The Structural Basis of Enzyme Catalysis: Mutagenesis of β-lactamase at Ala 172, Glu 166, and Ala 237

Thesis by

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Acknowledgements

I dedicate this work lovingly to my wife, Lynn, and my daughter, Auden. You keep things in perspective for me. Without you, none of this would matter.

Many thanks go to Marc Labgold: for many hours of work on alanine 237; for directing, producing, and editing this thesis; and most of all, for being a great friend.

Abstract

RTEM-1 β-lactamase is one of a group of enzymes that confer resistance to penam and cephem antibiotics by hydrolizing the β-lactam bond. In order to elucidate the structural basis for its catalytics, site saturation mutagenesis was performed on alanine 172, glutamate 166, and alanine 237. Site saturation is the replacement of any codon with a mixture of codons to produce all possible mutations at a single location.

Alanine 172 was found to have no effect on penam hydrolysis, but seven mutants--Arg, Asp, Glu, Gln, Ile, Leu, and Lys--were inactive on cephalothin. Although no specific structural role is hypothesized for this residue, some mutations (including those that change the charge) are able to affect activity. This altered activity is most likely due to propagated structural changes.

Glutamate 166 was chosen for mutagenesis because of its probable role as a general base in the deacylation step of the catalytic mechanism. Only three mutants (Asp, His, and Tyr) exhibited low activity. The K_M was measured for several mutants and found to be the same as wild-type (20 μM), indicating that mutations at this site do not affect substrate binding. Two mutants, Lys and Arg, yielded proteins that were unstable at 37°C, possibly because of an unfavorable interaction with lysine 73. A double mutant was constructed to replace Lys 73 with Glu while keeping Lys at 166; full cellular stability was restored.

Finally, the series of mutants at alanine 237 was produced; this residue is important to catalysis since it makes up part of the oxyanion hole that stabilizes a catalytic intermediate. Of all mutants at this site, only proline was inactive. Two mutants showed increased activity on cephems, asparagine (which had 380% of wild-type activity) and threonine (150%), revealing that this site is involved in substrate specificity.

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Nomenclature and Abbreviations

Using the one-letter amino acid codes, a mutant is specified as A237N if alanine at 237 has been changed to asparagine. The one-letter codes are: alanine, A; arginine, R; asparagine, N; aspartate, D; cysteine, C; glutamate, E; glutamine, Q; glycine, G; histidine, H; isoleucine, I; leucine, L; lysine, K; methionine, M; phenylalanine, F; proline, P; serine, S; threonine, T; tryptophan, W; tyrosine, Y; valine, V.

Chapter 1

Introduction:
RTEM-1 β-lactamase and Mutagenesis

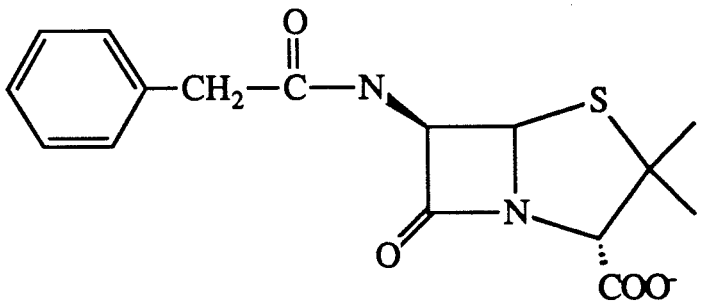
β-lactamase

RTEM-1 β-lactamase is one of a group of related enzymes characterized by their ability to confer resistance to penam and cephem antibiotics such as penicillin and cephalothin[1] (Figure I-1). This resistance is a result of the hydrolysis of the β-lactam ring of these antibiotics, which renders them inactive[2]. Until recently, little has been known of the mechanism of this RTEM-1 enzyme except that an active site serine is directly responsible for the attack of the β-lactam carbonyl[3]. With the aid of new techniques in mutagenesis, this laboratory has been investigating the structural basis for catalysis by β-lactamase. Several important residues to enzyme function have been studied, including Ser 70, Thr 71, Lys 73, and, in this work, Ala 172, Glu 166, and Ala 237.

Penicillin and its related compounds block bacterial growth by inhibiting cell wall biosynthesis. This is thought to be due to their resemblance to D-alanine-D-alanine, which is cleaved in formation of crosslinks in cell wall peptidoglycans by D-ala-D-ala carboxypeptidase[4]. This enzyme is one of a number of so-called penicillin-binding proteins (PBP's) since it binds penicillin essentially irreversibly (forming an acyl-enzyme). It is theorized that β-lactamase evolved from these PBP's as a response to antibiotics, developing the ability to deacylate the acyl-enzyme and render penicillin harmless. Evidence for a relationship between the two enzymes consists mainly of regional sequence homology (including the active site serine) and a strong similarity of low-resolution crystal structure[5].

The β-lactamases are classified based upon molecular weight, their requirement of metal ions, substrate profile, and sequence homology[6]. RTEM-1 is a member of the class A β-lactamases, which consist of enzymes of molecular weight of approximately 29,000 and have serine as an active site nucleophile[1]. Sequence homology is high among this class, ranging from 30 to 50%. Class A

A.



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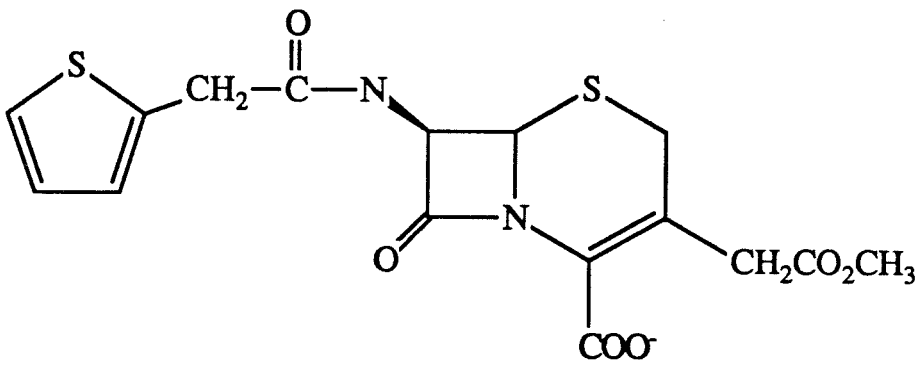

Figure I-1. Representative β-lactam antibiotics. Benzylpenicillin (A) is a penam, characterized by the 5,4 ring system. Cephalothin (B) is a cephem, with a 6,4 ring system containing a double bond.

includes the β-lactamases of *Staphyloccus areus* (PC1)[7], *Bacillus licheniformus* (749/C)[8], *Bacillus cereus* (569/H)[9] and *Escherichia coli* (RTEM-1 and RTEM-2)[10].

RTEM-1 β-lactamase is the enzyme encoded by the ampicillin resistance gene in the *E. coli* plasmid pBR322. It is synthesized as a 286 amino acid preprotein; a leader sequence of 23 amino acids is cleaved during export to the periplasm, yielding a molecular weight of 28,500[11]. It differs from RTEM-2 at only a single site: Glutamine 39 in RTEM-1 is lysine in RTEM-2. RTEM-1 has been the subject of much study both for medical interest in its role in penicillin resistance and as a convenient system for studying many biochemical processes. Its gene is easily manipulated and has been used to examine protein secretion[12], expression of fusion proteins[13], and as a selectable marker[14]. This laboratory has studied β-lactamase primarily as a model enzyme for examining the structural basis of catalysis (the relation between protein structure and function).

Mutagenesis

The goal of mutagenesis in the study of enzymes is to relate changes in protein function to the structural role of the amino acid that has been changed[15]. Through this type of experiment, investigators hope to expand knowledge of the basic principles of protein organization, as well as to elucidate specific mechanistic origins. Although many mutations are either silent or lethal, enough productive changes have been made that many new insights into the nature of enzyme catalysis are emerging[16].

In the recent past, the only way to discover the role of specific residues was through screening large numbers of randomly generated mutants for certain changes in phenotype and then discovering the molecular cause. With the advent of oligonucleotide-directed mutagenesis[17], site-specific single or multiple base changes could finally be produced, allowing the targeting of key residues for study.

In this method, an oligonucleotide primer containing the desired mutation is hybridized to single-stranded DNA, creating a mismatched duplex. Polymerization of this primer and subsequent screening produces a gene altered at a specific site. Thus, any amino acid can be changed to any other amino acid to study the effects of this change on enzyme function.

Automated DNA synthesis enabled the routine production of long segments of designed DNA. As a result, cassette mutagenesis was developed so that mutations of any length could be made by replacing a portion of a gene with synthetic double-stranded DNA[18]. This method relies on the existence of closely placed restriction sites, but these are now routinely placed into genes via site-directed mutagenesis. A derivation of cassette mutagenesis developed in the Richards laboratory is site saturation, wherein a degenerate codon producing all nineteen mutations at one site is inserted into a gene[19] Typically, three restriction sites are used in order to limit the chances of regenerating the wild-type gene. The great advantage of site saturation is that all mutations are produced without prejudice; therefore, one can get interesting results from substitutions that might not have been chosen if site-directed mutagenesis has been used.

Once mutations have been generated, available modes of studying them are numerous. Among these are: phenotypic characterization; determination of kinetic parameters relating change to binding, catalysis, or both; measurement of changes in pH profile or thermal stability; and examination of protein structure with standard methods. A danger in analyzing the results, however, is the assumption that a mutation affects only the site at which it occurs. A change in a residue not directly interacting with the substrate could nevertheless cause structural perturbations felt throughout an enzyme, directly affecting catalysis. This issue was examined in a series of mutations of T4 lysozyme, all of which were subjected to x-ray crystal analysis. Multiple mutants of threonine 157 all produced

only local changes in structure. Most of the mutants were stable, although one change (isoleucine) yielded a temperature-sensitive mutant[20]. Mutants of proline 86 in the same enzyme, however, produced structural changes that were propagated throughout the protein[21]. Lysozyme accommodated this change, though, and no altered stability was detected for any mutant. Therefore, although there is no guarantee that a mutation will affect only the site that is being changed, these results prove that single changes do not necessarily disturb the entire protein structure.

