THE EFFECTS OF A SINGLE AMINO ACID SUBSTITUTION ON THE SUBSTRATE PROFILE OF *ESCHERICHIA COLI* β-LACTAMASE

by

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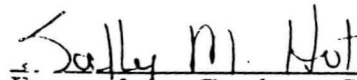

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ABSTRACT

THE EFFECTS OF A SINGLE AMINO ACID SUBSTITUTION ON THE SUBSTRATE PROFILE OF *ESCHERICHIA COLI* β-LACTAMASE

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Master of Science

Youngstown State University, 1991

Site-specific mutagenesis is one of the most powerful tools for the study of protein structure and function to date. The introduction of methods using small chemically synthesized oligonucleotide primers to target mutations to individual nucleotides has made it possible to examine the effects of even single amino acid substitutions on the catalytic properities of an enzyme. *Escherichia coli* RTEM β-lactamase, an enzyme which hydrolyzes the cyclic amide bond in β-lactam antibiotics, provides the bacteria with resistance to both penicillins and cephalosporins. In this study, a single amino acid substitution was introduced at the active site of this enzyme. This procedure utilized oligonucleotide-directed mutagenesis to substitute adenine for thymine at the codon for amino acid 70 resulting in the replacement of the wild-type serine residue by cysteine. *E. coli* cells producing the new cysteine mutant β-lactamase showed decreased resistance to penicillin series antibiotics as compared to cells producing the wild-type enzyme. However, these same cells

showed increased resistance to cephalosporins over the wild-type. These results suggest that the cysteine mutant β-lactamase was not simply decreased in activity but has unique catalytic properties which result in an altered substrate profile when compared to the wild-type enzyme.

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TABLE OF CONTENTS

TABLE OF CONTENTS (CONT'D)

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Mutagenesis

Mutagenesis is a process by which a change, either spontaneously or artificially generated, is induced in the natural occurring nucleotide sequence of the DNA of an organism. If this change is propagated such that a gene or phenotype different from the wild type is seen, a mutant has been created (Freifelder,1985). Even before Francis Crick in 1956 proposed the Central Dogma which states that DNA serves as a template for the transcription of RNA which then is translated into the amino acid sequence of a protein, the series nature of decoding of the genetic information suggested that a change in the DNA sequence would lead to a subsequent and necessary change in the final protein product.

Initial attempts to alter the nucleotide sequence of DNA, via the use of chemical or physical agents, lead to random non-specific mutagenesis. The usefulness of these agents in studying the effects of change to a specific gene or protein is limited since no specific portion of the DNA could be targeted. In last few decades, the advancement of modern recombinant DNA technology, most importantly the availability of nucleotide sequences of the genes of interest, has made *in vitro* site-specific mutagenesis possible. Mutations induced by these techniques are able to be directed toward a single gene or even a single nucleotide. This feature of site-specific mutagenesis has made it not only an ideal tool to study protein structure and function but also one of the most important advances in modern

genetics to date (Smith, 1985).

Site-specific mutagenesis of DNA was initiated with the work of Mueller et al. in 1978. This technique used unique restriction endonuclease sites to generate small single-stranded nicks in the DNA near the desired target of the mutation. These nicks could then be lengthened by single-strand specific exonucleases to reach targets short distances from the restriction sites. After the target site is reached, the mutagenesis is effected by the addition of N^6-hydroxydeoxycytidine, a nucleotide analog in place of deoxycitidine, and DNA polymerase I *in vitro*. As DNA pol I catalyzes the complementary base paring *in vitro*, the N^6-hydroxydeoxycytidine is able to pair as either deoxycytidine or deoxythymidine altering the wild-type base sequence in one strand (Falvell et al.,1974).

Original studies simply transformed bacterial cells with the single-strand enzymatically altered DNA; however, *in vivo* repair mechanisms employed by the cell upon completion of second-strand synthesis, frequently excised the nucleotide analog in favor of the original nucleotide or inactivating the entire strand (Mueller et al.,1978). In subsequent studies, complementary strand synthesis was completed *in vitro* with normal nucleotides bypassing the cellular repair mechanisms and increasing the frequency of a stable mutation (Wieringa et al.,1985).

Other studies where nucleotide substitution was accomplished at targets in areas near restriction endonuclease sites, utilized *in vitro* chemical modification of nucleotides. A study by Shortle et al. used sodium bisulfite to deaminate deoxycitidine nucleotides in a viral genome (1978). The deamination of the

deoxycytidine results in uracil in the DNA which after *in vivo* replication causes a CG to TA substitution. As the sodium bisulfite acts preferentially on single-stranded DNA, this technique could be targeted by producing single-strand nicks at unique sites with restriction endonuclease digestion and elongating the gap with single-strand specific exonucleases (Kulter et al.,1984).

Other chemical modifications of nucleotides utilizing similar procedures involved mispairing of nucleotides by treatment of DNA, specifically the deoxycytidine residues with methoxylamine, hydroxylamine, and nitrous acid (Smith, 1985). Non-specific pairing of purine and pyrimidines could also be induced by the treatment DNA with hydrazine (Meyers et al.,1985).

Restriction endonuclease digestion as a means of targeting mutation sites also led to mutations caused not by nucleotide substitution but by deletion of a segment of DNA at or near the restriction site. An example of this type of approach was seen in the work of Guo et al. (1983) who used restriction endonucleases to digest, or "cut", a segment of DNA at specific locations through both strands. The 3′ ends were then blocked by enzymatic addition of an α-triphosphoryl nucleoside. This blocked the 3′ ends from digestion by exonuclease III, a double-stranded specific enzyme which removes nucleotides from the ends of DNA not involved in phosphodiester linkage. The newly digested and shorter DNA fragment was then ligated together and transformed into a particular host cell, thus eliminating a portion of DNA from being transcribed.

The techniques of site-specific mutagenesis, including enzymatic incorporation

of nucleotide analogs, chemical modification of nucleotides, or deletions of segments of DNA, which relied on the use of restriction endonuclease sites to target the desired mutation were of limited usefulness. These procedures had a requirement for the mutation site to be near a restriction site which may not necessarily be the case. Also, chemical modifications of nucleotides to induce mispairing can result in a number of bases substituted opposite the modified site. A major step forward in the advancement of site-specific mutagenesis was the development of techniques which utilized small, chemically synthesized and purified sequences of DNA called oligodeoxyribonucleotides (Craik,1985).

Oligonucleotide synthesis of DNA is based upon the Merrifield solid-phase peptide synthesis technique. This technique was modified for use in the synthesis of DNA by Itakura et al. (1975). The chemical reaction involves the addition of nucleotides to the 5′ hydroxyl by the blocking of all reactive sites, with the exception of the 5′ hydroxyl, of the nucleotide and the binding of the newly synthesized chain to the solid support column by 3′ hydroxyl linkage. Initially, chemical synthesis of oligonucleotides was achieved manually, but now, computer-controlled oligonucleotide synthesizers are able to complete the chemical reactions and chromatography column purification necessary to oligonucleotide before its use in site-specific mutagenesis reactions (Craik,1985).

The first report of the potential use of oligonucleotides to serve in site-specific mutation reactions was proposed by Lederberg in 1960, before the recombinant DNA technology to attempt such a procedure was available (1960). Heteroduplex

formation or binding of an oligonucleotide to a similar sequence in the native DNA is the basis for most of the oligonucleotide directed mutagenesis procedures and was first established experimentally by Hutchison et al. (1971) and Weisbeek et al. (1970). These studies independently found that a mutation in the genone of a single-stranded bacteriophage could be reversed by the annealing of the appropriate wild-type sequence to the mutated site. This led Hutchison to suggest and finally prove experimentally that an oligonucleotide could be annealed to a phage genome in such a way as to create a desired mutation at any given site (Hutchison et al.,1978). Additional studies were published in support of this hypothesis including the work of Kossel et al. (1978) which introduced mutations into replicative sites of phage ϕX174. These investigations demonstrated that the most efficient method for generation of a site-specific mutation involves the annealing and subsequent substitution of the synthesized oligonucleotide, containing the desired mutated sequence, in place of the wild-type sequence (Craik,1985).

Most oligonucleotide-directed mutagenesis protocols share six common criteria which must be met in order for sucessful mutation at the target site. These criteria include: 1) the sequence or gene to be mutated must be cloned into a vector, 2) the sequence of the gene must be known, 3) the site of mutation must be available in single-stranded form, 4) an oligonucleotide must be synthesized such that it can anneal to the original sequence and also contain the desired mutation, 5) the remainder of the original sequence that is not meant for mutation must be able to be replicated accurately, 6) upon completion of the mutation, the wild-type sequence

must be separated from the mutated sequence (Craik,1985). Basic gene manipulation techniques made it relatively easy for the gene sequence of interest to be isolated (Maniatus et al.,1982), and the Maxam and Gilbert method of DNA sequencing (Maxam et al.,1980) made the nucleotide sequence available of nearly any gene which could be isolated. The requirement for the mutation site to be available in single-stranded form still relied on restriction endonuclease sites to create single-strand nicks and subsequent single-strand specific exonuclease digestion to create a single-strand gap at the mutation site, which was a major drawback of initial site-specific mutation techniques. This major disadvantage was overcome with the work of Messing using the filamentous bacteriophage M13 (Messing,1983).

