in the Ye laboratory, but we could not repeat their results at ESU.

Figure 1. Agarose gel electrophoresis of the 750 bp amplified *map* from *S. aureus*, *B. anthracis*, *O. iheyensis*, *T. maritima*, *H. influenzae*, *E. coli* O157:H7. Reaction conditions are described in the Methods and Materials. Lane 1, molecular weight markers; Lane 2, *S. aureus map*; Lane 3, *B. anthracis map*; Lane 4, *O. iheyensis map*; Lane 5, *T. maritima map*; Lane 6, *H. influenzae map*; Lane 7, *E. coli* O157:H7 *map*.

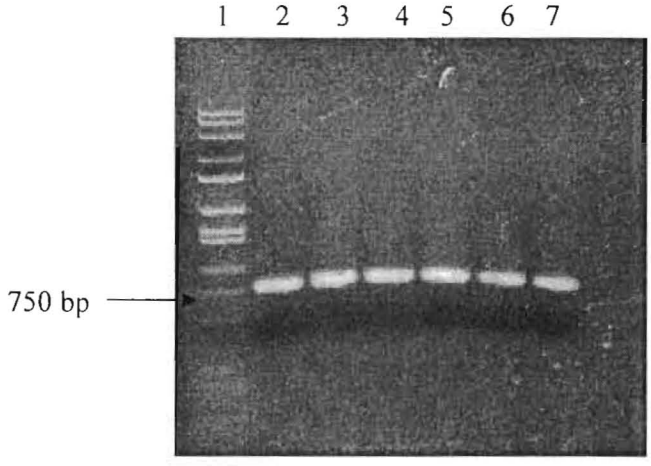

1 2 3 4 5 6 7
750 bp

Figure 2. Nucleotide sequence of the MetAP coding sequence from *B. anthracis* (747 bp).

ATGATTATTCGTAATGAACAAGATTTAGAAGGCTTACGAAAAATCGGCCGC
ATCGTTGCGCTTGCACGTGAAGAAATGAAAAAAGAAGCGAAGCCAGGTA
TGACAACGAAAGAGCTTGATTTGATCGGTAAAAAAGTATTAGATGAGCAT
GGTGCAATTTCTGCACCTGAAAAAGAATATGATTTCCCTGGTGTAACTTGC
ATCAGTGTAAACGAAGAAGTTGCTCACGGTATTCCAGGTGATCGCGTATTA
AAAGAAGGCGACCTTGTAAATGTCGACGTATCTGCAGCACTTGATGGTTAT
TATGCAGATACAGGTATTTCATTTGTACTTGGAGAAGATGAAGCAAAAGAA
AAGCTTTGCCAAGCAGCTGTTGATGCCTTTTGGGCAGCAATGAAAAAAGT
GAAAGCTGGTTCAAAACAAAACCAAATTGGTCGTGCTGTTTCAAACTTTG
CACATAAAAATGGATACAATGTTATTCAAAATTTAACTGGTCACGGTATTG
GTCTTAGCTTACATGAAGCACCAAACCATATCTTAAGTTACTTTGACCCAA
TGGATAATGCGCTTCTAAAAGACGGTCTTGTTATCGCTGTAGAACCATTTAT
TTCTATGAAAGCTGATCACATTATCGAACGTGGCGATGATGGTTGGACATT
CGTTACACCTGATAAAAGTCTTGTTGCACAATGTGAACATACAGTTGTCGT
AACTCGCGGCGAGCCGATTATTTTAACAGAAATATAA

Figure 3. Nucleotide sequence of the MetAP coding sequence from *E. coli* O157:H7 (795 bp).

ATGGCTATCTCAATCAAGACCCCAGAAGATATCGAAAAAATGCGCGTCGC
TGGCCGACTGGCTGCCGAAGTGCTGGAGATGATCGAACCATATGTTAAAC
CGGGCGTCAGCACCGGCGAGCTGGATCGCATCTGTAATGATTACATTGTTA
ATGAACAACACGCGGTTTCAGCCTGCCTCGGCTATCACGGTTATCCGAAAT
CCGTTTGCATTTCTATTAATGAAGTGGTGTGCCACGGTATTCCGGACGATG
CTAAGCTGCTGAAAGATGGCGATATCGTTAACATTGATGTCACCGTAATCA
AAGATGGTTTCCACGGCGATACCTCGAAAATGTTTATCGTCGGTAAGCCGA
CCATCATGGGCGAACGTCTGTGCCGTATCACGCAAGAAAGCCTGTACCTG
GCGCTACGCATGGTAAAACCAGGCATTAATCTGCGCGAAATCGGTGCGGC
GATTCAGAAATTTGTCGAAGCAGAAGGCTTCTCCGTCGTTCGTGAATATTG
CGGACACGGTATTGGTCGCGGCTTCCATGAAGAACCGCAGGTGCTGCACT
ATGACTCCCGTGAAACCAACGTCGTACTGAAACCTGGGATGACGTTCACC
ATCGAGCCAATGGTCAACGCGGGTAAAAAAGAGATCCGCACCATGAAAG
ATGGCTGGACGGTAAAAACCAAAGATCGCAGCTTGTCTGCACAATATGAG
CATACTATTGTGGTGACTGATAACGGCTGCGAAATTCTGACGCTACGCAAG
GATGACACCATCCCGGCGATAATCTCGCACGACGAATAA

Figure 4. Nucleotide sequence of the MetAP coding sequence from *H. influenzae* (807 bp).