Mutagenesis of β-lactamase

Early work in this laboratory used oligonucleotide-directed mutagenesis to introduce point mutations at specific sites in β-lactamase. The initial target was the active site serine 70; it was changed to Thr and Arg, and the double mutant Thr 70 Ser 71 was also created[22,23]. None of these was active; the only change allowed at residue 70 is cysteine, another nucleophile[24]. Another mutant, changing Cys 77 to Ser, was used to study the effect of a loss of the disulfide bond on secretion, processing and stability[25]. This mutant was phenotypically identical to wild-type except for a marked loss of stability at high temperature.

The technique of site-saturation mutagenesis was used to study the importance to β-lactamase function of two residues: threonine 71 and lysine 73. Both sites were chosen based on their proximity to the active site serine 70 and also because, like Ser 70, they are fully conserved among the class A β-lactamases.

Of all mutants produced at threonine 71, fourteen displayed appreciable resistance to penam antibiotics at 30°C[19]. The only inactive mutants were Asp, Arg, Lys, Trp and Tyr. Low activity resulted with Asn, Glu, Gln, and Phe, so residue 71 appears to be susceptible to charged or very large side chain substitutions. It appears that Thr 71, although conserved, is not essential for

binding and catalysis. However, all mutants showed a considerable loss of stability at 37°C (as seen on Western blots of protein gels) compared to 30°C. Therefore, although Thr 71 is not necessary to the function of β-lactamase, it is important for stability of the protein.

All mutants of lysine 73 were inactive on even the lowest levels of penam antibiotics tested[26]. Western blots of these mutants showed that each was stable at 37°C and was produced at levels equal to wild-type β-lactamase. The Cys 73 mutant was purified and shown to have a K_M close to that of wild-type (20 μM), but only 0.01% catalytic activity. Therefore, it appears that lysine 73 is essential for catalysis, but doesn't affect binding or stability. Treatment of the cysteine mutant with ethylenimine to produce aminoethylcysteine at 73 restored 62% of the activity, showing that it is the ε-amino group that is necessary for catalytic function.

Neither of the above experiments was designed based on the actual structure of β-lactamase, since none existed at the time. Recent work, however, has benefited from the publication of the three-dimensional structure of β-lactamase from *S. aureus* PC1 at 2.5 Å resolution[27]. This enzyme is a class A β-lactamase which has 30% identity with RTEM-1, although the percentage increases if conservative substitutions are considered. As Figure I-2 shows, *S. aureus* β-lactamase consists of eleven α-helices and a five-stranded, antiparallel β-sheet.

The similarity of the two β-lactamases justifies the use of this crystal structure in targeting residues for mutagenic study. For this reason, glutamate 166 was chosen for site-saturation mutagenesis to probe its importance to β-lactamase catalysis. The crystal structure shows that this residue is in an ideal position to participate in catalysis, and its functional group is appropriate for its hypothesized role as a general base. Site saturation was also performed on Ala 172 and Ala 237, but this work was initiated prior to publication of the structure.

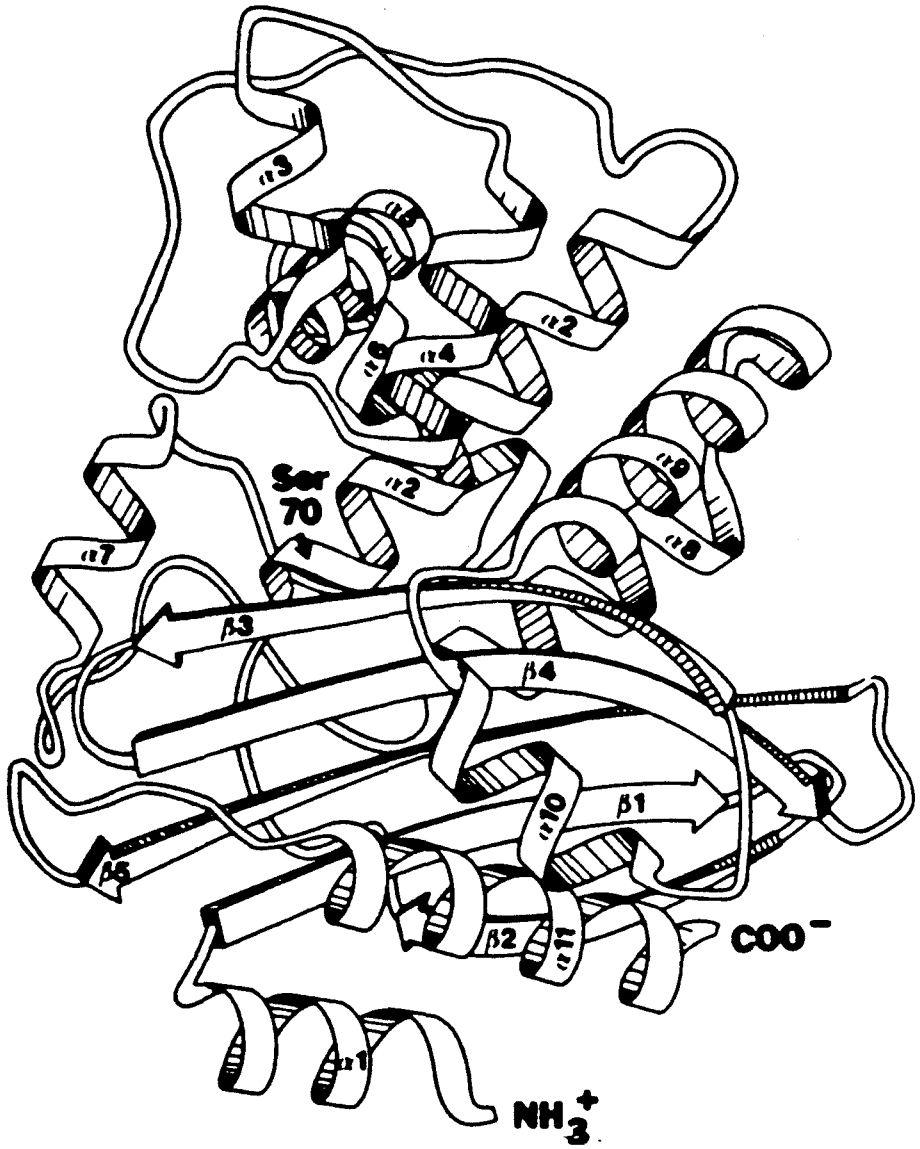

Figure I-2. Three-dimensional structure of *S. aureus* PC1 β-lactamase.

However, the structure shows that Ala 237 is also in a position to participate in catalysis; its amide backbone forms part of the oxyanion hole that stabilizes the acyl-enzyme intermediate. This thesis summarizes the mutagenesis of these three residues--Ala 172, Glu 166, and Ala 237--and attempts to explain how they contribute to the structural basis for catalysis by RTEM-1 β-lactamase.

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Chapter 2

Site saturation of β-lactamase at alanine 172

INTRODUCTION

Prior to publication of the x-ray crystal structure of the closely related *Staphylococcus aureus* β-lactamase[1], residues of RTEM-1 β-lactamase were chosen for site-directed mutagenesis based on 1) their linear proximity to the active site serine 70 and 2) their conservation among the four, known class A β-lactamases. Using these criteria, site saturation mutagenesis was performed on Thr 71 and Lys 73 to probe their importance to enzyme function[2,3]. An additional method to select residues for site saturation is based on the results of random mutagenesis. Random mutants that have altered activity identify important residues that may be targeted for further study. Additional mutations at that site could produce interesting changes that would not have been generated during chemical or other types of mutagenesis. In this work, alanine 172 was chosen for site saturation based on the discovery of a random mutant--threonine 172--which had higher activity on cephem antibiotics than did wild-type β-lactamase[4].

The Knowles lab at Harvard produced a mutant, termed *h1*, which was generated by directed selective pressure of RTEM-2 β-lactamase to increased levels of cephalosporin C[5]. This mutant had increased activity because of a two-fold increase in k_{cat}, and also because of an order of magnitude increase in the amount of enzyme produced. Therefore, *h1* is a double mutant, producing more of an enzyme modified at a single location: alanine 172. Site saturation at this residue was undertaken because 1) it would be the first such experiment in β-lactamase far from the active site in linear sequence, 2) it is not a conserved residue, but is replaced by valine or tyrosine in other class A β-lactamases, and 3) if an alanine-to- threonine mutation produced such an altered phenotype, some other mutation produced by site saturation might also produce a significant change in function.

With the publication of the crystal structure of *S. aureus* β-lactamase, the location of residue 172 (which is tyrosine in this species) could be pinpointed

(Figure II-1). As part of the Ω-loop, which includes residues 163 to 178, residue 172 is thought to form a narrow exit to the solvent, along with lysine 177. Although it defines part of the large cavity of β-lactamase, residue 172 appears to be too far from the actual binding site to make any contribution to binding or catalysis. Therefore, it is not readily apparent how a mutant at position 172 could have such a marked effect on enzyme specificity.

Following introduction of a silent mutation to give a new Sac I site in the β-lactamase gene, site saturation was performed at alanine 172. In addition, a separate ligation was performed to put threonine at 172 , since *h1* was identified as A172T. The resultant mutant colonies were screened on agar plates containing varying concentrations of penam and cephem antibiotics, and then sequenced. No mutant (including threonine) grew on higher concentrations of cephem than did wild-type. Subsequently, it was learned that *h1* had been misidentified and was actually alanine 237; investigation of that site is the topic of Chapter 4. However, the study of Ala 172 is still important since, although it is not conserved and is not part of the active site, it still produces variations in activity among different mutants.