M13 is a DNA bacteriophage which enters an *E. coli* cell by way of the F pilus. The viral gemone consists of a single strand of DNA called the (+) or non-coding strand. Upon entry into the host cell, the bacteriophage is stripped of its major coat protein and begins synthesis of a (-) strand on the (+) strand template, using only the polymerase enzymes of the host cell. The complex of (+) and (-) strands stays associated in double-stranded form known as the replicative form (RF). The two major functions of the (-) strand synthesized in the bacterial cell are: 1) this strand is the sense strand and contains all the coding information for the viral proteins, and 2) this strand serves as the template for all viral (+) strand DNA synthesis (Messing,1983). The replicative form is therefore a necessary requirement for the production of progeny viral particles.

Several features of the M13 system make it a favorable choice for use in

oligonucleotide directed mutagenesis reactions. First, two separate types of DNA can be isolated from a bacterial cell infected with M13 as a cloning vector. After centrifugation, the cell pellet contains double-stranded RF DNA in much the same form as a plasmid; while in the supernatant, single-stranded DNA of the same sequence can be obtained. This fulfills the condition of oligonucleotide directed mutageneis reactions which requires the desired sequence to be mutated to be available in single-stranded form, but the RF form still allows cloning into the double-stranded form as in a plasmid. Second, there is no lysis of the bacterial cell upon infection with M13 as with bacteriophage λ. This allows the cell to remain intact while still actively producing viral particles. Third, M13 can package DNA sequences larger than the actual viral DNA (Messing,1983). This allows for the cloning of large genes into the M13 system. Forth, after the gene of interest has been cloned in to the M13 viral DNA, the recombinant can then be introduced either by infection of the cell with the viral particles or the transformation of either the single-stranded or double-stranded forms by standard procedures (Maniatus et al.,1982). These features make M13 an ideal vector for use in oligonucleotide directed mutagenesis reactions.

A typical mutagenesis protocol using M13 and oligonucleotides was established first by Zoller and Smith (1983). The gene of interest is cloned in double-stranded form into the replicative form of the M13 virus by restriction endonuclease digestion and subsequent annealing of the chimeric DNA. This chimeria is then transformed into the *E. coli* host cell by any number of

transformation methods. After transformation, the replicative form of the viral DNA is recognized and transcription of viral proteins begins. Release of mature virus particles with single-stranded copies of the gene of interest follows.

Template DNA is prepared by extracting the protein coat from the virus and purifying the single-stranded DNA. A mutagenic oligonucleotide is chosen such that the desired mutation, be it a base substitution, deletion, or insertion, is flanked by a number of nucleotides complementary to the sequence on the template DNA. This is necessary to permit the oligonucleotide to anneal to the template via base pair matching and hold throughout the *in vitro* synthesis reaction. Second strand synthesis is accomplished by the addition of all four deoxynucleotide triphosphates, The Klenow fragment of *E. coli* DNA polymerase I, and bacteriophage T4 ligase (Zoller et al.,1983). The DNA polymerase, in the presence of the deoxynucleotide triphosphates, synthesizes the second-strand complementary to the first using the mutagenic oligonucleotide as a primer; thus, the new strand will be wild-type in all areas with the exception of the mutated site. The second-strand is then ligated to the opposite end of the primer with T4 ligase, and the mutated replicative form is then transformed into the bacterial cell.

As is evident from this technique, with one wild-type strand and one mutant strand, the expected efficiency that would be obtained upon transformation is 50%. In actual mutagenesis procedures, the efficiency is usually 20-40%. It is unclear why there is preferential synthesis of the wild-type sequence. Many methods have been developed to increase the efficiency of mutant strand replication and proliferation

in the oligonucleotide directed mutagenesis reaction using M13 vectors. Each of the techniques involves a mechanism by which the mutant strand can be distinguished and separated from the wild-type strand, while the wild-type strand is preferentially selected against by the host cell (Geisselsloder et al.,1987). A technique has been developed by Kunkel by which mutation efficiency can be increased to nearly 100% (Kunkel,1985). In this technique, the chimeric replicative form of the viral DNA is introduced into a *dut⁻ ung⁻* double mutant *E. coli*. The *dut⁻* mutation inactivates the enzyme deoxyuraciltriphosphatase in the cell which results in high intracellular levels of deoxyuraciltriphosphate which are randomly incorporated into newly synthesized DNA. This does not interrupt the coding sequence because the uracil both codes like and subsitutes for thymine (Kunkel,1985). The uracil remains stable in the DNA because of the *ung⁻* mutation which inactivates uracil N-glycosylase, the error correct enzyme responsible for the removal of misincorporated uracils. Thus, the single-stranded viral DNA will have a number of uracils in its sequence. When this is used as a template for an oligonucleotide directed mutation reaction, the wild-type strand is inactivated when transformed into a wild-type *E. coli* with functional deoxyuraciltriphosphatase and uracil N-glycosylase enzymes (Geisselsloder et al.,1987). This results in nearly all the remaining functional DNA being the mutated strand.

Another advance in oligonucleotide directed mutagenesis using M13 as a vector was introduced by Messing (1987). There were some negative aspects of using M13 as a vector for mutation reactions, particularly with respect to lack of stability

of certain inserts and difficulty of manipulation of the replicative form. These problems were overcome by the incorporation of the intergenic region of bacteriophage f1 into a plasmid (phagemid) which resulted in the production of single-stranded copies of the plasmid when the cell was infected with a helper phage (Messing et al.,1987). Until the work of Messing, a major disadvantage in using phagemid vectors was the low amount of single-stranded phagemid that was produced due to interference from superinfection by two separate viruses. This was overcome by the construction of M13K07 helper phage (Messing et al.,1987). The virus has deletions in its DNA such that it does not interfere on superinfection and packages the phagemid into single-stranded virus particles at the expense of itself, making large amounts of single-stranded template DNA conveinently available for mutatagenesis reactions.

With the advances in mutatagenesis reactions that allowed easy and reliable nucleotide substitutions, insertions, and deletions targeted to any specific nucleotide in the gene sequence of any protein, the study of protein structure and function at the amino acid level has become possible.

β-Lactamase

Beta-lactamase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6) is a naturally occurring enzyme in many gram positive and gram negative bacterial species, actinomycetes, yeasts, and blue-green algae (Sykes et al,.1982). It was found to hydrolyze the cyclic amide bond in β-lactam (penicillins and cephalosporins)

containing antibiotic molecules. Hydrolysis of this bond eradicates the bacteriocidal properities of these antibiotics and provides resistance to the organism when challenged (Sykes et al.,1982). The action of β-lactamase on penicillin produces a non-bacteriocidal penicilloate in stoichiometric proportions. The action on cephalosporins is similar; however, the cephalosporoate is very unstable and rapidly degraded.

Gram positive β-lactamase was first described in 1940 by Abraham and Chain shortly after the introduction of penicillin. The selection pressure applied by penicillin to gram positive cocci, the first bacteria to be treated with the antibiotic, resulted in the isolation of a β-lactamase producing and penicillin-resistant *Staphylococcus aureus* from hospitalized patients (Sykes et al.,1982). By the mid-1950s, 80% of nosocomial gram positive cocci were resistant to penicillin (Sykes et al.,1982). The rise and proliferation of such strains precipitated additional reasearch into other types of β-lactam antibiotics, including semi-synthetic penicillins and additional cephalosporins. The use of these antibiotics led to genetic modifications resulting in the production of novel β-lactamases by a wide variety of organisms and led to the discovery and characterization of the β-lactamase producing gram negative bacteria (Sykes et al.,1982).

Two main classes of β-lactamases, classes A and B, have been described based on molecular weight, conformation, and amino acid sequence analysis. Most β-lactamases belong to class A with molecular weights around 29,000 A.M.U. and includes both gram negative and positive organisms (Ambler,1980). There is very little sequence diversity, usually limited to 3 to 6 amino acid substitutions, in strain

specific enzymes; however, these changes lead to a considerable difference in subtrate profile when the enzymes are tested with a variety of antibiotics (Ambler.1980). This seems to suggest that the amino acid substitutions are possibly the a result of selection pressure applied to each specific species of bacteria when challenged with certain β-lactam antibiotics. The clinical importance of β-lactamase producing bacteria resistant to treatment with penicillins and cephalosporins makes the study of the structure and function of β-lactamase significant. Since the individual amino acid substitutions in this enzyme cause different kinetic properities and different substrate profiles (Ambler,1980), site-specific mutation of the β-lactamase gene to induce individual amino acid changes in β-lactamase structure is an excellent method for study of the structure and function of this enzyme.