ATGGCTATTCCAATTAGAACTGAAAAAGAAATTGTAAAACTGCGTGAGGC
CTGCAAATTGGCTTCGGATGTGCTGGTGATGATTGAACCTTACGTAAAAGC
AGGTGTAACTACAGGTGAACTTGATCGCATTTGCCATGAATATATAGTAAAT
GAACAAAAGGTTATTCCTGCTTGTTTGAATTATCACGGTTTCCCAAAGGCG
ACCTGTATTTCCATTAACGAAGTCGTTTGTCACGGTATTCCAAGTGACGAT
AAAGTGTTAAAAAATGGCGATATTGTGAATATTGATGTTACCGTGATTAAA
GACGGTTATTTTGGCGATAACTCAAAAATGTATATCGTGGGCGGAGAAACA
AATATTCGTAGTAAAAAGTTAGTGGAAGCGGCACAAGAGGCATTATATGTT
GGGATACGCACTGTAAAACCCGATATTCGTTTAAATGAAATAGGTAAAGCC
GTGCAAAAATATACTGAAAGCCAGACTTTCAGCGTTGTGCGTGAATATTGC
GGACATGGCGTTGGTACGGAATTTCACTGCGAACCTCAAGTATTGCATTAT
TACGCCGATGATGGCGGTGTAATTTTAAAACCCGGAATGGTGTTTACCATT
GAGCCAATGATTAACGCAGGCAAAAAAGAAGTGCGAGTTATGGGAGATG
GTTGGACAGTAAAAACCAAAGATCGTAGCCATTCAGCACAATATGAACAT
CAACTTGTTGTCACGGAAACTGGTTGTGAAGTGATGACTATTCGCGATGA
AGAAATTGCAGAAGGCCGAATTTCGCGGATTATGGTAAATGTGTAA

Figure 5. Nucleotide sequence of the MetAP coding sequence from *O. iheyensis* (741 bp).

ATGATTGCAAAGACAGAAGCGGATTTCAACGGATTAAAAGAAATGGGTAA
AATTTGTGGAGCGATTCGGGATGAATTGGTTCGTTCTACCAAACCGGGAAT
GACTACAAAAGAACTTGATGAAATGGCAGGCACGATGTTTGCACAAGCA
GGAGCTCAATCGGCGCCAAAAGGAGAGTATGATTTCCCAGGATATACATG
CATTAGCATTAATGAAGAAGTAGCGCACGGGATTCCCGGCGAACGGGTAA
TTGAGGAAGGCGATATTGTTAACATAGATGTTTCCGGGTCGAAAAATGGTT
ATTTTGCAGATACGGGAATTTCCTTTGTAGCTGGGCAAGGGGAGGAAATG
TCACAGAAAGTATGTGATGTGGTAAAAGAAGCATTCGAAGCTGGGTTGGA
GAAGGCAAAACCAGGTGTGAGCACAAGTGCGCTTGGGAAAGCTGCACAC
AATGTGGCAAAAAAACATGGCTTAACCGTGATCAAGAACCTTACCGGACA
CGGAATTGGCCGCTCGATACATGAAGCGCCAGATCATATTTTCAGTTACTT
CTCTCGTTGGGATAATGAGATTTTAAAAGATGGCATGGTTATCGCCTTTGA
GCCATTTATCTCAACATTTGAAGAGCAAGTCTATCAAGGAGAAGACGGCT
GGACCTTCTTAACGGTGGAAAGCGCGGTCGCTCGATACGAACATACTATTA
TTGTAACGGAAGATGGCCCAATTATTACGACATTGTAA

Figure 6. Nucleotide sequence of the MetAP coding sequence from *S. aureus* (759 bp).

ATGATTGTAAAAACAGAAGAAGAATTACAAGCGTTAAAAGAAATTGGATA
CATATGCGCTAAAGTGCGCAATACAATGCAAGCTGCAACCAAACCAGGTA
TCACTACGAAAGAGCTTGATAATATTGCGAAAGAGTTATTTGAAGAATACG
GCGCTATTTCAGCGCCAATTCATGATGAAAATTTTCCTGGTCAAACGTGTA
TTAGTGTTAATGAAGAGGTGGCACATGGGATTCCAAGTAAGCGTGTCATTC
GTGAAGGAGATTTAGTAAATATTGATGTATCGGCATTGAAGAATGGCTATTA
TGCAGATACAGGCATTTCATTTGTCGTTGGAGAATCAGACGATCCAATGAA
ACAAAAAAGTATGTGACGTAGCAACGATGGCATTTGAGAATGCAATTGCAA
AAGTAAAACCGGGTACTAAGTTGAGTAACATCGGTAAAGCGGTGCATAAT
ACAGCTAGACAAAATGATTTGAAAGTCATTAAAAACTTAACAGGTCATGG
TGTTGGTTTATCATTACATGAAGCGCCAGCACATGTACTTAATTACTTTGAT
CCAAAAGACAAAACATTATTAACTGAAGGTATGGTATTAGCTATTGAACCG
TTTATCTCATCAAATGCATCATTCGTTACAGAAGGTAAAAATGAATGGGCT
TTTGAAACGAGCGATAAAAGTTTTGTTGCTCAAATTGAGCATACAGTTATC
GTGACTAAGGATGGTCCGATTTTAACGACAAAAATTGAAGAAGAATAG

Figure 7. Nucleotide sequence of the MetAP coding sequence from *T. maritima* (753 bp).