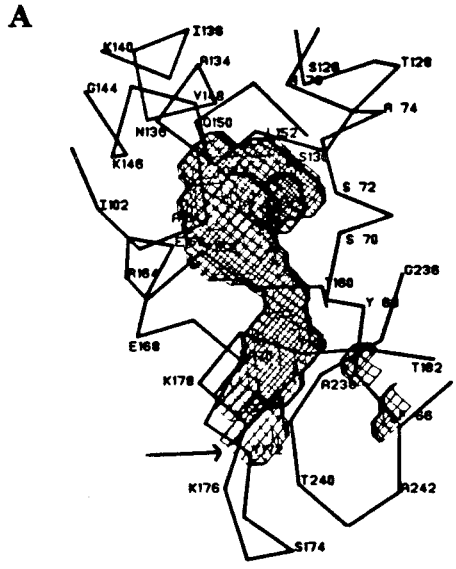


Figure II-1. **A.** Large cavity in *S. aureus* β-lactamase; residue 172 (marked with an arrow) is part of the Ω-loop, which consists of residues 162-178. **B, C.** Closeup of the active site with and without ampicillin bound. Ala 172 (not shown) is clearly not in the near vicinity of the substrate.

MATERIALS AND METHODS

Enzymes and Chemicals

All enzymes were purchased from Boehringer Mannheim Biochemicals. Antibiotics were from Sigma Chemical Company. Radioactive materials were supplied by Amersham. Isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal) were purchased from International Biotechnologies, Inc. (IBI) Molecular biology grade reagents agarose, phenol and chloroform were also from IBI.

Bacterial Strains

Escherichia coli were used in all experiments. Plasmid DNA was harbored in strain HB101[6]; bacteriophage were propagated in strain JM101[7]. Culture medium was L broth (10 g tryptone, 5 g yeast extract, 5 g NaCl in 1 l; add 15 g bacto-agar to make L plates), unless otherwise indicated.

Transformation

Cells were made competent for transformation of plasmid DNA using a process adapted from Hanahan[8]. A single colony of *E. coli* was grown in 100 ml of SOB media (20 g tryptone, 5 g yeast extract, 0.6 g NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ in 1 l) at 37°C to OD_{550}~0.3. At this point, cells were divided into two polypropylene tubes and chilled. Following centrifugation at 2500 rpm for 10 minutes at 4°C, each pellet was drained thoroughly and resuspended in 16 ml transformation buffer 1 (12 g RbCl, 9.9 g $MnCl_2 \cdot H_2O$, 1.5 g $CaCl_2 \cdot H_2O$, 30 ml of 1M KOAc, pH 7.5 in 1 l). This suspension was incubated on ice for 15 minutes, centrifuged again at 2500 rpm, and resuspended in 4 ml transformation buffer 2 (1.2 g RbCl, 11 g $CaCl_2 \cdot H_2O$, 20 ml of 0.5 M MOPS, pH 6.8 in 1 l). An aliquot of 10-20 µl DNA was added to 300 µl of these competent cells with gentle mixing. These

were left on ice for 40 minutes, then heat-shocked at 42°C for 45 seconds. After addition of 700 µl SOB, the mixtures were incubated for 1 h at 37 °C and then plated onto L agar containing an antibiotic.

DNA

Oligonucleotides were synthesized by the Caltech Microchemical Facility using phosphoramidite chemistry on the Applied Biosystems automated DNA synthesizer, Model 380A. Degenerate oligonucleotides were made equimolar in A, C, G, and T at positions 1 and 2 of the codon, and in C and G at position 3. They were then purified by preparative polyacrylamide gel electrophoresis (20%, 40 cm gel; 900 volts; 12 h); DNA was visualized with UV light reflected from a fluorescing silica plate. The appropriate bands were excised and DNA eluted in 0.2 M NaCl for 4-6 h at 55°C. Samples were then desalted using G-25 Sephadex spin columns.

Wild-type plasmid pBR322 and bacteriophage M13 mp18 replicative form (RF) DNA were purchased from Bethesda Research Laboratories. Mutant plasmids and RF phage were purified from *E. coli* by the alkaline lysis method[9]. Large-scale preparations were further purified using ultracentrifugation in cesium chloride/ ethidium bromide gradients (single spin: 20 h, 45,000 rpm)[10]. Single-stranded phage DNA was prepared from phage supernatant by precipitation in 20% polyethylene glycol-6000/2.5 M NaCl, followed by phenol/chloroform extraction and ethanol precipitation[11].

Restriction digests typically used 20 µg plasmid DNA, 2-5 units of restriction enzyme, and 2 µl 10X digest buffer in 20 µl at 37°C for 1-2 h. DNA restriction fragments were typically run on 1.2% agarose gels, visualized with ethidium bromide and isolated with either a UEA electroeluter (IBI) or an elutrap (Schleicher & Schuell), according to manufacturers' instructions. Recovery was typically 40-60% following phenol/chloroform extraction and ethanol precipitation.

All DNA concentrations were estimated from absorbance at 260 nm.

Oligonucleotide-directed Mutagenesis

Before cassette mutagenesis for site saturation at alanine 172 was possible, a new Sac I restriction site had to be added at position 3651 in pBR322. First, the EcoR I/Pst I 752 base-pair fragment from pBR322 was cloned into M13 mp18 RF DNA (which had also been digested with EcoR I and Pst I, then treated with alkaline phosphatase). The fragments were ligated using standard conditions and then transformed into competent JM101. Plasmid-containing cells were then plated along with saturated JM101, IPTG, and X-gal. Clear plaques on a lawn of JM101 represented successful clones containing the pBR322 EcoR I to Pst I insert; blue plaques indicated wild-type phage.

Single-stranded phage were isolated from a clear plaque and annealed with the septadecamer 5'-GCTTCATTGAGCTCCGG (mismatch underlined) by heating to 95°C in 10mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, and then slowly cooling to room temperature. This was followed by chain extension with DNA polymerase I (Klenow fragment) and all four dNTP's (1.25 mM each) for one hour at 37°C to synthesize the complementary DNA strand[12]. Transformation and plating yielded several thousand plaques. Replicas of the plates were made by placing a nitrocellulose filter onto each surface; these were then baked in a vacuum oven at 80°C for one hour. The same heptadecamer was radioactively labelled with [γ-^{32}P]-dATP and T4 DNA kinase and used to probe for mutants: filters were incubated in a solution containing this probe and then washed at increasing temperatures. After the 54°C wash, only those plaques containing the mutant were visible following autoradiography. Potential positives were replated; several plaques from each were picked for minipreps of phage DNA for dot blots (in which phage is placed directly onto the nitrocellulose). These were also screened

with the septadecamer at increasing temperatures. Positives were verified by Sanger dideoxynucleotide sequencing[13].

To subclone the Sac I site into pBR322, Pvu I and Pst I were used to minimize the size of DNA taken out of M13. This was done to avoid transferring any second site mutations which sometimes occur in SS M13 phage. Since there are multiple Pvu I sites in M13mp18, it was first cut with Pst I and EcoR I (see Figure II-2). The 752 base-pair fragment was isolated and then cut with Pvu I; the 126 base-pair fragment contained the new Sac I site. Separate samples of pBR322 were then digested with either Pvu I/Sal I or Pst I/Sal I and the 1279 and 2958 base pair fragments (respectively) were isolated. These were then combined in a three fragment ligation to produce pBR322-Sac. The presence of a new restriction site was confirmed by restriction mapping with Sac I and EcoR I.

Cassette mutagenesis: site saturation at 172

The three-fragment ligation scheme used is outlined in Figure II-3. Complementary synthetic oligonucleotides--one set containing a degenerate codon for residue 172 and one coding for Thr 172 (Figure II-4)--were left unkinased; 50 pmoles of each were annealed by heating to 95°C in 100 µl of 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, and then slowly cooled to room temperature over a period of two hours. One pmole of the annealed oligonucleotides was combined with approximately 0.2 pmole each of Sac I/Sal I 1363 base-pair fragment and Pst I/Sal I 2958 base-pair fragment, to give a 5:1 insert to vector ratio and approximately 30 µg/ml total DNA concentration in a 20 µl reaction. This was incubated in a mixture of 25 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 5 mM DTT, 0.5 mM ATP, and 1 unit T4 DNA ligase for 12-14 h at 16°C; 10 µl were transformed directly into competent HB101 and spread onto 15 mg/l tetracycline plates. Control ligations were performed in the absence of insert DNA to test for contamination by