Escherichia coli RTEM plasmid mediated β-lactamase has a nucleotide sequence identical to that of *E. coli* cells with β-lactamase coded for by chromosomal genes (Sutcliffe,1978). The gene is included as a selectable marker in many plasmids, for example pBR322 and pTZ18U. This makes the gene readily accessible without the need for considerable cloning from chromosomal genes. Using the nucleotide sequence of pBR322 β-lactamase gene provided by Sutcliffe (1978), the serine residue at position 70 and its corresponding codon, AGC, have been implicated as the active site of the enzyme acting by an acyl-enzyme intermediate before the hydrolysis of the cyclic amide bond of the β-lactam ring (Fisher et al., 1980). Several amino acid substitutions have been made at the active site of the enzyme using site-specific mutation including serine-70 to arginine-70 (Dalbadie-McFarland et al.,1984),

serine-70 and threonine-71 to threonine-70 and serine-71 (Foster et al.,1987), and serine-70 to cysteine-70 (Sigal et al.,1982).

Only the cysteine-70 mutant enzyme was shown to have any hydrolytic activity. This activity has been reported to be 1/30 the activity of the wild-type enzyme when tested with benzylpenicillin and comparing the enzyme kinetics *in vitro* (Foster et al.,1987). However, it was observed that the mutant enzyme had an increased K_m *in vitro* to the cephalosporin nitrocephin (Sigal et al.,1984). Thus, the net substitution of a sulfhydryl group in cysteine for an hydroxyl group in serine seems to generate a new class of enzyme with a unique substrate profile.

As the appearance of β-lactamase was first described in clinical isolates responding to selection pressure from penicillin type antibiotics, the selection pressure from the cephalosporin type antibiotics could possibly select for substitutions such as the cysteine-70 active site β-lactamase due to the increased hydrolytic activity of the enzyme against cephalosporin. It would be of interest to generate a clinical *in vivo* substrate profile in terms of antibiotic resistance provided to a bacteria producing the mutant enzyme.

This study utilizes a M13 derived phagemid vector, pTZ18U, with a selectable marker for ampicillin resistance provided by the RTEM β-lactamase gene from pBR322 (Messing,1983). This phagemid was used in the oligonucleotide-directed mutagenesis procedure modified by Kunkel (1985) to generate a mutant β-lactamase with the amino acid substitution at the active site serine-70 to cysteine-70. This is a unique method for generating this particular amino acid change by the substitution

of an adenine for a thymine at the codon for serine-70 using an M13 derived uracil-containing template DNA and a synthetic oligonucleotide. The mutant β-lactamase was then challenged with a number of β-lactam antibiotics to generate an *in vivo* clinical substrate profile to determine the effects of the mutant enzyme in terms of altered antibiotic resistance as compared to the wild-type enzyme.

CHAPTER II

MATERIALS AND METHODS

Bacterial and Phage Strains

Escherichia coli strain MV1190 is Δ(lac-pro AB), thi, sup E, (Δsr1-rec A)306::Tn10 (tet^r)[F′:tra D36, pro AB, lac $I^qZ\Delta M15$]. This strain, as well as all other bacterial strains, phage, and DNA, were obtained from Bio-Rad Laboratories, Richmond, California. In order to ensure pili formation which is necessary for subsequent infection with bacteriophage M13, this strain contains a deletion in the chromosomal DNA coding for proline biosynthesis which is complemented by the proline synthesis genes in the F plasmid. Thus, this bacteria is grown in glucose minimal media (6g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCl, 1g NH_4Cl, 1mL of 1M $MgSO_4 \cdot 7H_2O$, 0.5mL of 2% thiamine HCl in deionized H_2O, 10mL of 20% glucose, deionized water to 1L) without proline to ensure only cells harboring the F plasmid and pili will be present.

E. coli strain CJ236 is dut-1, ung-1, thi-1, rel A-1; pCJ105 (Cm^r). The *dut*$^-$ and *ung*$^-$ mutations result in the substitution of uracil for thymine at random locations in all DNA synthesized in this strain. In order to ensure pili formation in this strain, the F plasmid pCJ105 (Joyce et al.,1984) provides chloramphenicol resistance. CJ 236 is grown in LB medium (10g Bactotryptone, 5g yeast extract, 5g NaCl, deionized H_2O to 1L) with the addition of chloramphenicol at the concentration of 30μg/mL to ensure pili formation.

For long term storage of bacteria, each strain was grown in its respective medium, and 8.5mL of an overnight culture was added to 1.5mL of sterile glycerol. The cells were mixed thoroughly and stored at -20°C (Maniatus et al.,1982).

M13K07 helper phage (Vieira et al.,1987) has been constructed so that interference by superinfection is partially disabled allowing for the packaging of the phagemid DNA at the expense of the helper phage upon infection. The M13K07 genome also includes a gene for kanamycin resistance so that all cells grown in the presence of kanamycin will be infected by the helper phage. Stock cultures of the helper were prepared using a modified method by Messing (1982). A culture of *E. coli* MV1190 was grown overnight and infected with M13KO7 at a concentration of 1-5 $\times$ 10^{3-5} P.F.U./mL using an overlay of soft LA (LB medium with 7.5g of agar per liter) onto LA (LB medium with 15g of agar per liter). Individual plaques were picked with a sterile pipette and used to innoculate 2mL of 2×YT (16g Bactotryptone, 10g yeast extract, 5g NaCl, deionized H_2O to 1L) which was grown for 3 hours with agitation at 37°C. A culture of uninfected MV1190 was grown under the same conditions and at the end of the incubation, was inoculated with 250μL of the M13KO7 infected cells. The culture was incubated overnight, and M13KO7 was found in the supernatant after centrifugation.

Phagemid DNA

Phagemid pTZ18U (Mead et al.,1986) was constructed from the pUC plasmid series (Norander et al.,1983) such that the single-stranded DNA origin of replication

from bacteriophage f1 was inserted within the β-galactosidase gene. This allows for the packaging of the phagemid in single stranded form when a cell containing the phagemid is infected with helper phage M13KO7. The β-lactamase gene of interest is contained in the phagemid as a selectable marker, and as such, no cloning was required to isolate this gene.

Oligonucleotide DNA

A 5′-phosphorylated 15 base oligonucleotide was obtained from Oligos Etc. Inc., Guilford, Connecticut with the sequence 5′-d(pAAAAGTGCACATCAT)-3′. This oligonucleotide corresponds to the the sequence complementary to the codons for amino acids 68-72 in the β-lactamase gene of phagemid pTZ18U with the exception of a one base substitution at position 7. This base change, adenine for thymine, results in the substitution of the amino acid cysteine for serine at the active site of β-lactamase.

Chemical Reagents

Unless otherwise noted, all chemicals and reagents, including antibiotics, were obtained from Sigma Chemical Company, St. Louis, Missouri.

Extraction and Purification of DNA

A. Double-stranded Plasmid DNA

Plasmid DNA was extracted from the cells using a modification of a technique

provided by Dr. Al Steggles of the Northeastern Ohio Universities College of Medicine. A total of 1.4mL of an overnight culture was transferred to a 1.5mL microcentrifuge tube and harvested by centrifugation for 5 minutes at room temperature. The supernatant was discarded, and an additional 1.4mL of the overnight culture was added to the microcentrifuge tube. The sample was then centrifuged for an additional 5 minutes and the supernatant discarded. The cell pellet was then resuspended in 250μL of STE buffer (100mM NaCl, 10mM Tris pH 7.5, 1mM EDTA).

The plasmid DNA was released from the bacterial cells by the disruption of the cell membrane by the addition of 250μL of a 25:24:1 mixture of phenol/chloroform/isoamyl alcohol to the tube. The contents of the microcentrifuge tube were then vortexed for 2 minutes and immediately microcentrifuged for 10 minutes at 4°C. After centrifugation, the top, aqueous phase containing the DNA was removed and transferred to a new microcentrifuge tube.

The plasmid DNA was precipitated from the aqueous phase by the addition of 0.1 volume of 7.5M ammonium acetate and 2 volumes of ice-cold absolute (100%) ethanol. The solution was stored at -20°C overnight and was centrifuged the next day in a microcentrifuge for 15 minutes at 4°C to pellet the DNA. The supernatant was discarded, and the DNA pellet was thoroughly dried and resuspended in 20μL of TE buffer (10mM Tris pH 7.5, 1mM EDTA). No further purification of the plasmid DNA was found to be necessary.

B. Single-stranded Phagemid DNA

Protein coated single-stranded phagemid DNA was extracted by adding 500μL of phagemid solution in a microcentrifuge tube to an equal volume of STE saturated phenol. The solution was then vortexed for 1 minute and microcentrifuged at room temperature for 2 minutes. The top, aqueous phase was removed and transferred to a new microcentrifuge tube where the phenol extraction was repeated a second time. Three additional extractions were performed, two with a 1:1:1/48 mixture of phenol/chloroform/isoamyl alcohol and one with chloroform alone.