ATGATAAGAATAAAGACACCCTCTGAGATCGAGAAAATGAAAAAAGCCG
GAAAAGCGGTCGCAGTAGCTTTGAGGGAAGTTAGAAAGGTGATCGTTCC
AGGAAAAACCGCATGGGATGTTGAAACACTTGTTCTGGAGATCTTCAAAA
AACTCAGAGTCAAACCAGCTTTCAAGGGATACGGTGGTTACAAATACGCA
ACTTGTGTTTCTGTGAACGAAGAGGTGGTACACGGTCTTCCTTTGAAGGA
GAAGGTTTTTAAAGAAGGAGACATTGTATCTGTAGATGTCGGAGCGGTATA
TCAGGGACTTTACGGTGATGCGGCCGTTACATACATCGTTGGAGAAACCG
ATGAAAGAGGAAAAGAACTGGTGAGAGTAACAAGAGAGGTTCTGGAAA
AGGCTATAAAGATGATAAAACCCGGCATCAGACTTGGAGATGTTTCGCAC
TGTATTCAAGAAACAGTTGAATCGGTAGGTTTCAACGTGATCAGGGATTAT
GTGGGTCATGGAGTGGGGAGAGAACTCCACGAAGACCCTCAAATTCCAA
ACTACGGAACACCCGGAACGGGTGTGGTATTAAGAAAGGGTATGACGCTG
GCCATAGAACCCATGGTGAGTGAAGGAGACTGGAGAGTTGTTGTGAAAG
AAGACGGATGGACAGCCGTTACAGTAGACGGTTCCAGATGCGCTCATTTT
GAACACACGATTCTGATAACAGAAAACGGGGCGGAGATATTGACCAAGG
AGGGATGA

Figure 8. Comparison of *B. anthracis* MetAP and *E. coli* MetAP. Conserved amino acids implicated in binding metals are underlined. Identical amino acids: 95/247 (38%); Positive amino acids: 143/247 (57%); Gaps created for proper alignment: 5/247 (2%). "+" means the amino acid is similar, but not identical. (Ecol, *E. coli;* Bant, *B. anthracis*)

```
Ecol   IKTPEDIEKMRVTGRLAAEVLEMIEPYVKPGVSTGELDRICNDYIVNEQHAVSACLGYHG
       I+  +D+E +R   GR+ A   E ++    KPG++T ELD I     +++E  A+SA    +
Bant   IRNEQDLEGLRKIGRIVALAREEMKKEAKPGMTTKELDLI-GKKVLDEHGAISAPEKEYD

Ecol   YPKSVCISINEVVCHGIPDDAKLLKDGDIVNIDVTVIKDGFHGDTSKMFIVGKPTIMGER
       +P   CIS+NE V  HGIP D ++LK+GD+VN+DV+   DG++ DT    F++G+     E+
Bant   FPGVTCISVNEEVAHGIPGD-RVLKEGDLVNVDVSAALDGYYADTGISFVLGEDEAK-EK

Ecol   LCRITQESLYLALRMVKPGINLREIGAAIQKFVEAEGFSVVREYCGHGIGRGFHEEP-QV
       LC+   ++  + A++ VK G    +IG A+  F    G++V++   GHGIG   HE P  +
Bant   LCQAAVDAFWAAMKKVKAGSKQNQIGRAVSNFAHKNGYNVIQNLTGHGIGLSLHEAPNHI

Ecol   LHYDSRETNVVLKPGMTFTIEPMVNAGKKE-IRTMKDGWTVKTKDRSLSAQYEHTIVVTD
       L Y     N +LK G+   +EP ++       I   DGWT   T D+SL AQ EHT+VVT
Bant   LSYFDPMDNALLKDGLVIAVEPFISMKADHIIERGDDGWTFVTPDKSLVAQCEHTVVVTR

Ecol   NGCEILT
           ILT
Bant   GEPIILT
```