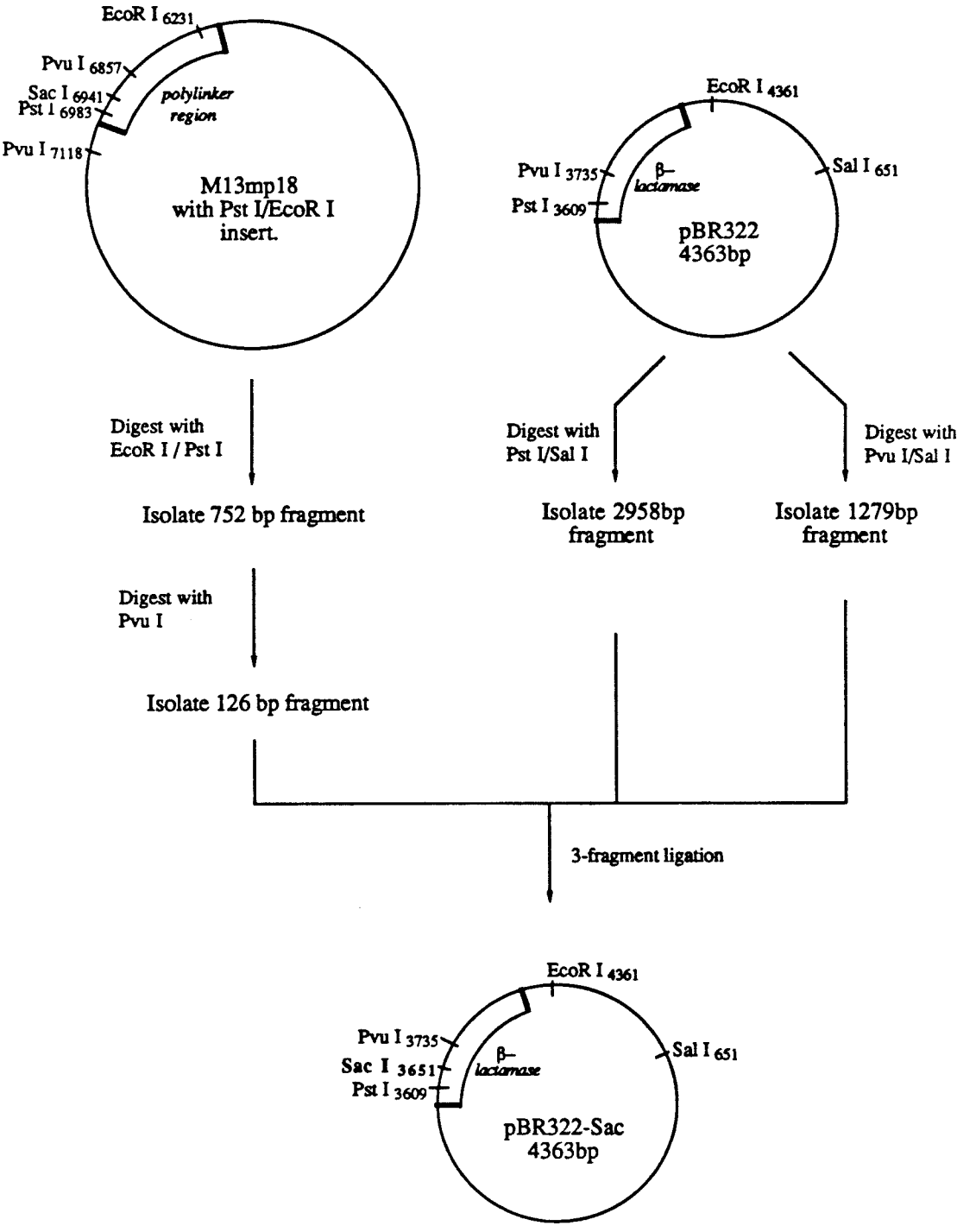


Figure II-2. Cloning scheme used to transfer Sac I restriction site from M13 into pBR322 to create pBR322-Sac.

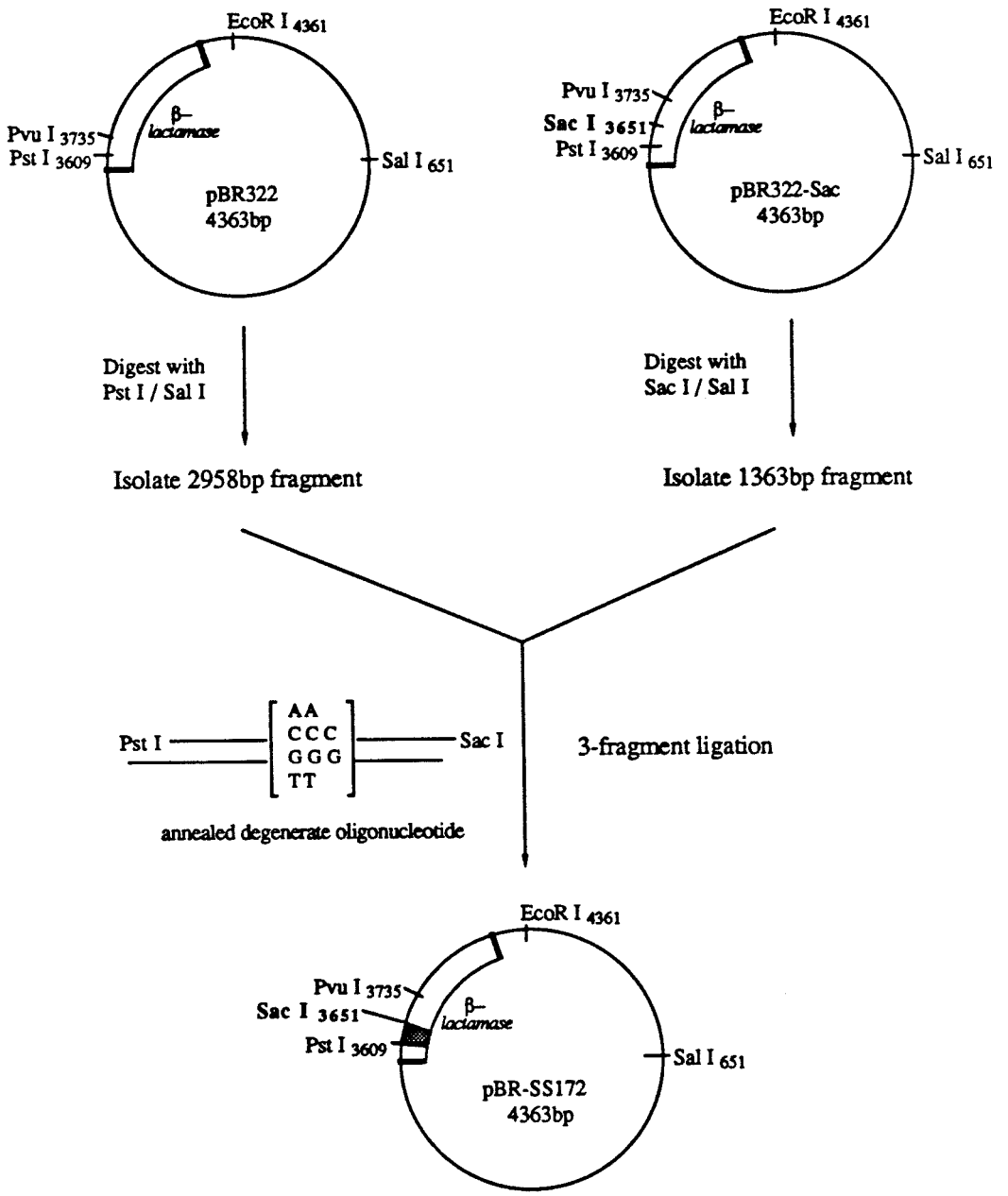


Figure II-3. The three-fragment ligation scheme for site saturation at alanine 172.

Figure II-4. Oligonucleotide inserts used for three-fragment ligation. **A** contains a degenerate codon at position 172 to produce all substitutions of alanine at that site. **B** contains the codon for threonine at 172 (underlined).

wild-type pBR322. At least four plates of each were made in order to generate sufficient numbers of colonies for sequencing. A 2 ml culture of each colony was grown to saturation and then frozen to preserve the mutants.

Phenotypic screening

One hundred forty-five colonies from the site saturation, four from the threonine ligation, and one wild-type colony were picked, using sterile toothpicks, onto a "master" tetracycline plate. These colonies were then picked onto plates containing ampicillin (50-2000 mg/l), benzylpenicillin (50-2000 mg/l) or cephalothin (25-2000 mg/l) to screen for levels of resistance to penam and cephem antibiotics. Wild-type β-lactamase grew at the highest level tested on the penams and up to 100 mg/l on cephalothin.

DNA Sequencing

To reveal the identities of the mutants, plasmid DNA was prepared from each colony and sequenced.

Maxam-Gilbert Chemical Sequencing. Plasmid DNA was first digested with Pst I, then 3'-labelled with [α-^{32}P]-ddATP and terminal deoxynucleotydyl transferase (TdT), since there were no 5'-overhangs. (TdT adds nucleotides to the 3'-overhang of DNA.) This was then digested with Sal I, and the small fragment was isolated from an agarose gel. Standard Maxam-Gilbert sequencing reactions were performed[14] and then run on an 8% polyacrylamide gel at 1200 volts for approximately 3 h.

Double-stranded Plasmid Sequencing. Five μg of CsCl-purified plasmid were denatured in 20 μl 0.2 M NaOH for 5 minutes, then neutralized with 2 μl of 2 M NH_4OH, pH 4.5 and ethanol precipitated[15]. The sequencing primer used was 5'-TAGTTCGCCAGTTAATAG; this is located about 60 base pairs downstream from

the codon for 172 and is complementary to the sense strand . The DNA pellet was resuspended along with 5 pmoles of sequencing primer in 6.6 mM each of Tris-HCl, pH 7.5, NaCl, and $MgCl_2$ in a volume of 10 µl, and annealed at 37°C for 15 minutes. Sequencing reactions were carried out by adding premixed deoxy-/dideoxy-nucleotide solutions (Pharmacia), 2 µl [α-^{35}S]-dATP, and 2 units of DNA polymerase I (Klenow fragment) for 20 minutes at 37°C. Reactions were stopped by adding 4 µl 0.03% (w/v) bromophenol blue in deionized formamide. Samples were loaded onto 8% polyacrylamide gels, run at 1200 volts for approximately 5 h, and autoradiographed.

Western blots

Colonies harboring mutants were grown to late log phase (OD_{600}~1.0); a 1.5 ml sample of each was pelleted by centrifugation and resuspended in 100 µl protein sample buffer (10% v/v glycerol, 5% v/v 2-mercaptoethanol, 3% w/v sodium dodecyl sulfate, 62.5 mM Tris-HCl, pH 6.8, 1.0 mM EDTA, 0.05% w/v bromophenol blue) and heated to 95°C for 10 minutes to lyse the cells. Aliquots of 20 µl each were loaded onto a 15 cm, 12% polyacrylamide stacking gel and run at a constant current of 5 mA for 12-16 h. Protein was then transferred from the gel onto DEAE nitrocellulose using a Bio-Rad Transblot cell for 6 h at 12 volts. β-lactamase was visualized following binding of rabbit anti-β-lactamase, using the highly sensitive Vectastain® ABC immunoperoxidase system (Vector Laboratories)[16].

RESULTS

Initially, a Sac I site was added to the β-lactamase gene in pBR322, using oligonucleotide-directed mutagenesis to introduce a silent mutation at base number 3654. Out of more than 5000 plaques screened, six were found to contain the desired mutation through hybridization screening. This was confirmed by both DNA sequencing and restriction mapping with Sac I.