The DNA in the aqueous phase from the last extraction was precipitated by the addition of 0.1 volume of 7.5M ammonium acetate and 2.5 volumes of ice-cold absolute (100%) ethanol. The DNA solution was stored overnight at -20°C and was recovered the next day by centrifugation for 15 minutes at 4°C. The supernatant was discarded, and the DNA pellet was thoroughly dried and resuspended in 20μL of TE buffer. No further purification of the phagemid DNA was found to be necessary.

Analysis of Plasmid DNA Extractions

A. Agarose Gel Electrophoresis

DNA samples were mixed with loading buffer (0.1% bromophenol blue, 1mM EDTA, 1% sodiumdodecyl sulfate, 50% glycerol) and loaded into 10μL wells pre-cast in a 1% agarose gel with the addition of ethidium bromide at a concentration of 1μg/mL. The samples were electrophoresed at 55 milliamps for approximately 1 hour using TAE electrophoresis buffer (40mM Tris acetate, 5mM sodium acetate,

1mM EDTA pH 7.8, 1μg/ml ethidium bromide). After electrophoresis the gel was destained in running distilled water for 15 minutes and photographed using Polaroid Type 667 film. The DNA concentration of each of the samples was estimated by comparing the intensity of the bands generated to known DNA samples (Hackett et al.,1988). Standards were run where necessary to confirm identity of the DNA samples.

B. Spectrophotometry

DNA samples in TE buffer were diluted with distilled water and scanned with a Beckman DU-50 spectrophotometer across a wavelength range of 200-300nm. An appropriate amount of TE buffer diluted similarly with distilled water in the same cuvette was used as a blank in all scans.

Transformation of Plasmid DNA

Bacterial cells were made competent to accept DNA using a modified calcium chloride technique (Bio-Rad). Bacterial cells were grown, CJ236 in LB media supplemented with chloramphenicol at 30μg/mL and MV1190 in glucose minimal media, and approximately 1mL of an overnight culture was added to 40mL LB media to give an OD_{600} of 0.1. The diluted culture was then incubated with shaking at 37°C until an OD_{600} of 0.8-0.9 was reached.

The entire culture was harvested by centrifugation at 0°C for 5 minutes at 4,000 × g. The supernatant was drained, discarded, and replaced with 1mL of ice-

cold 50mM $CaCl_2$ and then resuspended by gentle pipetting in an ice water bath. An additional 19mL of ice-cold 50mM $CaCl_2$ was added, and the cells were held on ice for 1 hour.

At the end of the incubation on ice, the cells were again harvested by centrifugation at 0°C for 5 minutes at 4000 × g. The supernatant was discarded and replaced with 1mL of ice-cold 50mM $CaCl_2$. After gentle resuspension, an additional 3mL of ice-cold 50mM $CaCl_2$ was added. The dilute cells were then stored overnight at 0°C. The cells were then considered to be competent to accept the plasmid DNA.

To initiate transformation of the cells, 0.3mL of cells prepared as described above were transferred to a microcentrifuge tube on ice, and to these competent cells, 1-10ng of plasmid DNA was added. The cells were then incubated on ice for 90 minutes to allow for complexing of the DNA with the cells. The microcentrifuge tube was then heat shocked by transferring it to a water bath at 42°C for 3 minutes and then returned to ice for 15 minutes.

The cells, with the addition of 1mL of LB media, were grown at 37°C with shaking for 1 hour to allow for production of β-lactamase and its accompanying ampicillin resistance. Ten μL of the cells were then spread on the surface of a LA plate supplemented with ampicillin at 50μg/mL and incubated overnight.

Growth of Uracil-Containing Phagemids

E. coli CJ236 was grown overnight in LB medium with the addition of chloramphenicol at 30μg/mL. One mL of the overnight culture was added to 50mL

2xYT and incubated with shaking at 37°C for 3 hours or until the culture reached an OD_{600} of 0.3.

M13KO7 helper phage were then added to the culture with a multiplicity of infection of 50 phage per cell. The infected culture was then incubated for an additional hour before 70μL of a 50mg/mL kanamycin stock was added. This was then incubated under the same conditions overnight.

To harvest the phagemids, 45mL of the culture was centrifuged at room temperature for 15 minutes at 4000 × g. The supernatant was collected and transferred to another tube where the centrifugation was repeated under similar conditions. The supernatant from the second centrifugation was then transfered to a fresh centrifuge tube where 200μg of DNase-free RNase A was added. The tube was then allowed to remain at room temperature for 30 minutes.

The phagemids were precipated from the liquid by the addition of NaCl to a concentration of 1M and PEG-6000 to a concentration of 10%. The solution was stirred at 4°C until the NaCl and PEG were dissolved and then stored in an ice-water bath for 2 hours. The phagemids were collected by centrifugation at 0°C for 20 minutes at 17,000 × g. The supernatant was discarded, and the phagemid pellet was then resuspended in 250μL of high salt buffer (300mM NaCl, 100mM Tris at pH 7.5, 1mM EDTA). The suspension was transferred to a microcentrifuge tube and held in an ice-water bath for 1 hour, and was then microcentrifuged for 2 minutes to remove insoluables from the supernatant.

Determination of Uracil Content in Phagemid DNA

In order to ascertain the effectiveness of the *dut*⁻ *ung*⁻ mutations of *E. coli* CJ236 in substituting uracil for thymine at various locations in the double-stranded plasmid, the phagemid was titered on both *E. coli* CJ236 and MV1190 (Bio-Rad).

Cultures of both MV1190 and CJ236 were grown overnight with shaking at 37°C, MV1190 in glucose minimal media and CJ236 in LB media supplemented with chloramphenicol at the concentration of 30μg/mL . Two 50mL aliquots of 2xYT were inoculated, one with 0.5mL of MV1190 and one with 1mL of CJ236 and incubated with shaking at 37°C until the OD_{600} of both cultures reached 0.3. Ten μL of phagemid stock was added to each culture and grown in a similar manner for an additional 2 hours. Each culture was then diluted 10-fold, 100-fold, and 1000-fold with LB medium. LA plates were then inoculated with 100μL samples of each of the dilutions and allowed to incubate overnight at 37°C.

In vitro Mutagenesis

The mutagenesis protocol used was a combination of methods from Geisselsoder et al. (1987), Gilliam et al. (1979), and Kunkel et al. (1985). It was necessary to modify the original procedures used as the reaction conditions change with variables such as the site of mutation, length of the oligonucleotide, and number of base substitutions (Bio-Rad). To anneal the oligonucleotide to the single-stranded phagemid DNA template, 200-1000ng of uracil-containing template DNA and oligonucleotide to a concentration of 9-20pM was added to a sterile 500μL

microcentrifuge tube containing 1μL of 10X annealing buffer (200mM Tris-HCl at pH 7.4, 20mM $MgCl_2$, 500mM NaCl). The volume of the reaction was then adjusted to 10μl with sterile distilled H_2O. A second reaction similar to the above was also prepared without the oligonucleotide to serve as a negative control for spontaneous synthesis of double-stranded DNA. The tubes were then placed in a water bath at 70°C and allowed to cool to room temperature over a 90 minute period.

The complementary strand was synthesized on the template from the oligonucleotide primer by the addition of 1μL of 10X synthesis buffer (4mM of each dNTP, 7.5mM ATP, 175mM Tris-HCl at pH 7.4, 37.5mM $MgCl_2$, 5mM dithiothreitol), 5 units of T4 DNA ligase, and 2 units of T4 DNA polymerase. The reaction was held on ice for 15 minutes, at room temperature for 5 minutes, and then incubated at 37°C for 2 hours. At the end of the incubation, 90μL of TE buffer was added, and the tubes were frozen to stop the reaction.

Evidence of Cysteine Substitution in the *β*-lactamase

A. Osmotic Shock Release of *β*-lactamase

E. coli MV1190 with the mutant or wild-type plasmid was cultured overnight in 2xYT medium with the addition of ampicillin at the concentration of 50μg/mL. The cells were harvested by centrifugation at 4°C for 5 minutes at 4,000 × g. The supernatant was discarded and the cells washed in 25mL of sterile 30mM Tris-HCl at pH 7.0. The cells were then resuspended by vortexing and pelleted by centrifugation in a similar manner. The supernatant was again discarded and the

cells resuspended in 25mL of a 20% solution of sterile sucrose in 30mM Tris-HCl at pH 7.0 and 1mM EDTA. The suspension of cells in sucrose was transferred to an erlynmeyer flask and mixed with shaking for 15 minutes at room temperature. The cells were then harvested by centrifugation and the supernatant was discarded.

The cell pellet was chilled in an ice-water bath for 15 minutes before the addition of 4mL of ice-cold sterile, distilled H_2O. The suspension was then vortexed and held on ice for 10 minutes before the addition of 0.4ml of 100mM KH_2PO_4 at pH 7.0. The cells were then held on ice for an additional 10 minutes and centrifuged at 4°C for 20 minutes at 12,000 × g. The supernatant was decanted, frozen at -70°C and subsequently tested for β-lactamase activity.