Figure 9. Comparison of *H. influenzae* MetAP and *E. coli* MetAP. Conserved amino acids implicated in binding metals are underlined. Identical amino acids: 87/252 (34%); Positive amino acids: 144/252 (57%); Gaps created for proper alignment: 5/252 (1%). "+" means the amino acid is similar, but not identical. (Ecol, *E. coli*; Hinf, *H. influenzae*)

```
Ecol   IKTPEDIEKMRVTGRLAAEVLEMIEPYVKPGVSTGELDRICNDYIVNEQHAVSACLGYHG
       +KT E+++ ++  G + A+V    ++   KPG++T  ELD I  + +  E   A+SA +
Hinf   VKTEEELQALKEIGYICAKVRNTMQAATKPGITTKELDNIAKE-LFEEYGAISAPIHDEN

Ecol   YPKSVCISINEVVCHGIPDDAKLLKDGDIVNIDVTVIKDGFHGDTSKMFIVGKPTI-MGE
       +P   CIS+NE V HGIP    +++++GD+VNIDV+ +K+G++ DT    F+VG+    M +
Hinf   FPGQTCISVNEEVAHGIPSK-RVIREGDLVNIDVSALKNGYYADTGISFVVGESDDPMKQ

Ecol   RLCRITQESLYLALRMVKPGINLREIGAAIQKFVEAEGFSVVREYCGHGIGRGFHEEP-Q
       ++C +   +    A+  VKPG   L  IG A+           V++    GHG+G   HE P
Hinf   KVCDVATMAFENAIAKVKPGTKLSNIGKAVHNTARQNDLKVIKNLTGHGVGLSLHEAPAH

Ecol   VLHYDSRETNVVLKPGMTFTIEPMVNAGKKEIRTMKDGWTVKTKDRSLSAQYEHTIVVTD
       VL+Y   +    +L  GM    IEP +++     +   K+ W   +T D+S  AQ EHT++VT
Hinf   VLNYFDPKDKTLLTEGMVLAIEPFISSNASFVTEGKNEWAFETSDKSFVAQIEHTVIVTK

Ecol   NGCEILTLRKDD
       +G  ILT + ++
Hinf   DG-PILTTKIEE
```

Figure 10. Comparison of *O. iheyensis* MetAP and *E. coli* MetAP. Conserved amino acids implicated in binding metals are underlined. Identical amino acids: 89/246 (36%); Positive amino acids: 140/246 (56%); Gaps created for proper alignment: 4/246 (1%). "+" means the amino acid is similar, but not identical. (Ecol, *E. coli*; Oihe, *O. iheyensis*)

```
Ecol   KTPEDIEKMRVTGRLAAEVLEMIEPYVKPGVSTGELDRICNDYIVNEQHAVSACLGYHGY
       KT  D    ++  G++    + + +     KPG++T ELD +        +  A SA   G + +
Oihe   KTEADFNGLKEMGKICGAIRDELVRSTKPGMTTKELDEMAGTMFA-QAGAQSAPKGEYDF

Ecol   PKSVCISINEVVCHGIPDDAKLLKDGDIVNIDVTVIKDGFHGDTSKMFIVGKPTIMGERL
       P   CISINE V HGIP + +++++GDIVNIDV+   K+G+  DT    F+ G+   M +++
Oihe   PGYTCISINEEVAHGIPGE-RVIEEGDIVNIDVSGSKNGYFADTGISFVAGQGEEMSQKV

Ecol   CRITQESLYLALRMVKPGINLREIGAAIQKFVEAEGFSVVREYCGHGIGRGFHEEP-QVL
       C + +E+    L    KPG++    +G A      +  G +V++    GHGIGR  HE P  +
Oihe   CDVVKEAFEAGLEKAKPGVSTSALGKAAHNVAKKHGLTVIKNLTGHGIGRSIHEAPDHIF

Ecol   HYDSRETNVVLKPGMTFTIEPMVNAGKKEIRTMKDGWTVKTKDRSLSAQYEHTIVVTDNG
        Y SR  N +LK GM     EP ++  ++++    +DGWT   T + S  A+YEHTI+VT++G
Oihe   SYFSRWDNEILKDGMVIAFEPFISTFEEQVYQGEDGWTFLTVE-SAVARYEHTIIVTEDG

Ecol   CEILTL
         I TL
Oihe   PIITTL
```

Figure 11. Comparison of *S. aureus* MetAP and *E. coli* MetAP. Conserved amino acids implicated in binding metals are underlined. Identical amino acids: 87/252 (34%); Positive amino acids: 144/252 (57%); Gaps created for proper alignment: 5/252 (1%). "+" means the amino acid is similar, but not identical. (Ecol, *E. coli*; Saur, *S. aureus*)

```
Ecol   IKTPEDIEKMRVTGRLAAEVLEMIEPYVKPGVSTGELDRICNDYIVNEQHAVSACLGYHG
       +KT E+++ ++  G + A+V   ++    KPG++T ELD  I  + +   E  A+SA +
Saur   VKTEEELQALKEIGYICAKVRNTMQAATKPGITTKELDNIAKE-LFEEYGAISAPIHDEN

Ecol   YPKSVCISINEVVCHGIPDDAKLLKDGDIVNIDVTVIKDGFHGDTSKMFIVGKPTI-MGE
       +P   CIS+NE V HGIP    +++++GD+VNIDV+ +K+G++ DT    F+VG+    M +
Saur   FPGQTCISVNEEVAHGIPSK-RVIREGDLVNIDVSALKNGYYADTGISFVVGESDDPMKQ

Ecol   RLCRITQESLYLALRMVKPGINLREIGAAIQKFVEAEGFSVVREYCGHGIGRGFHEEP-Q
       ++C +   +    A+   VKPG  L  IG A+           V++    GHG+G    HE P
Saur   KVCDVATMAFENAIAKVKPGTKLSNIGKAVHNTARQNDLKVIKNLTGHGVGLSLHEAPAH

Ecol   VLHYDSRETNVVLKPGMTFTIEPMVNAGKKEIRTMKDGWTVKTKDRSLSAQYEHTIVVTD
       VL+Y   +    +L   GM   IEP +++     +    K+ W  +T D+S  AQ EHT++VT
Saur   VLNYFDPKDKTLLTEGMVLAIEPFISSNASFVTEGKNEWAFETSDKSFVAQIEHTVIVTK

Ecol   NGCEILTLRKDD
       +G  ILT + ++
Saur   DG-PILTTKIEE
```