This Sac I site and an existing Pst I site were then used for cassette mutagenesis in which all twenty amino acid codons were to be placed at the site of alanine 172 (site saturation). Cassette mutagenesis was also used in a separate reaction to insert threonine at 172 by itself. A three-fragment ligation scheme (employing another restriction site: Sal I) was used to reduce the number of background, wild-type colonies. This ligation was successful, resulting in four tetracycline plates with a total of 600 colonies. The ligation of the cassette containing the threonine codon produced two plates with 100 colonies each. Control ligations, which lacked insert DNA, yielded between zero and three colonies--an acceptable range.

One hundred fifty colonies were picked from tetracycline plates for phenotypic screening: 145 from the site saturation ligation, four from the A172T mutation, and one wild-type. Wild-type β-lactamase grew on the highest levels of ampicillin and benzylpenicillin tested (2000 mg/l), and up to 100 mg/l cephalothin. Of the 145 site saturation colonies, 18 (12%) were phenotypically inactive on all antibiotics, 35 (24%) were fully active on ampicillin and benzylpenicillin, but not at all active on cephalothin, and the remaining 92 (64%) had identical activity to wild-type β-lactamase. The four A172T mutants also showed identical activity to wild-type. No colonies showed increased resistance to cephalothin, contrary to earlier expectations.

Sequencing plasmid DNA from these colonies was attempted using the Maxam-Gilbert chemical method. However, this sequencing was difficult and tedious because of the process of labelling, cleaving, and isolating of DNA, made even more difficult with 3'-overhangs. Eventually, this method was abandoned in favor of double-stranded DNA dideoxy sequencing--a modification of the Sanger method in which the template is single-stranded DNA produced by regional base denaturation of supercoiled plasmid. This method was most effective when CsCl-purified DNA was used, since high levels of background and streaking were seen on sequences from less pure DNA. Figure II-5 shows an autoradiogram of a typical sequencing gel; the background is minimal and the sequence is easily read. A total of 76 large-scale preps were completed; these proved sufficient to find all available mutants at position 172.

Three of the independently generated A172T mutants were sequenced and found to have the correct codon: ACC. Early on in the sequencing of the site-saturation colonies, it was noticed that the third base of the codon for position 172 was consistently A or T. This was thought to be impossible since the oligonucleotides ordered contained only C and G at that position. Apparently, an error occurred during synthesis of the degenerate oligonucleotides. The result was that only eighteen residues could be produced at this site; methionine (ATG) and tryptophan (TGG) were unobtainable. All eighteen of these mutants were found after sequencing fifty-nine colonies. In addition, both stop codons, three wild-type codons (GCC), and one secondary mutation (a single base pair insertion) were discovered. Overall, 27 out of 32 possible codons were found; only ACT, CCT, CTA, GCT, and TCA were missing. Distributions of the bases at each position in the codon (Table II-I) were close to equal at the first and third positions, but cytosine was seriously deficient at position two. Four of the five missing codons contained cytosine in this position. Fortunately, this did not affect discovery of all possible

Figure II-5. Autoradiogram of a representative sequencing gel for mutants at 172. Double-stranded plasmid dideoxy sequencing was used; labelling was with [α-^{35}S]-dATP. The codon at 172 for each mutant is bracketed.

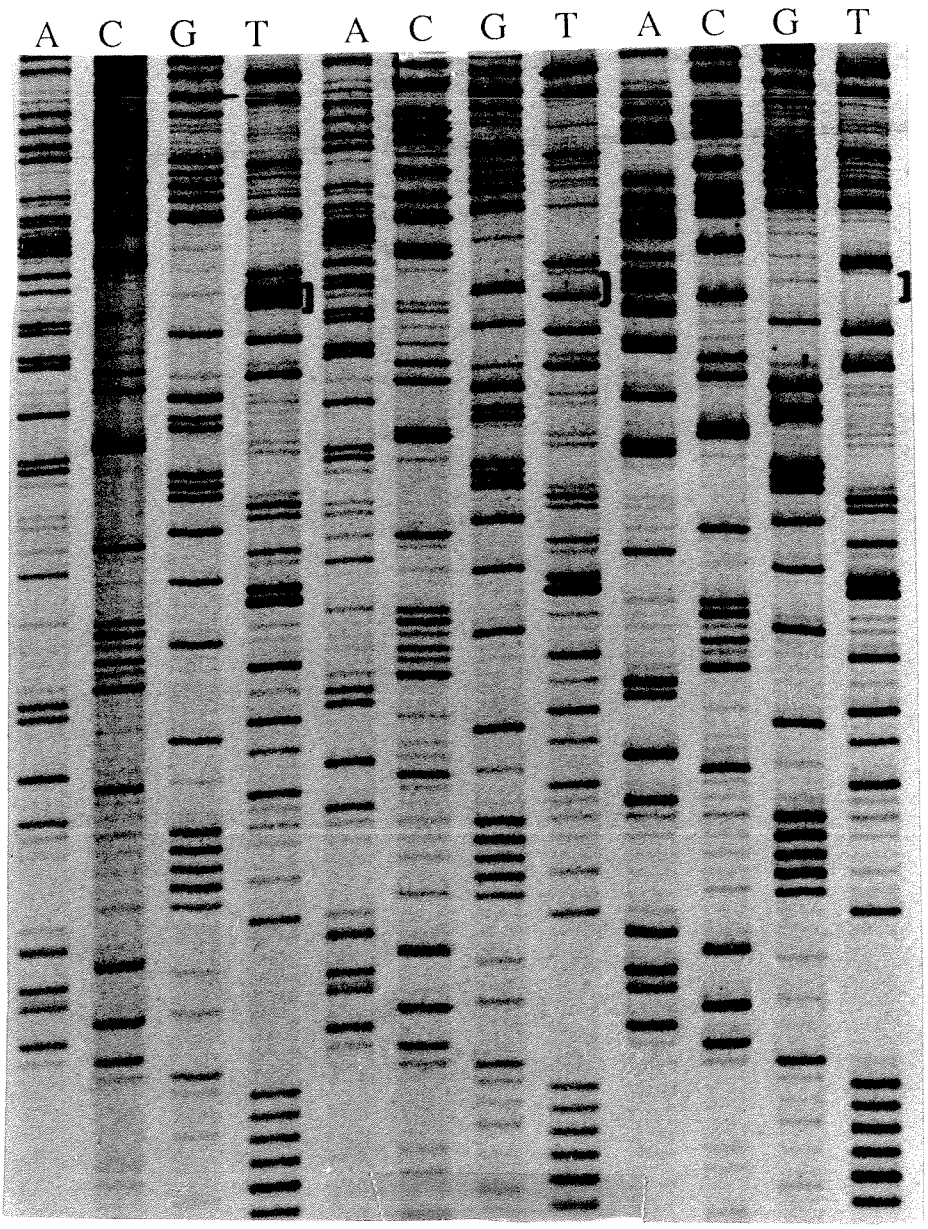

A C G T A C G T A C G T
Phe (TTT)
Stop (TGA)
Gln (CAA)

Table II-I Distribution of bases at each position for the codon for Ala 172.

	1st position	2nd position	3rd position
A	27%	25%	50%
C	23%	15.4%	- -
G	25%	32.6%	- -
T	25%	27%	50%

mutants, since there are no unique codons containing cytosine in position two. The eighteen plasmids containing all mutants at position 172 were then resequenced to confirm their identities.

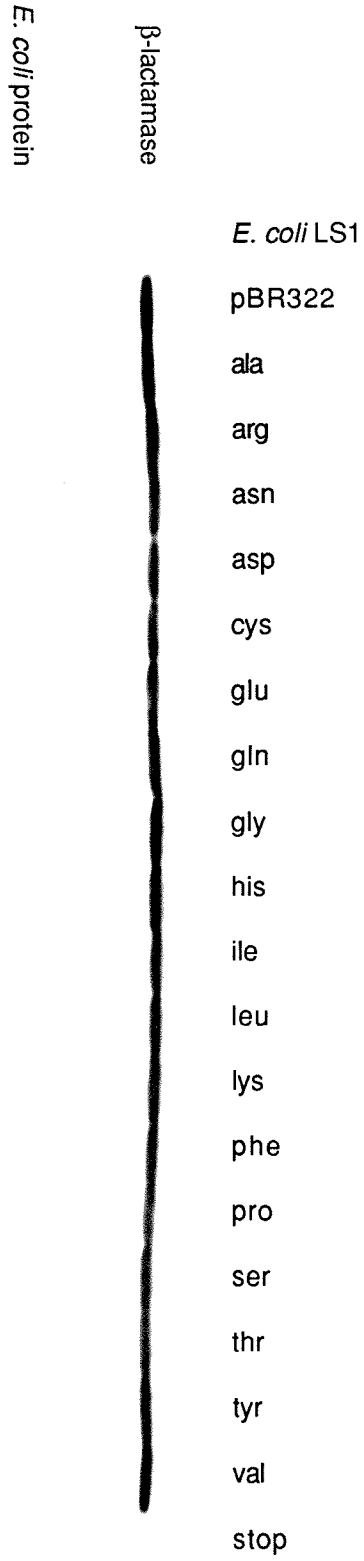
Table II-II matches the activity of each mutant on ampicillin, benzylpenicillin, and cephalothin to its sequence. Numbers listed are the highest concentration of each antibiotic on which the mutant grew; the highest level tested for each was 2000 mg/l. All mutants except one were active on the highest levels of ampicillin and benzylpenicillin tested; Lys 172 grew to only 500 mg/l benzylpenicillin. On cephalothin, seven mutants (Arg, Lys, Asp, Glu, Gln, Leu and Ile) were inactive. The other mutants (Gly, Asn, Cys, Val, Ser, Thr, His, Phe, Tyr, and Pro) had activity equal to wild-type.

Since all mutants showed activity, it could be assumed that each is a stable enzyme. To confirm this, Western blots were run. Figure II-6 shows that all mutant β-lactamases are stable and present in the cells at levels equal to that of wild-type.

Table II-II Activity Table--Ala 172 mutants. The number indicates the highest concentration of antibiotic on which the colonies grew. 2000 mg/l is highest tested.