B. Iodophore Test for β-lactamase Activity

The mutant and wild type β-lactamases were tested using a modification of the iodophore reaction described by Ross et al. (1973). This reaction utilizes a change in color of an iodine solution from yellow to clear when iodine reacts with penicillionic acid, the product of penicillin hydrolysis by β-lactamase.

In a 15mL test tube, 1ml of ampicillin at the concentration of 3mg/mL was added to 3mL of aqueous medium (100mM KH_2PO_4, 0.5% gelatin). One mL of iodine reagent (25mM I_2 in 125mM KI) was added, and the tube was transferred to a water bath at 30°C for 5 minutes. While in the water bath, 1mL of β-lactamase extracted as described above was added. A positive test is indicated by a color change from a yellow solution to clear. Two negative controls were always run

parallel to the sample tube, one with all the contents except the β-lactamase and one with all the contents except the ampicillin.

C. Inhibition of Mutant β-lactamase Activity

To confirm the active site substitution of cysteine for serine, p-chloromercuriobenzoic acid, a cysteine modifying agent and inhibitor of the mutant enzyme, was used in the above protocol. A total of 100μL of a 5mM stock solution of p-chloromercuriobenzoate was added to each tube prior to the addition of the β-lactamase.

D. Inhibition of Wild-Type β-lactamase Activity

To confirm the active site substitution of cysteine for serine, boric acid, an inhibitor of the wild-type enzyme, was used in the above protocol. Boric acid was added to each tube to a final concentration of 10mM prior to the addition of the β-lactamase.

Microdilution Sensitivity Assay

To generate a substrate profile for the mutant β-lactamase, microdilution sensitivity tests were performed using 4 concentrations of each antibiotic to be tested. The procedure was performed in a sterile, 96 well microdilution plate (Falcon #3042). Fifty μL of LB media was added to wells 2 through 11 of the plate. Fifty μL of a stock antibiotic solution was added to wells 1, 2, and 12 in each of the four

rows. The antibiotic solution was then serially diluted 2-fold by mixing and transferring 50μl from well 2 to well 3 via the use of a 50μL tulip diluter. Well 3 was then mixed and 50μl was then transferred to well 4. This process was continued until well 9, where 50μl was discarded after mixing.

Overnight cultures of *E. coli* MV1190, both with the mutant and wild-type plasmid, were grown in LB medium with shaking at 37°C and subsequently diluted 1000-fold before being used to inoculate wells 1 through 9 and 11 in each of the four rows. The microdilution plates were incubated overnight at 37°C, and the OD_{600} of each of the wells was read using a Bio-Tek Instruments EL311 Microplate Reader. The minimal inhibitory concentration (MIC) was considered to be the lowest concentration of antibiotic at each dilution at which growth was inhibited with respect to the positive growth control.

CHAPTER III

RESULTS

Extraction of pTZ18U from *E. coli* MV1190

Double-stranded pTZ18u phagemid DNA was extracted with a mini-prep procedure utilizing a single cell membrane lysis and extraction step with 25:24:1 mixture of phenol:chloroform:isoamyl alcohol. A 10μL sample of the extracted phagemid in TE buffer was loaded onto a 1% agarose electrophoresis gel along with appropriate controls and electrophoresed at 55 milliamps for approximately 1 hour. A photograph of the gel is shown as figure 1. The wells and positive electrode are positioned to the left and the negative electrode the right. The DNA samples electrophoresed in each lane on the gel are as follows: Lane 1, HindIII digested λ DNA/Hinc III digested ϕX174 RF DNA; Lane 2, plasmid pBR322; Lane 3, Bio-Rad standard phagemid pTZ18U; Lanes 4 and 5, extracted samples of phagemid pTZ18u (10μL in each well). The estimated phagemid DNA concentration, when compared to standard amounts of DNA electrophoresed in the same manner (Hackett et al.,1988), was determined to be approximately 50ng/μL.

A 1:10 dilution of the phagemid DNA sample was then examined for contaminants by spectrophometry using an OD_{260}/OD_{280} ratio. This value was calculated to be 1.9 indicating the phagemid DNA sample was free from any major protein or phenol contaminants (Maniatus et al.,1982), and the DNA was acceptable for subsequent use.

Transformation of CJ236 with Phagemid pTZ18U

E. coli CJ236 cells, made competent by a modified calcium chloride procedure (Bio-Rad), were transformed with 10ng of phagemid DNA. A 1:100 and 1:1000 dilution of these cells was then plated on the surface of LA plates with the addition of ampicillin at the concentration of 50μg/mL to select for transformed cells. After an overnight incubation, 44 colonies were found on the plate corresponding to the 1:1000 dilution. A single colony was picked and grown in LB media with ampicillin (50ug/mL). This culture of CJ236/pTZ18U was used for subsequent procedures.

Analysis of Uracil Content in Single-Stranded Phagemid DNA

The uracil content of single-stranded phagemid particles produced by infection of CJ236/pTZ18U cells with M13KO7 helper phage was analyzed by titering the phagemid particles on both CJ236 and MV1190. If the single-stranded DNA contains a sufficient amount of uracils substituted for thymines in the DNA, the phagemid titer will be approximately 10^4 higher on the bacteria (CJ236) with an inactive uracil N-glycosylase and deoxyuraciltriphosphatase than on the bacteria (MV1190) able to synthesize both of these enzymes. The titer yielded 6.6 x 10^7 ampicillin resistant colonies upon infection of CJ236 and 2 x 10^2 ampicillin resistant colonies upon infection of MV1190. This indicates a sufficient uracil content was obtained in the single-stranded phagemid DNA and is suitable for use as a template in the oligonucleotide-directed mutagenesis procedure once extracted and purified.

Extraction of Single-Stranded pTZ18U Phagemid DNA

The protein coat was removed from the phagemid particles with phenol, and the single-stranded DNA was concentrated by ethanol precipitation. A 10μL sample of phagemid DNA resuspended in TE buffer was loaded into wells of a 1% agarose electrophoresis gel along with the appropriate controls and electrophoresed at 55 milliamps for approximately 1 hour. A photograph of the gel is seen as figure 2. The wells and positive electrode are positioned to the left in the photograph and negative electrode the right. The DNA samples electrophoresed in each well of the gel are as follows: Lane 1, Hind III digested λ DNA; Lane 2, plasmid pBR322; Lane 3, double-stranded phagemid pTZ18U; Lane 4, Bio-Rad standard single-stranded pTZ18U; Lane 5, extracted sample of single-stranded pTZ18U. The estimated single-stranded phagemid DNA concentration, when compared to standard amounts of DNA electrophoresed in the same manner (Hackett et al.,1988), was found to be about 25ng/μL.

A 1:10 dilution of the single-stranded phagemid DNA sample was then examined for contaminants by spectrophometry using an OD_{260}/OD_{280} ratio. This value was calculated to be 2.0 indicating the extracted single-stranded uracil-containing phagemid DNA sample was free from any major protein or phenol contaminants (Maniatus et al.,1982) and was suitable for use as a template in the mutagenesis reaction.

Electrophoretic analysis of *in vitro* Mutagenesis Products

After the completion of the *in vitro* mutagenesis and synthesis of the complementary strand to return the phagemid to its original double-stranded form, the mutated sample was electophoresed against both single and double stranded forms of the phagemid pTZ18U. If complete synthesis of the second strand took place, the mutated sample should migrate to a position similar to the double-stranded form of pTZ18U. A 10μL sample of two separate *in vitro* mutagenesis reactions were loaded into individual wells in a 1% agarose gel along with the appropriate controls, and the gel was electrophoresed for approximately 1 hour at 55 milliamps. A photograph of the gel is seen as figure 3. The wells and negative electrode are positioned to the left in the photograph and the positive electrode toward the right. The DNA samples electrophoresed in each of the wells are as follows: Lane 1, double-stranded pTZ18U; Lane 2, mutagenesis reaction using 6pmol of synthetic oligonucleotide; Lane 3, mutagenesis reaction using 9pmol of synthetic oligonucleotide; Lane 4, single-stranded pTZ18U. A band migrating the same distance as the double-stranded phagemid standard can be seen in both experimental samples (Lanes 2 and 3). The highest concentration of double-stranded form found in well 2. This sample was used to transform *E. coli* MV1190.

Transformation of Mutant Phagemid into *E. coli* MV1190

E. coli MV1190 cells, made competent by a modified calcium chloride procedure (Bio-Rad), were transformed with 10μL of the phagemid from the

mutagenesis reaction. Ten μL of the cells were then plated of the surface of LA plates with the addition of ampicillin at the concentration of 10μg/mL. This concentration of antibiotic is sufficient to eliminate untransformed cells from cells containing both the mutant and wild-type phagemid. After an overnight incubation, 77 colonies were found on the plate.