Figure 12. Comparison of *T. maritima* MetAP and *E. coli* MetAP. Conserved amino acids implicated in binding metals are underlined. Identical amino acids: 106/247 (42%); Positive amino acids: 157/247 (62%); Gaps created for proper alignment: 1/247 (0%). "+" means the amino acid is similar, but not identical. (Ecol, *E. coli*; Tmar, *T. maritima*)

```
Ecol   ISIKTPEDIEKMRVTGRLAAEVLEMIEPYVKPGVSTGELDRICNDYIVNEQHAVSACLGY
       I IKTP +IEKM+ G+ A  L  +  + PG +  +++ +  + I +    A  GY
Tmar   IRIKTPSEIEKMKKAGKAVAVALREVRKVIVPGKTAWDVETLVLE-IFKKLRVKPAFKGY

Ecol   HGYPKSVCISINEVVCHGIPDDAKLLKDGDIVNIDVTVIKDGFHGDTSKMFIVGKPTIMG
        GY  + C+S+NE V  HG+P   K+ K+GDIV++DV  +   G +GD +  +IVG+    G
Tmar   GGYKYATCVSVNEEVVHGLPLKEKVFKEGDIVSVDVGAVYQGLYGDAAVTYIVGETDERG

Ecol   ERLCRITQESLYLALRMVKPGINLREIGAAIQKFVEAEGFSVVREYCGHGIGRGFHEEPQ
       + L R+T+E L  A++M+KPGI L ++   IQ+ VE+ GF+V+R+Y GHG+GR  HE+PQ
Tmar   KELVRVTREVLEKAIKMIKPGIRLGDVSHCIQETVESVGFNVIRDYVGHGVGRELHEDPQ

Ecol   VLHYDSRETNVVLKPGMTFTIEPMVNAGKKEIRTMKDGWTVKTKDRSLSAQYEHTIVVTD
       + +Y +  T VVL+ GMT  IEPMV+  G  +   +DGWT  T D S  A +EHTI++T+
Tmar   IPNYGTPGTGVVLRKGMTLAIEPMVSEGDWRVVVKEDGWTAVTVDGSRCAHFEHTILITE

Ecol   NGCEILT
       NG EILT
Tmar   NGAEILT
```

Table 4. Protein similarity of MetAPs with *E. coli* MetAP.

B. anthracis	*H. influenzae*	*O. iheyensis*	*S. aureus*	*T. maritima*	*E. coli* O157:H7
38%	34%	36%	34%	42%	100%

Figure 13. SDS-PAGE gel of induced proteins at 37°C (A) and room temperature (B). The lanes in both (A) and (B) are identical.

Lane 1: molecular weight marker;

Lane 2: *E. coli* BL21(DE3) pET30 EK/LIC:: *S. aureus* MetAP uninduced protein;

Lane 3: *E. coli* BL21(DE3) pET30 EK/LIC:: *S. aureus* MetAP induced protein;

Lane 4: *E. coli* BL21(DE3) pET30 EK/LIC:: *B. anthracis* MetAP uninduced protein;

Lane 5: *E. coli* BL21(DE3) pET30 EK/LIC:: *B. anthracis* MetAP induced protein;

Lane 6: *E. coli* BL21(DE3) pET30 EK/LIC:: *O. iheyensis* MetAP uninduced protein;

Lane 7: *E. coli* BL21(DE3) pET30 EK/LIC:: *O. iheyensis* MetAP induced protein;

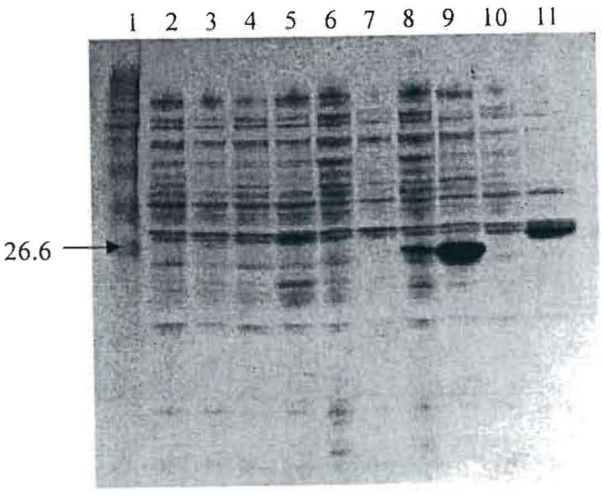
Lane 8: *E. coli* BL21(DE3) pET30 EK/LIC:: *T. maritima* MetAP uninduced protein;

Lane 9: *E. coli* BL21(DE3) pET30 EK/LIC:: *T. maritima* MetAP induced protein;

Lane 10: *E. coli* BL21(DE3) pET30 EK/LIC:: *H. influenzae* MetAP uninduced protein;

Lane 11: *E. coli* BL21(DE3) pET30 EK/LIC:: *H. influenzae* MetAP induced protein.