Amino Acid	Codon	Penicillin G (mg/l)	Ampicillin (mg/l)	Cephalothin (mg/l)
Alanine (wt)	GCC	2000	2000	100
Arginine	CGA	2000	2000	0
Asparagine	AAT	2000	2000	100
Aspartate	GAT	2000	2000	0
Cysteine	TGT	2000	2000	100
Glutamate	GAA	2000	2000	0
Glutamine	CAA	2000	2000	0
Glycine	GGT	2000	2000	100
Histidine	CAT	2000	2000	100
Isoleucine	ATT	2000	2000	0
Leucine	TTA	2000	2000	0
Lysine	AAA	500	2000	0
Methionine	not found (A T G)			
Phenylalanine	TTT	2000	2000	100
Proline	CCA	2000	2000	100
Serine	AGT	2000	2000	100
Threonine	ACA	2000	2000	100
Tryptophan	not found (T G G)			
Tyrosine	TAT	2000	2000	100
Valine	GTT	2000	2000	100
STOP	TGA	0	0	0

Figure II-6. Western blots of all residues at position 172 of β-lactamase. The enzyme was visualized following binding of rabbit anti-β-lactamase using the highly sensitive Vectastain ABC immunoperoxidase system. A background lane of *E. coli* protein that cross-reacts with the antibody confirms that the same amount of cells was loaded in each lane. Two gels were run separately and then combined for this figure.


E. coli protein
β-lactamase
E. coli LS1
pBR322
ala
arg
asn
asp
cys
glu
gln
gly
his
ile
leu
lys
phe
pro
ser
thr
tyr
val
stop

DISCUSSION

Site-saturation mutagenesis was used to examine the role of alanine 172 in the function of β-lactamase. Although it was initially thought that a mutant at 172 produced increased resistance to cephalothin, it was subsequently learned that the mutation was actually at alanine 237. However, continued study of Ala 172 mutants gave the opportunity to investigate the effect of mutagenesis at an apparently nonessential amino acid, which is not part of the active site of β-lactamase.

Cassette mutagenesis using a three-fragment ligation scheme was successful, although only eighteen mutants were found because of an error in degenerate oligonucleotide synthesis. Since alanine 172 turned out not to be the site of the mutant *h1*, it was decided that the remaining two mutants would not be made separately. Out of 59 plasmids sequenced, only one contained a secondary mutation (a single base pair insertion), compared to eleven errors found during site saturation at lysine 73. The roughly equal distribution of bases at the first and third positions of the codon for residue 172 allowed the discovery of all possible mutants in the first 59 plasmids. Although position two had a low percentage of cytosine (15.4 %), it did not affect the rapid discovery of all mutants since there are no unique codons with cytosine as the second base. The double-stranded sequencing method that was used allowed rapid determination of large numbers of sequences, which is an asset in site saturation where so many plasmids must be sequenced before finding all mutants. The time advantage of this method over chemical sequencing is enormous.

Phenotypic screening revealed that all mutants had some activity, showing that in all cases viable enzymes were produced. Western blots confirmed that all seventeen mutants were produced at levels comparable to that of wild-type and were stable to thermal denaturation and proteolysis. The only mutants that

showed zero activity were those with stop codons (which did not produce viable proteins on the Western blots) and the insertion mutant.

Although Ala 172 is not a conserved residue and is not a participant in the active site, mutants showed differential activity between the two penams tested and cephalothin. Ten of the seventeen mutants grew to the same levels of the antibiotics tested as did wild-type β-lactamase, including valine and tyrosine, which are the residues found at position 172 in the β-lactamases from *Bacillus licheniformis* and *Staphyllococcus aureus*, respectively. Seven mutants exhibited activity equal to wild-type on ampicillin and benzylpenicillin (growing to levels of 2000 mg/l), but were inactive on cephalothin. These included the charged residues Arg, Lys, Asp, and Glu, as well as Gln, Leu and Ile. Since cephalothin is a poor substrate, it is a better indicator for loss of activity than ampicillin. The penams are such good substrates for β-lactamase that no phenotypic difference can be detected by plating unless there is a major loss of activity. Kinetic studies would have to be performed to quantitate the change in activity, if any, on penams.

It is significant that viable, functioning enzymes with altered phenotypes are produced at a residue apart from the active site. Alanine 172 does not have an "important" structural role in binding or catalysis, but its mutants apparently can have great effect on β-lactamase activity for some substrates. Since these effects cannot be occurring directly at the active site, they must be due to the interaction of amino acids at 172 with surrounding residues. From the crystal structure, it can be seen that possible neighboring residues include: Glu 239, Glu 177, and Pro 67. Studies on crystal structures of multiple mutants of T4 lysozyme have shown that while some sites when mutated have only local effects, mutations at other sites can propagate structural changes throughout the enzyme[17,18]. It is the latter case that could be occurring in several of these mutants of alanine 172.

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Chapter 3

Site saturation of β-lactamase at glutamate 166

INTRODUCTION

With the publication of the crystal structure of a Class A β-lactamase--that of *Staphylococcus aureus*--it was possible to choose residues for study based on both their locations in the enzyme and on their hypothesized roles in catalysis[1]. Although no structure of an enzyme-substrate cocrystal has been solved, model building with ampicillin clearly showed that only a very few modes of substrate binding were possible. Thus, it was confirmed that serine 70 is in the precise position necessary to attack the carbonyl of the β-lactam ring. Although a catalytic triad as in the serine proteases was not apparent, Reference 1 proposed that two other amino acids were involved in the catalytic mechanism: Lys 73 and Glu 166. These residues are separated from each other by 2.2 Å--the ideal distance for forming a salt-bridge--and both are fully conserved smong class A β-lactamases.

In the serine proteases, deacylation is simply a reversal of acylation[2]. Once the acyl-enzyme intermediate is formed, departure of the leaving group allows the binding of a water molecule. In contrast, when β-lactamase forms an acyl-enzyme intermediate, the leaving group is covalently attached, making replacement by water possible. Instead, water must bind and attack from the opposite direction. This is supported by the fact that the rate of deacylation is unaffected by the addition of external nucleophiles[3], suggesting that some internal nucleophile is involved. The crystal structure shows that this water could hydrogen-bond to the main chain carbonyl of residue 237, the side-chain carbonyl of Asn 170, and the carboxyl group of Glu 166. Figure III-1 clearly shows Glu 166 positioned opposite Ser 70, the site of acylation, at the active site. It is probable that the glutamate enhances the nucleophilic attack of the water molecule. A possible mechanism showing this role for Glu 166 is given in Figure III-2. This role for an active site glutamate is also seen in S1 nuclease, where Glu 43 is postulated to act as a general base[4]. Site saturation of glutamate 166 was undertaken to substitute all

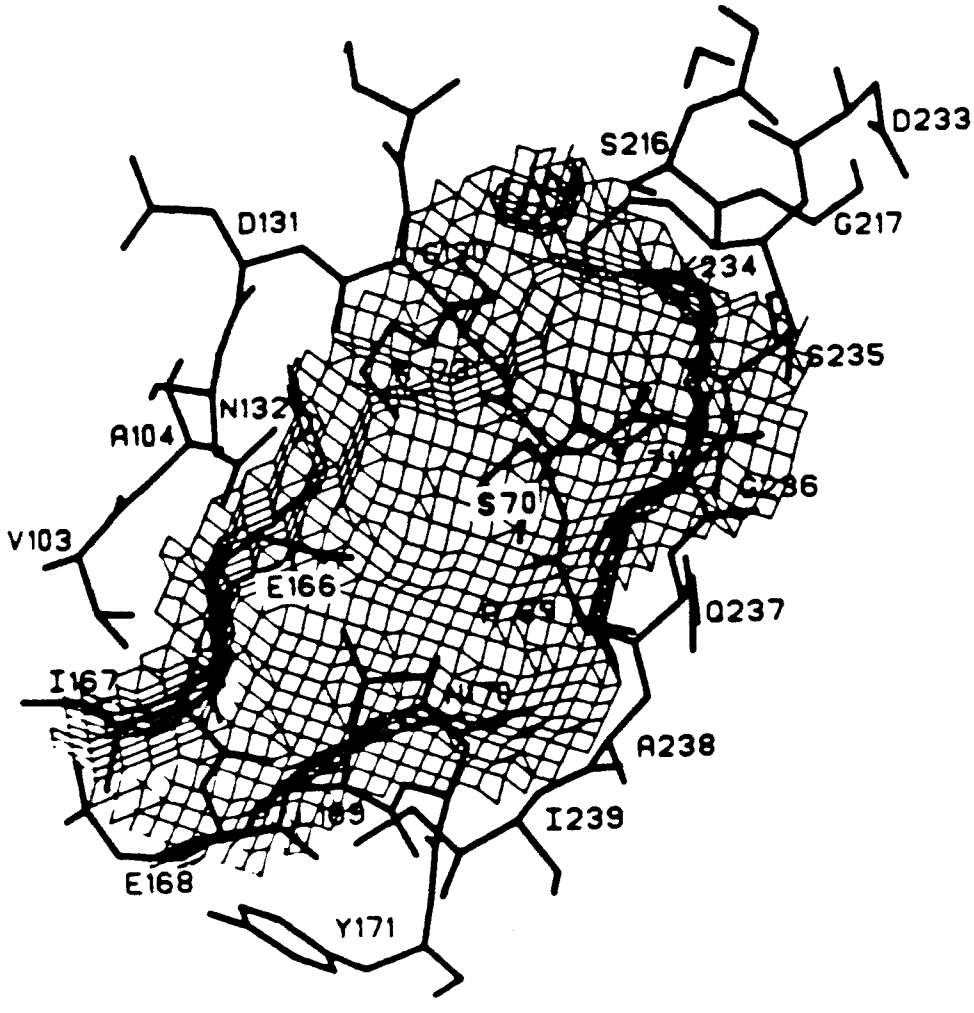


Figure III-1. Crystal structure of the active site of *S. aureus* β-lactamase. E166 is located opposite S70 in the binding pocket.