To separate the cells containing the mutant phagemid from the wild-type, 30 cells at random were chosen and replicate plated onto LA plates containg ampicillin at the concentrations 10μg/mL, 50ug/mL, 100μg/mL, 500μg/mL, 1000μg/mL, and 5000μg/mL. The plates were then incubated overnight. Seven replicates were found to grow on the plates with ampicillin concentrations of 10 and 50μg/mL only while all others grew on the plates at all concentrations. In view of a previous mutagenesis study (Sigal et al.,1982) which stated that ampicillin resistance of cysteine mutant β-lactamase possessing bacteria is decreased with respect to that of the wild-type enzyme, the seven replicates were selected as suspected mutants to be tested further.

Evidence of the Cysteine Substitution in the Mutant β-Lactamase

It was found by Ross et al. (1973) that the hydrolytic activity of β-lactamase on penicillin type antibiotics could be detected *in vitro* by the decolorization of iodine in the presence of penicillionic acid, the hydrolysis product of β-lactamase action on penicillin. A replicate and wild-type MV1190 colony were chosen and grown overnight in 2xYT medium. The β-lactamase from each culture was released via osmotic shock by incubation in 20% sucrose then transfered to distilled water and

tested using the iodophore reaction. Both the mutant and wild-type β-lactamases showed decolorization of the iodine with the wild-type occurring nearly 10-fold faster.

The mutant and wild-type β-lactamases were then challenged with boric acid and reexamined via the iodophore test. Boric acid is a competitive inhibitor of the wild-type enzyme due to the serine residue at the active site. The acid should not compete with the cysteine active site of the mutant enzyme (Sigal et al.,1984). Only the mutant enzyme showed decolorization of the iodophore reagent in the presence of 10mM boric acid after 15 minutes. Finally, the mutant and wild-type β-lactamases were challenged with p-chloromercuriobenzoic acid and again reexamined via the iodophore test. P-chloromercuriobenzoic acid inactivates enzymes by modifying the cysteine residue at the active site (Sigal et al.,1982). Only the wild-type enzyme showed decolorization of the iodophore reagent in the presence of 1mM p-chloromercuriobenzoic acid.

Clinical Substrate Profile of Mutant β-Lactamase

Microdilution sensitivity tests were performed to determine the *in vivo* response of the bacteria to the mutant enzyme in terms of antibiotic resistance to both penicillin and cephalosporin type antibiotics. As can be seen from table 1, the resistance of *E. coli* MV1190 with the wild-type β-lactamase gene to ampicillin is considerably higher than that of MV1190 with the mutant β-lactamase gene. The mutant enzyme does in fact provide ampicillin resistance, albeit decreased with respect to the wild-type enzyme, over that of MV1190 without a β-lactamase gene

with and average minimal inhibitory concentration value of 10μg/mL of ampicillin. These values are qualitatively predicted by previous *in vitro* studies (Sigal et al.,1982,1984). Table 1 includes the minimal inhibitory concentration values obtained for several species of penicillin series antibiotics.

As can be seen from table 2, the mutant β-lactamase provides protection to the bacterial cell well above that of the wild-type enzyme when challenged with cephalosporins. The cysteine active site substitution does not just decrease the activity of the enzyme but generates a completely unique substrate profile. These values are consistent with the kinetics values for both the mutant and wild-type enzymes for hydrolysis of penicillin and cephalosporins (Sigal et al.,1984).

FIGURE 1
Agarose Gel Electrophoresis of Double-Stranded pTZ18U Phagemid DNA
Lane 1-Hind III digested λ DNA/Hinc III digested φX174 DNA (size standard)
Lane 2-plasmid pBR322 (size standard)
Lane 3-Bio-Rad standard phagemid pTZ18U
Lane 4-extracted sample of phagemid pTZ18U
Lane 5-extracted sample of phagemid pTZ18U

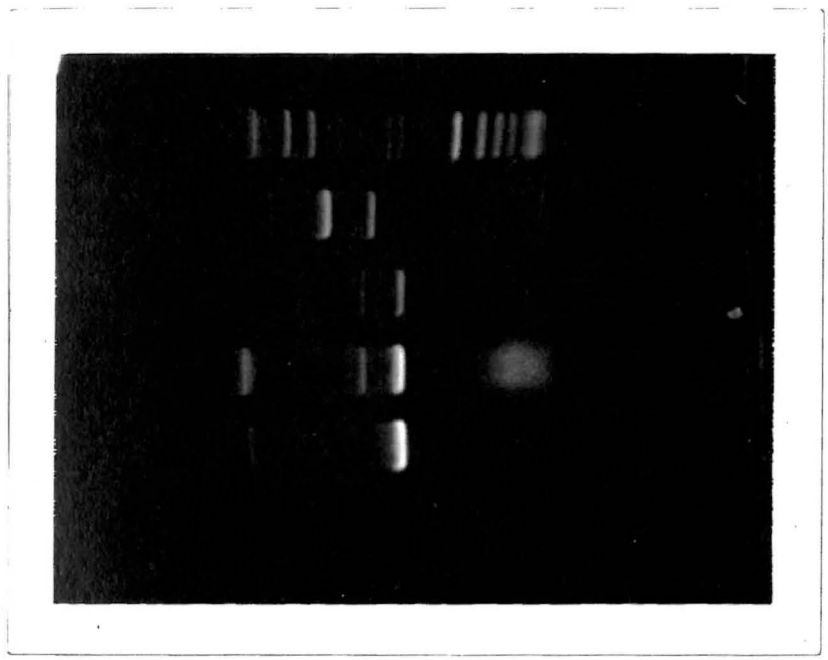
1
2
3
4
5

FIGURE 2
Agarose Gel Electrophoresis of Single-Stranded pTZ18U Phagemid DNA
Lane 1-Hind III digested λ DNA (size standard)
Lane 2-plasmid pBR322 (size standard)
Lane 3-double-stranded phagemid pTZ18U
Lane 4-Bio-Rad standard single-stranded phagemid pTZ18U
Lane 5-extracted sample single-stranded phagemid pTZ18U

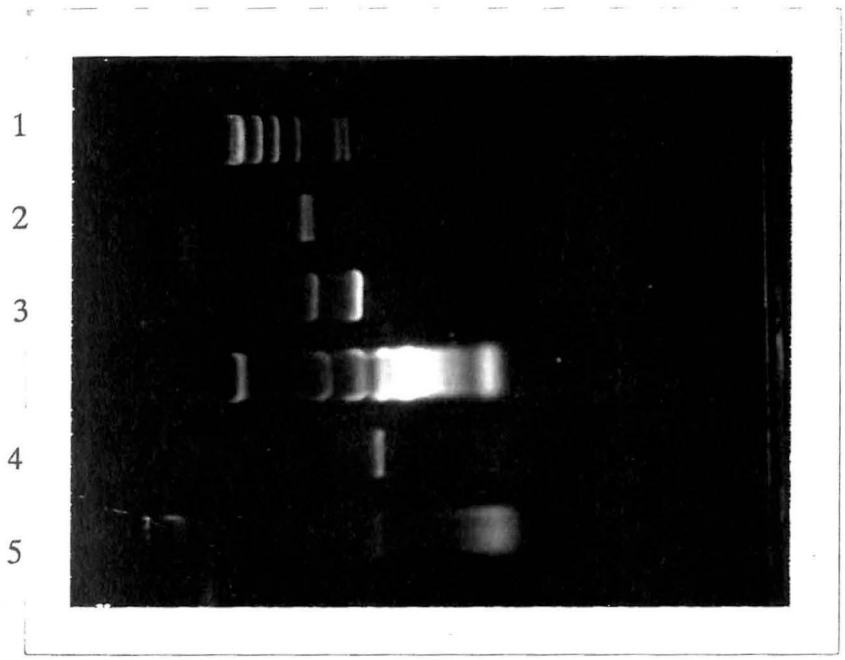
1
2
3
4
5

FIGURE 3
Agarose Gel Electrophoresis of *in vitro* Mutagenesis Products
Lane 1-double-stranded phagemid pTZ18U
Lane 2-mutagenesis product (6pmol of oligonucleotide)
Lane 3-mutagenesis product (9pmol of oligonucleotide)
Lane 4-single-stranded phagemid pTZ18U

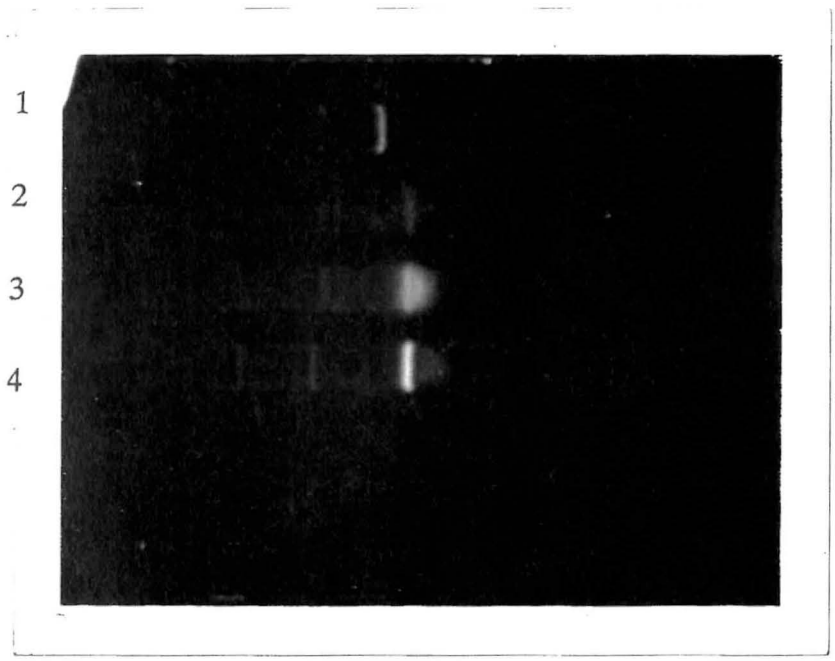
1
2
3
4

TABLE 1
Microdilution Sensitivity Test Results Reported as Minimal Inhibitory Concentration Values (in μg/mL) for Penicillin Series Antibiotics