A



B

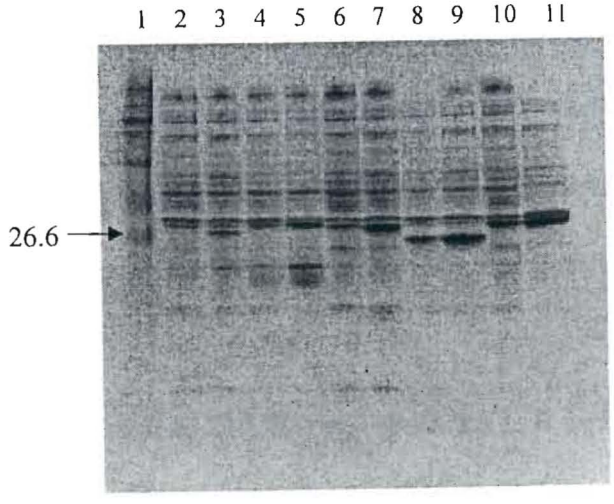


Figure 14. Western blot indicated induced proteins at 37°C (A) and room temperature (B).

Lane 1: molecular weight marker;

Lane 2: *E. coli* BL21(DE3) pET30 EK/LIC:: *S. aureus* MetAP uninduced protein;

Lane 3: *E. coli* BL21(DE3) pET30 EK/LIC:: *S. aureus* MetAP induced protein;

Lane 4: *E. coli* BL21(DE3) pET30 EK/LIC:: *B. anthracis* MetAP uninduced protein;

Lane 5: *E. coli* BL21(DE3) pET30 EK/LIC:: *B. anthracis* MetAP induced protein;

Lane 6: *E. coli* BL21(DE3) pET30 EK/LIC:: *O. iheyensis* MetAP uninduced protein;

Lane 7: *E. coli* BL21(DE3) pET30 EK/LIC:: *O. iheyensis* MetAP induced protein;

Lane 8: *E. coli* BL21(DE3) pET30 EK/LIC:: *T. maritima* MetAP uninduced protein;

Lane 9: *E. coli* BL21(DE3) pET30 EK/LIC:: *T. maritima* MetAP induced protein;

Lane 10: *E. coli* BL21(DE3) pET30 EK/LIC:: *H. influenzae* MetAP uninduced protein;

Lane 11: *E. coli* BL21(DE3) pET30 EK/LIC:: *H. influenzae* MetAP induced protein.

A

1 2 3 4 5 6 7 8 9 10 11

B

1 2 3 4 5 6 7 8 9 10 11

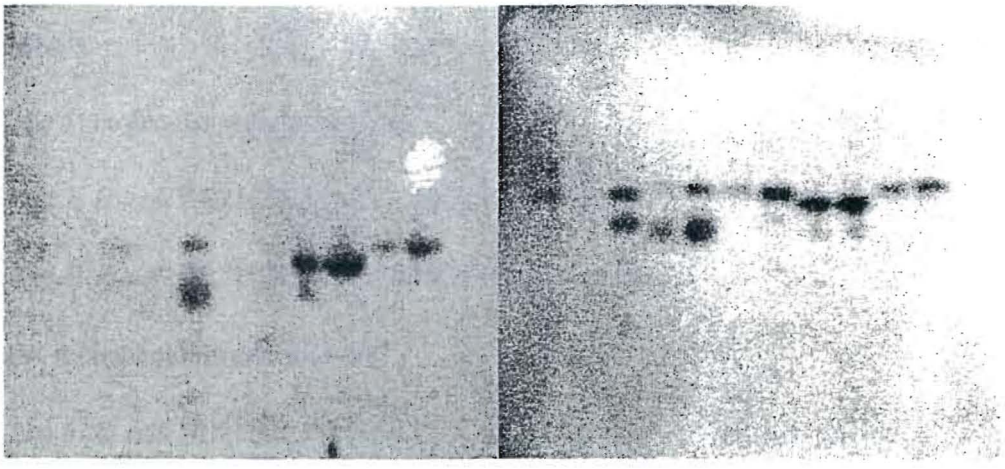


Figure 15. SDS-PAGE gel from pre- and post-Ni affinity Chromatography and dialysis.

Lane 1: molecular weight markers;

Lane 2: *E. coli* BL21(DE3) pET30EK/LIC :: *T. maritima* MetAP total extract;

Lane 3: *E. coli* BL21(DE3) pET30EK/LIC :: *T. maritima* MetAP total extract post-nickel column and dialysis;

Lane 4: *E. coli* BL21(DE3) pET30EK/LIC :: *H. influenzae* MetAP total extract;

Lane 5: *E. coli* BL21(DE3) pET30EK/LIC :: *H. influenzae* MetAP total extract eluted from nickel column;

Lane 6: *E. coli* BL21(DE3) pET30EK/LIC :: *H. influenzae* MetAP total extract post-nickel column and dialysis;

Lane 7: *E. coli* BL21(DE3) pET30EK/LIC :: *B. anthracis* MetAP total extract;

Lane 8: *E. coli* BL21(DE3) pET30EK/LIC :: *B. anthracis* MetAP total extract eluted from nickel column;

Lane 9: *E. coli* BL21(DE3) pET30EK/LIC :: *B. anthracis* MetAP total extract post-nickel column and dialysis.