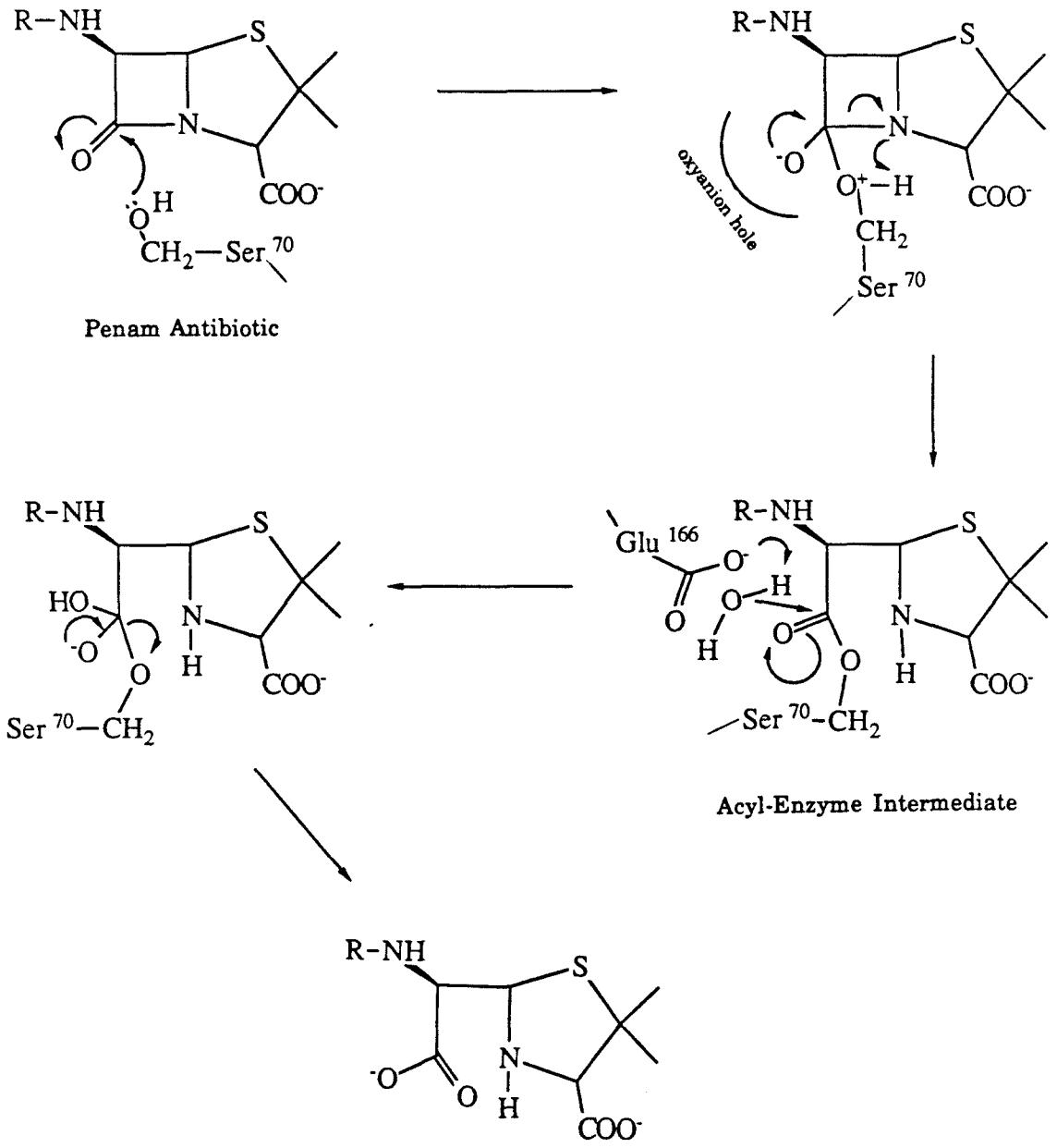


Figure III-2. A mechanism for the hydrolysis of a β-lactam by β-lactamase showing the proposed involvement of Glu 166 in deacylation.

other nineteen amino acids into that site in order to define its structural role in catalysis by β-lactamase. Lys 73 had previously been saturated by S. Carroll and no mutants with appreciable activity were found[5]. This could be due to the loss of the salt bridge. For the same reason, all mutants at 166 could be inactive because of a loss in stability rather than loss of a catalytic function.

All mutants replacing glutamate at this site were produced, and three were shown to confer resistance to β-lactam antibiotics: aspartate, histidine, and tyrosine. In addition, a double mutant that exchanges glutamate and lysine residues at 166 and 73, respectively, was constructed to attempt to rebuild the salt bridge. Several of these mutants were then expressed, purified, and subjected to kinetic analysis.

MATERIALS AND METHODS

Enzymes and Chemicals

All enzymes were purchased from Boehringer Mannheim Biochemicals. Antibiotics were from Sigma Chemical Company. Radioactive materials were supplied by Amersham. Isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal) were purchased from International Biotechnologies, Inc. (IBI). Molecular biology grade reagents agarose, phenol and chloroform were also from IBI.

Bacterial Strains

Escherichia coli were used in all experiments. Plasmid DNA was harbored in strain HB101[6]; the pJN expression vector was in strain D1210, which is lac i^Q. Bacteriophage were propagated in strain JM101[7]. Culture medium was L broth (10 g tryptone, 5 g yeast extract, 5 g NaCl in 1 l; add 15 g bacto-agar to make L plates), unless otherwise indicated.

Transformation

Cells were made competent for transformation of plasmid DNA using a process adapted from Hanahan[8]. A single colony of *E. coli* was grown in 100 ml of SOB media (20 g tryptone, 5 g yeast extract, 0.6 g NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ in 1 l) at 37°C to OD_{550}~0.3. At this point, cells were divided into two polypropylene tubes and chilled. Following centrifugation at 2500 rpm for 10 minutes at 4°C, each pellet was drained thoroughly and resuspended in 16 ml transformation buffer 1 (12 g RbCl, 9.9 g $MnCl_2 \cdot H_2O$, 1.5 g $CaCl_2 \cdot H_2O$, 30 ml of 1M KOAc, pH 7.5 in 1 l). This mixture was incubated on ice for 15 minutes, centrifuged again at 2500 rpm, and resuspended in 4 ml transformation buffer 2 (1.2 g RbCl, 11 g $CaCl_2 \cdot H_2O$, 20 ml of 0.5 M MOPS, pH 6.8 in 1 l). An aliquot of 10-20

μl DNA was added to 300 μl of these competent cells with gentle mixing. These were left on ice for 40 minutes, then heat-shocked at 42°C for 45 seconds. After addition of 700 μl SOB, the mixtures were incubated for 1 h at 37 °C and then plated onto L agar containing an antibiotic.

DNA

Oligonucleotides were synthesized by the Caltech Microchemical Facility using phosphoramidite chemistry on the Applied Biosystems automated DNA synthesizer, Model 380A. Degenerate oligonucleotides were made equimolar in A, C, G, and T at positions 1 and 2 of the codon, and in C and G at position 3. They were then purified by preparative polyacrylamide gel electrophoresis (20%, 40 cm gel; 900 volts; 12 h); DNA was visualized with UV light reflected from a fluorescing silica plate. The appropriate bands were excised and DNA eluted in 0.2 M NaCl for 4-6 h at 55°C. Samples were then desalted, using G-25 Sephadex spin columns.

Wild-type plasmid pBR322 and bacteriophage M13 mp19 replicative form (RF) DNA were purchased from Bethesda Research Laboratories. Mutant plasmids and RF phage were purified from *E. coli* by the alkaline lysis method[9]. Large-scale preparations were further purified using ultracentrifugation in cesium chloride/ ethidium bromide gradients (single spin: 20 h, 45,000 rpm)[10]. Single-stranded phage DNA was prepared from phage supernatant by precipitation with 20% polyethylene glycol-6000 (in 2.5 M NaCl)[11], followed by phenol/chloroform extraction and ethanol precipitation.

Restriction digests typically used 20 μg plasmid DNA, 2-5 units of restriction enzyme, and 2 μl 10X digest buffer in 20 μl at 37°C for 1-2 h. DNA restriction fragments were run on 1.2% agarose gels, visualized with ethidium bromide, and isolated with either a UEA electroeluter (IBI) or an elutrap (Schleicher & Schuell),

according to manufacturers' instructions. Recovery was typically 40-60%, following phenol/chloroform extraction and ethanol precipitation.

All DNA concentrations were estimated from absorbance at 260 nm.

Oligonucleotide-directed Mutagenesis

Before cassette mutagenesis was possible, a new restriction site had to be added to pBR322. Two possible sites--Mlu I and Xba I--were adjacent to each other at positions 3679 and 3674, respectively; to add either one required mutagenesis of two bases. However, to produce both at once required mutagenesis of only three bases. First, the EcoR I/Pst I 752 base-pair fragment from pBR322 was cloned into M13 mp19 RF DNA (which had also been digested with EcoR I and Pst I, then treated with alkaline phosphatase). The fragments were ligated using standard conditions and then transformed into competent JM101. Plasmid-containing cells were then plated along with saturated JM101, IPTG, and X-gal. Clear plaques on a lawn of JM101 represented successful clones containing the pBR322 EcoR I to Pst I insert; blue plaques indicated wild-type phage.

Then, a triple base-pair mutation was made using the Amersham oligonucleotide-directed *in vitro* mutagenesis kit[12] (which employs the Eckstein method of strand-specific selection[13]) as outlined in Figure III-3. Single-stranded phage were isolated and annealed to the 23-mer 5'-CATGTAACGCGTCTAGATCGTTG (mismatches underlined) by heating to 95°C in 10mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, and then slowly cooling to room temperature over a period of two hours. Chain extension from this oligonucleotide was accomplished with T4 DNA polymerase, T4 DNA ligase, and a mixture of dATP, dGTP, dTTP, and αS-dCTP. After completion, the reaction mixture was filtered to remove single-stranded DNA. Double-stranded DNA was saved and digested with the restriction enzyme Nci I. Only the native DNA strand was cleaved because of the presence of

A.