ANTIBIOTIC STOCK SOLUTION	Wild-Type β-Lactamase Minimal Inhibitory Concentration (μg/mL)	Mutant β-Lactamase Minimal Inhibitory Concentration (μg/mL)
Benzylpenicillin		
125μg/mL	15625	-
25.0μg/mL	12500	97.66
2.5μg/mL	+	78.13
0.25μg/mL	+	62.5
Ampicillin		
125μg/mL	15625	-
25.0μg/mL	12500	48.83
2.5μg/mL	+	39.06
0.25μg/mL	+	31.25
Carbenicillin		
125μg/mL	7812.5	244.14
25.0μg/mL	6250	195.31
2.5μg/mL	+	125
0.25μg/mL	+	+
Methicillin		
125μg/mL	7812.5	-
25.0μg/mL	6250	97.66
2.5μg/mL	+	78.13
0.25μg/mL	+	62.5

Values obtained in each row are from dilutions of a stock solution of 250μg/mL of each antibiotic.
+ corresponds to growth in all wells
- corresponds to no growth in all wells

TABLE 2
Microdilution Sensitivity Test Results Reported as Minimal Inhibitory Concentration Values (in μg/mL) for Cephalosporin Series Antibiotics

ANTIBIOTIC STOCK SOLUTION	Wild-Type β-Lactamase Minimal Inhibitory Concentration (μg/mL)	Mutant β-Lactamase Minimal Inhibitory Concentration (μg/mL)
Cephalosporin C		
125μg/mL	-	3906.25
25.0μg/mL	195.31	3125
2.5μg/mL	156.25	+
0.25μg/mL	125	+
Cephalothin		
125μg/mL	-	488.28
25.0μg/mL	244.14	390.62
2.5μg/mL	195.31	312.5
0.25μg/mL	+	+
Cefoperazone		
125μg/mL	-	-
25.0μg/mL	-	48.83
2.5μg/mL	4.88	39.06
0.25μg/mL	1.95	31.25

Values obtained in each row are from dilutions of a stock solution of 250μg/mL of each antibiotic.
+ corresponds to growth in all wells
- corresponds to no growth in all wells

CHAPTER IV

DISCUSSION

Site-specific mutagenesis is one of the most useful and powerful tools for the study of protein structure and function. Specific nucleotides can be targeted in such a way that individual and deliberate amino acid substitutions in the natural sequence of a protein can be studied. Altering the nucleotide sequence to induce protein modification has several advantages over chemical modification or *in vitro* synthesis of mutant proteins. First, since the nucleotide sequence corresponding to mutant protein is available, that sequence can be introduced into a bacterial cell where natural protein synthesis can generate an abundant supply of the mutant protein. Second, the phenotypic effect of the mutant protein can also be studied in the bacterial cell. With the availability of amino acid sequences of many proteins and the relative ease of synthesis of small sequences of DNA, oligonucleotide-directed mutagenesis as a means of targeting mutations has become routine.

Six basic criteria must be met in order to sucessfully complete any oligonucleotide-directed mutation. These include: 1) the sequence of the gene of interest must be cloned into a vector recognized by a bacterial cell, 2) the sequence of the gene must be known, 3) the sequence to be mutated must be available in single-stranded form, 4) an oligonucleotide that contains the desired mutation must be available, 5) the remainder of the sequence away from the mutation must be accurately replicated, 6) the wild-type sequence must be separated from the mutant

sequence (Craik,1985).

In this study, the sequence to be mutated was the active site of *Escherichia coli* RTEM β-lactamase. The active site, elucidated by Fisher et al. (1980), was found to be serine-70 in the linear amino acid sequence as described by the nucleotide sequence of Sutcliffe (1978). This enzyme functions through an acyl-enzyme intermediate at the nucleophilic active site to hydrolyze the β-lactam cyclic amide bond in β-lactam antibiotics, of which the cephalosporin and penicillin series are the most common (Sykes et al.,1982). Hydrolysis of the both types of antibiotics leads to the formation of nonbacteriocidal acids which in turn provides bacteria producing this enzyme with resistance to β-lactam antibiotics.

With the relative ease of purification and availability of nucleotide sequences, the class A gram negative β-lactamases, including *E. coli* RTEM β-lactamase, remain the most frequently studied (Ambler,1980). Many mutagenesis studies have been done at or near the active site of this enzyme to study its catalytic properities including: Dalbadie-McFarland et al., serine-70 to arginine-70 (1984); Dalbadie-McFarland et al., serine-70 and threonine-71 to threonine-70 and serine-71 (1987); Dalbadie McFarland et al., threonine-71 to serine-71 (1985); Sigal et al., serine-70 to cysteine-70 (1982). Of all the mutants constructed, only the mutants with a primary nucleophile, cysteine or serine, at the active site retain any hydrolytic activity. The serine-70 serine-71 mutant shows a 15% decrease in *in vitro* catalytic activity to penicillin and cephalosporin with respect to the wild-type (Dalbadie-McFarland et al.,1985) while the cysteine-70 mutant shows a similar K_m as the wild-type for

penicillin and 10-fold increase in the K_m for cephalosporin (Sigal et al.,1982). This suggests that the amino acid at the active site dictates the substrate specificity of the enzyme, and substitution of nucleophiles may generate a new class of β-lactamase instead of simply inhibiting activity.

The RTEM β-lactamase gene is often included as a selectable marker in many plasmid and phagemid vectors for cloning. The production of the enzyme confers ampicillin resistance on the bacterial cell; thus only cells carrying the vector will grow in media supplemented with antibiotic. Phagemid vector pTZ18U (Messing et al.,1987) contains the RTEM β-lactamase gene from plasmid pBR322 which is identical in sequence to the chromosomal *E. coli* β-lactamase gene (Sutcliffe et al.,1978), and thus fulfills the requirement of mutagenesis reactions that the sequence of interest be cloned into a vector capable of expression and independent replication.

The phagemid pTZ18U is included as a stable, "plasmid-like" replicon in *E. coli* strain MV1190 and accordingly, must be extracted from the bacteria in a manner identical to standard plasmid extraction techniques (Maniatus et al.,1982). A rapid and simple extraction protocol was used which only required one step to lyse and remove the cellular debris. The phagemid was subsequently concentrated by ethanol precipitation and subjected to agarose gel electrophoresis. The extracted phagemid sample showed little chromosomal DNA or RNA contamination and migrated similar to the control pTZ18U indicating the phagemid was sucessfully recovered and is suitable for further use.

The extracted phagemid sample was used to transform *E. coli* CJ236. This strain contains a *dut*$^-$ *ung*$^-$ double mutation and represents the important addition to the typical oligonucleotide-directed mutagenesis procedures, provided by Kunkel (1985). The mutations in CJ236 inactivate two important error correcting enzymes, uracil N-glycosylase and deoxyuraciltriphosphatase, which are responsible for the removal of erroneously incorporated uracils in newly synthesized DNA. Thus, the transformed phagemid, after replication in the bacteria, will have uracils substituted for thymines at various locations. This does not interrupt the coding sequence of the genes in the phagemid because uracil both codes as and substitutes for thymine (Kunkel,1985). The uracil substitutions provide a means to select against the parental wild-type strand when used in an *in vitro* mutagenesis reaction.

Single-stranded template DNA was produced from the double-stranded uracil-containing phagemid by the infection of the CJ236 with helper phage M13KO7. The pTZ18U phagemid contains the intergenic region of bacteriophage f1 which allows the phagemid to be packaged in single-stranded form upon infection with the helper phage (Messing et al.,1987). A good titer of the viral particles was obtained after infection of CJ236 with M13KO7, and uracil content of the single-stranded viral DNA was confirmed by a 10^5-fold higher titer on CJ236 than on MV1190, the strain wild-type for both uracil N-glycosylase and deoxyuraciltriphosphatase.

It then had to be established if the phagemid was indeed packaged in place of the original helper viral DNA which would yield a similar viral. The DNA was extracted from the viral protein coat with phenol and ethanol precipitated. Agarose

gel electrophoresis showed a band migrating to a position similar to that of the control single-stranded pTZ18U indicating that single-stranded pTZ18U was synthesized, and the phagemid was indeed packaged at the expense of the viral DNA.