1 2 3 4 5 6 7 8 9

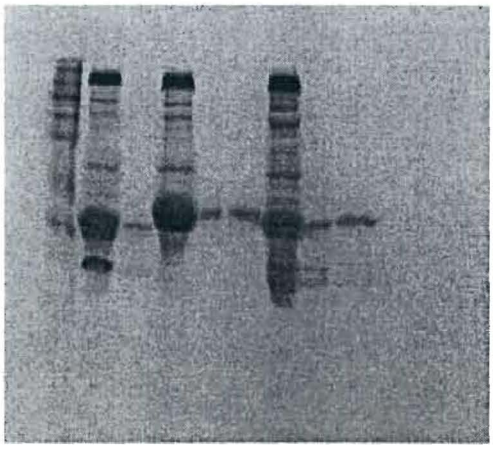
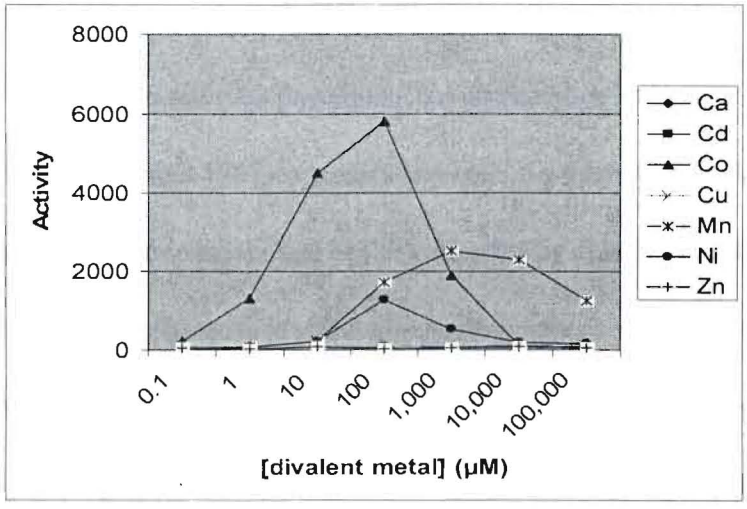


Figure 16. Activation of *E. coli* MetAP by Divalent Metals. Data points are the result of compiled data from nine different experimental trials for each protein. Activity values are a measure of fluorescence upon the cleavage of Met-AMC by MetAP.


8000
6000
4000
2000
0
Activity
0.1
1
10
100
1,000
10,000
100,000
[divalent metal] (µM)
Ca
Cd
Co
Cu
Mn
Ni
Zn

Discussion

Antibiotic resistance is becoming increasingly problematic in today's society. For example, penicillin-resistant pneumococcus strains have increased from 0.02 percent between 1979 and 1987, to today's level at 6.6 percent (12). In the U.S., drug resistant bacterial infections increase health care costs by at least $4 billion per year (27). They also indirectly cause problems in treating patients with fatal diseases. It's estimated that 25-30% of cancer patients die from infectious agents, half of which were drug resistant (24). Furthermore, the emergence of multiple-drug resistance in organisms such as *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Shigella dysenteriae*, has further indicated we are loosing the war against drug resistant organisms (11, 25). Although the search for new antibiotics is ongoing, if history repeats itself it is only a matter of time before drug resistance develops against the new antimicrobials.

An alternative approach to combating the problem of bacterial antibiotic resistance is to focus on the organism itself. Many proteins within the cell are essential for cell survival, and if their activity is inhibited, the cell will die. MetAP is one example of an essential protein and was the focus of this study. This enzyme exists in all bacteria and is essential for cell survival (11, 33). Since its function is to remove the initiator N-terminal methionine residue from a newly formed protein (11, 33, 7), inhibition of this process results in cell death. Likewise, deletion of *map* is lethal (2, 26). These facts illustrate the essentiality of the protein to the bacterial cell and its usefulness as a target for the development of new inhibitors.

To develop inhibitors against MetAP, this protein needs to be purified and its crystal structure determined. Indeed, the structure of *E. coli* MetAP has been solved (1, 4, 17, 20, 21). While all bacterial MetAPs' studied to date have similar conserved amino acids comprising the active site, finding species-specific differences could aid in the development of species-specific inhibitors. Thus, solving the crystal structure of MetAP from different organisms would be an important first step toward this goal.

In this study, we attempted to clone and overexpress MetAP from *S. aureus*, *H. influenzae*, *E. coli* O157:H7, *B. anthracis*, *T. maritima*, and *O. iheyensis*. Since the genomes of all six organisms had been previously sequenced, this facilitated our development of PCR primers. Four out of the six organisms, *S. aureus*, *H. influenzae*, *E. coli* O157:H7, and *B. anthracis* were selected because they are pathogens. The other two organisms, *T. maritima* and *O. iheyensis*, were selected since they are classified as extremophiles. Since adaptation to life at high temperatures involves protein structure changes, it is of interest to determine how the enzymatic active domains of MetAP from an extremophile interact with its metal ligand. This structural information could possibly lead to the design of novel MetAP inhibitions by providing a unique scaffold on which to build.

map was amplified and cloned into the overexpression plasmid pET30EK/LIC. This vector has several attributes which makes it a good choice for overexpression of *map*. Cloned inserts, when placed inframe, will contain a N-terminal His•Tag/S•Tag™ sequence upon expression. The option also exists for a C-terminal His•Tag sequence. This His•Tag sequence is translated into a stretch of six histidine

amino acids, which facilitates protein purification. Cloned inserts are placed under the control of the T7 promoter, which itself is controlled by viral T7 polymerase. Expression of T7 polymerase is induced by adding IPTG to growing cells. The LIC designation refers to "ligation-independent cloning," which is designed for directional cloning of PCR products without restriction enzyme digestion or ligation reactions. This attribute allowed us to directionaly clone *map* into this vector, thereby simplifying our cloning efforts. The EK denotes "enterokinase" which promotes removal of all vector-encoded sequences from the target protein by digestion with recombinant enterokinase.

Nucleotide sequencing was performed on all cloned *map* inserts contained in pET30EK/LIC. Since the genome of all six organisms used to amplify *map* was available, it was not surprising that DNA sequence obtained matched the corresponding *map* DNA sequence 100%. Since *E. coli* MetAP has been studied in depth, we compared all other translated protein sequences to the *E. coli* MetAP sequence. With the exception of *E. coli* O157:H7 which matched 100%, all MetAPs demonstrated relatively low protein homology (Table 4). However, conserved amino acids implicated as part of the catalytic domains which bind divalent metals, were present in the amino acid sequence of MetAP from each organism (18, 20, 28, 30).

Once the integrity of each amplified gene was confirmed, we transformed each recombinant plasmid into *E. coli* BL21(DE3). *E. coli* BL21(DE3) is a widely used strain used for gene overexpression. The designation (DE3) indicates the host is a lysogen of λDE3, and therefore carries a chromosomal copy of the viral T7 RNA

polymerase gene under control of the *lacUV5* promoter. Since *E. coli* O157:H7 MetAP indicated 100% identity with *E. coli* MetAP, we did not pursue *E. coli* O157:H7 MetAP further in the present study.

As shown in Figure 13, all five MetAPs were overexpressed. However, the level of expression varied considerably. These results are typical of these types of studies. Since many different factors affect overexpression, the conditions leading to maximal levels are often only found after considerable experimental trials. We chose *T. maritima*, *H. influenzae*, and *B. anthracis* for further study since MetAP from these three organisms was overexpressed at the highest level.

MetAP from *T. maritima*, *H. influenzae*, and *B. anthracis* were each purified via nickel affinity chromatography. Since elution of MetAP from the nickel column was accomplished using a buffer rich in imidazole, dialysis was used to remove this compound and replace the buffer with MetAP assay buffer. Dialysis is a protocol routinely used to adjust a protein sample from one buffer to another, in adjusting metal and salt ion concentrations, and in removing unwanted small molecules (36). Since our purified protein was in a buffer containing imidazole, which mimics the structure of histidine, this procedure was necessary since MetAP is catalyzed by divalent metals.

Purified MetAP from *T. maritima*, *H. influenzae*, and *B. anthracis* were initially assayed in the Ye laboratory at the University of Kansas. Dr. Qizhuang Ye is an expert on MetAP and has published several papers regarding this protein (5, 6, 9, 10, 13, 14, 15, 16, 22, 23, 35). In his laboratory, only MetAP from *T. maritima* showed

enzymatic activity. Enzymatic activity of MetAP from *H. influenzae* and *B. anthracis* could not be detected. Unfortunately, we could not detect enzymatic activity from any of the MetAPs when they were assayed at ESU. To rule out variations with the MetAP assay itself, we obtained purified *E. coli* MetAP from the Ye laboratory. Enzymatic analysis of *E. coli* MetAP assayed at ESU confirmed a similar mode of action (Fig. 15) as previously documented by the Ye laboratory (16).

Since it seemed our protein lost activity during storage at -20°C, MetAP from *T. maritima* was purified again. Activity still could not be detected. Although this observation cannot be explained, it is possible that since we did not purify the protein using the exact same conditions, as published previously (7, 14, 16, 18), we may be co-purifying some inhibitor of enzymatic activity. This seems unlikely, however, since activity of *T. maritima* MetAP was demonstrated in the Ye laboratory. Another possibility is that the fusion His•Tag protein affects the enzymatic activity of *T. maritima* MetAP. Indeed, Halliwell *et al.* (8) indicated inclusion of a His•Tag might induce misfolding of the target protein. However, inclusion of a His•Tag has been used by countless investigators with no effect on protein structure due to its small size. Another possibility is that *T. maritima* MetAP has very low enzymatic activity which we could not detect.

In conclusion, *map* was successfully cloned and overexpressed from *B. anthracis*, *T. maritima*, *H. influenzae*, *S. aureus*, *O. iheyensis*, and *E. coli* O157:H7. Although MetAP from *B. anthracis*, *T. maritima*, and *H. influenzae* MetAPs were successfully purified, enzymatic activity could not be reproducibly detected. Since

the MetAP assay is functional in the Crupper laboratory at ESU, future studies could examine if the His•Tag is indeed interfering with protein activity by generating genetic constructs devoid of this tag.

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Cloning, Overexpression, and Purification of Bacterial Methionine Aminopeptidase

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