The uracil-containing single-stranded phagemid was then used as a template in the *in vitro* mutagenesis reaction. This reaction utilizes a mutagenic oligonucleotide responsible for the substitution of thymine for adenine in the codon for the active site serine-70 residue of β-lactamase. This substitution replaces the serine (AGC) with cysteine (TGC) at the active site; however, the net change in the final protein is only the replacement of the active site hydroxyl group of serine with the sulfhydryl group of cysteine. Since only a one base substitution took place, the relative size and conformation of the mutant and wild-type double-stranded phagemids should be similar. Agarose gel electrophoresis of the reaction products showed a band that migrated to a position on the gel comparable to the wild-type double-stranded phagemid; thus, double-stranded DNA was synthesized *in vitro* from the mutagenic oligonucleotide primer on the uracil-containing template. The newly synthesized mutant/wild-type heteroduplex phagemid was then used to transform *E. coli* MV1190. This strain is wild-type with respect to the production of uracil N-glycosylase and deoxyuraclitriphosphatase. These error correcting enzymes cleave the misincorporated uracils and inactivate the parental wild-type strand thus selecting for the survival and replication of the mutant sequence (Kunkel,1985).

Although nucleotide sequence analysis is often employed to confirm suspected mutants, in this study, phenotypic selection and biochemical traits of the mutant β-

lactamase were employed. The primary screening of bacteria possessing the mutant phagemid was done on the basis of resistance of the cells to ampicillin. Sigal et al. (1984) reported in a study of the enzyme kinetics properities of cysteine-70 β-lactamase, that ampicillin resistance of cells expressing the mutant enzyme is decreased nearly 100-fold. When the ampicillin resistance of the wild-type and cysteine-70 mutant β-lactamase expressing cells developed in this investigation was tested, minimal inhibitory concentration values of 15625-12500μg/mL and 97.66-62.5μg/mL respectively were obtained. This is a decrease in resistance of the mutant of approximately 100-fold suggesting that the intended mutation did indeed occur. An *in vitro* assay was also used to further establish if the amino acid substitution did occur. Ross et al. (1973) described a technique by which β-lactamase activity could be detected by the decolorization of iodine in the presence of the product of hydrolysis of penicillins, penicillionic acids. Extracted β-lactamase from wild-type cells was shown to decolorize the iodine almost instantaneously upon its addition while the mutant enzyme required nearly 10-fold longer time period for decolorization. This suggests that the rate of hydrolysis of the mutant enzyme was decreased with respect to the wild-type, consistent with the k_{cat} values for both obtained by Sigal et al. (Sigal et al.,1984).

Further proof of the cysteine-70 substitution was established by the use of the iodophore test to detect penicillin hydrolysis in the presence of inhibitors specific for either the wild-type or mutant enzymes. Boric acid was shown previously to inhibit the action of wild-type β-lactamase while having only a small noncompetitive

inhibitory effect on the mutant enzyme (Sigal et al.,1984). Decolorization of iodine in the presence of boric acid showed that the enzyme was indeed not wild-type with respect to the serine active site. To determine if the cysteine was now in place at the active site, the enzyme was then challenged with a specific cysteine-modifying agent, p-chloromercuriobenzoic acid, and tested again with the iodophore reaction. The p-chloromercuriobenzoic acid blocked the decolorization of iodine by the mutant enzyme but had no effect on the wild-type enzyme. Taken together these tests confirm that the oligonucleotide-directed mutagenesis procedure changed the active site serine-70 to that of cysteine-70, and suggest that this substitution is a direct result of the nucleotide substitution of adenine for thymine at the codon for amino acid 70 changing from AGC (serine) to TGC (cysteine).

Cysteine mutant β-lactamase has been extensively characterized with respect to enzyme kinetics (Sigal et al.,1984); however, this study focuses on the phenotypic response of the bacteria, in terms of antibiotic sensitivity, to the cysteine-70 mutant β-lactamase. Sigal et al. (1984), noted a decrease in k_{cat} values for the mutant β-lactamase with ampicillin, 2% activity with respect to that of the wild-type, while the K_m values for both are the same. This would correspond to the decrease in resistance to ampicillin, in terms of minimal inhibitory concentration values, of approximately 100-fold with the cysteine active site enzyme. The 100-fold decrease is also consistent with reported decrease ampicillin resistance (Sigal et al.,1984). The net decrease of resistance of the bacteria with mutant β-lactamase when compared to the wild-type is also seen with other penicillin series antibiotics, approximately

100-fold decrease with methicillin and 50-fold decrease with carbenicillin. It should be kept in mind that antibiotic resistance is also a function of outer membrane permeability of the bacterial cell to the antibiotic (Nikaido et al.,1987), as well as the folding properities of the β-lactamase in the periplasmic space (Challes et al.,1982). Thus, relationships between kinetics values and minimal inhibitory concentration values for each antibiotic must only be treated on a qualitative basis.

It is interesting to note that the ampicillin resistance reported here, 15625-12500μg/mL, is over 10-fold greater than that reported by Sigal et al. (1984) for the wild-type β-lactamase. Similarly, the mutant β-lactamase also shows nearly a 10-fold greater resistance than reported, 97.66-62.5μg/mL. Several factors may be involved in this difference. First, the strain of *E. coli* used in each study is different. The antibiotic resistance of strains HB101 and MM294 without a β-lactamase gene, the strains used by Sigal et al., were reported to be less than 5μg/mL while that of MV1190, used in this study, was 10μg/mL. This may be due in part to a difference in the fine structure of the cell membrane from strain to strain leading to altered reactivity in response to plasmid mediated β-lactamase production. Different strains of bacteria within a species have been shown to have different membrane permeabilities to β-lactam antibiotics which directly effect antibiotic resistance (Nikaido et al.,1987). Also, even though the β-lactamase genes used in each study share identical nucleotide sequences, the vectors used are different. Phagemid pTZ18U was used in this study while plasmid pBR322 was used by Sigal. As the copy number of different vectors is often different (Challes et al.,1982), this may also

be correlated to antibiotic resistance since RTEM β-lactamase is produced constitutively from the all available sequences, not inducibly in response to antibiotic concentration (Ambler et al.,1980).

The antibiotic resistance of the mutant β-lactamase to the cephalosporin series was increased compared to that of the wild-type enzyme particularly with cephalosporin C. Sigal et al. (1984) reports the K_m of the mutant enzyme with the cephalosporin nitrocephin to be nearly 10-fold greater than that of the wild-type. The k_{cat} value obtained is also increased, but definitive values were not reported. It appears from the kinetic data that the cysteine substitution at the active site has an increased affinity for cephalosporins. However, as the rate limiting step has not been determined for the cysteine-70 mutant enzyme (1984), it is difficult to correlate kinetics data with any observed antibiotic sensitivity toward cephalosporins. Perhaps an increase in k_{cat} value may correspond to a qualatative increase in antibiotic resistance. Cephalosporin antibiotics have different membrane permeabilities than those of the penicillin series (Nikaido et al.,1987). This may be involved in the antibiotic resistance and in any differences noted between the *in vitro* kinetics data and *in vivo* antibiotic sensitivity responses.

The studies of mutant β-lactamases to date have focused on the altering of amino acid sequence as a method to study the change in kinetic properities of the wild-type enzyme; however, the clinical implications of mutant β-lactamases have gone relatively unstudied. The clinical importance of β-lactamase is evident in the fact that the enzyme destroys the bacteriocidal properities of the β-lactam antibiotics,

penicillins and cephalosporins. With the introduction of penicillin as an antibiotic in 1940, the selection pressure applied to the gram positive cocci soon lead to the emergence of gram positive β-lactamase (Sykes et al.,1982). As the antibiotics changed to respond to the resistance of the bacteria, the bacteria also changed by spontaneous mutation and selection for new classes of β-lactamases and the emergence of gram negative β-lactamases (Sykes et al.,1982). A study by Dalbadie-McFarland (1985) in which the active site of *E. coli* RTEM β-lactamase was inactivated by a double amino acid substitution at positions 70 and 71, introduction of ampicillin to the media at the concentration of 10μg/mL, led to seven colonies producing an active mutant β-lactamase after only an overnight incubation. These rapid responses to selection pressure by the bacteria may suggest a substitution at the active site, such as cysteine-70, could possibly be propagated due to its unique substrate profile and altered antibiotic resistance.

Oligonucleotide-directed mutagenesis is not only an excellent method for the study of protein structure and function but also has clinical significance. The application of this technique to determine the antibiotic resistance afforded to *Escherichia coli* by a cysteine-70 mutant β-lactamase, illustrates the usefulness of analysis of specific mutations in the DNA sequence of enzymes and the phenotypic traits provided by their production.

One additional observation, as a result of this investigation, a new vector, pMM151, was created with a relatively high resistance to cephalosporins and might be useful where ampicillin resistance is undesireable.

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