5'-CATGTA**ACGCGT**CTAGA**T**CGTTG-3'
Mlu I Xba I

B.

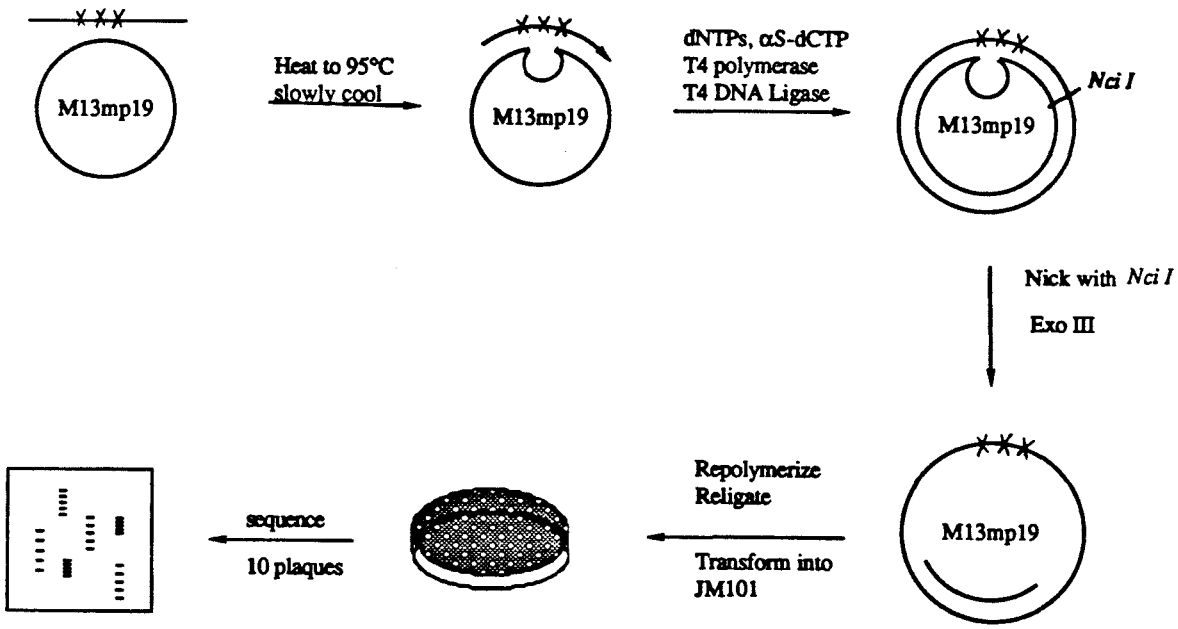


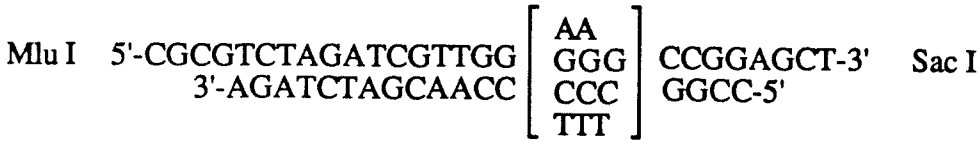
Figure III-3. **A.** Primer used to add two overlapping restriction sites (mismatches in bold face). **B.** Outline of the Amersham *in vitro* mutagenesis scheme using the Eckstein selection method (xxx=mismatches).

αS-dCTP (which interferes with cutting by Nci I) in the daughter strand. Following this, the nicked duplex DNA was digested using exonuclease III to remove the native DNA in the region of the mismatch. Repolymerization with all dNTP's, DNA polymerase I (Klenow fragment), and T4 DNA ligase yielded double-stranded DNA in which both strands carried the correct mutated base. Because of this selection method, screening of thousands of plaques by hybridization following transformation was unnecessary. Instead, ten plaques were chosen for sequencing using the chain extension method[14]. Eight out of ten tested positive for both of the desired mutations. These were then subcloned back into pBR322, again using EcoR I and Pst I, to produce pBR322-Mlu. The presence of the Xba I and Mlu I restriction sites was confirmed by restriction mapping. It was decided to use Mlu I in subsequent mutagenesis since Xba I required DNA grown in a *dam*$^-$ strain of *E. coli*.

Site saturation at residue 166: cassette mutagenesis

The complementary synthetic oligonucleotides containing a degenerate codon for residue 166 (Figure III-4A) were kinased (using T4 DNA kinase and ATP) and 50 pmoles of each were annealed by heating to 95°C in 100 µl of 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, and then slowly cooled to room temperature over a period of two hours. A three-fragment ligation was then carried out, as shown in Figure III-5, using pBR322-Mlu and (from Chapter 2) pBR322-Sac. One pmole of the annealed oligonucleotides was combined with approximately 0.12 pmole each of the Mlu I/Sal I 1335 base-pair fragment and the Sac I/Sal I 3000 base-pair fragment to give an insert to vector ratio of 8:1 and approximately 25 µg/ml total DNA in a 20 µl reaction. This mixture was incubated in a mixture of 10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, and 1 unit T4 DNA

a.)



b.)

Figure III-4. Synthetic oligonucleotides used in three-fragment ligations for a.) site-saturation at 166 and b.) placing Cys and Tyr at 166.

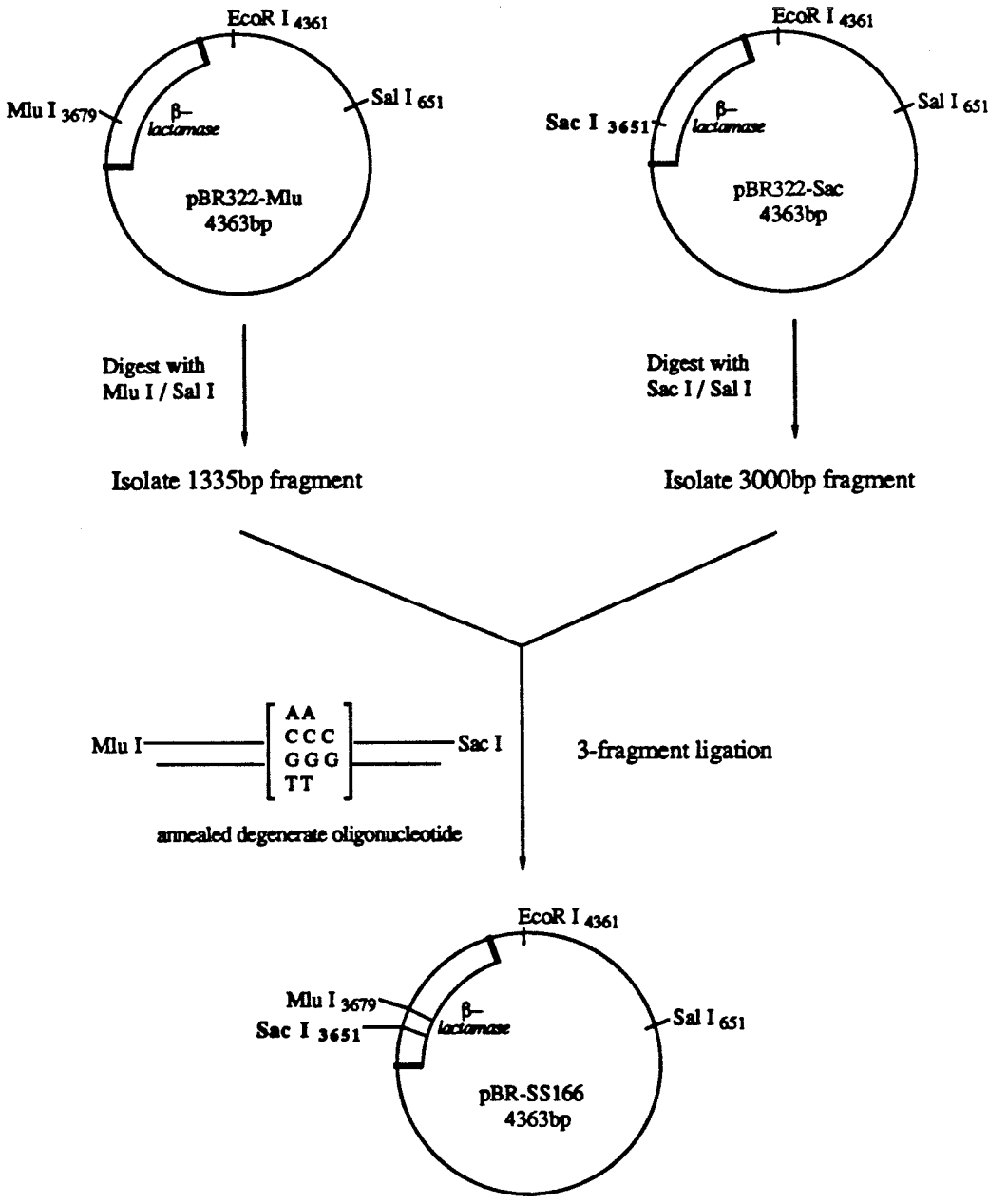


Figure III-5 Three-fragment ligation scheme for site saturation at residue 166.

ligase for 12-14 h at 16°C; 10 µl were transformed directly into competent HB101 and plated onto 15 mg/l tetracycline plates. Six reactions were performed in order to generate sufficient numbers of colonies for sequencing. In addition, control reactions were run in the absence of insert DNA to test for contamination by wild-type pBR322.

After sequencing all plasmids that had been prepared (see below), two mutants--cysteine and tyrosine--still were not found. A second three-fragment ligation was performed with a doubly degenerate oligonucleotide (Figure III-4B) to produce these two final mutants.

Phenotypic screening

One hundred seventy-seven colonies from six tetracycline plates were picked, using sterile toothpicks, onto "master" tetracycline plates. These colonies were then picked onto plates containing ampicillin, benzylpenicillin, or cephalothin to screen for levels of resistance to penam and cephem antibiotics. Two sets of plates were prepared--one grown at 30°C and one at 37°C. Wild-type β-lactamase grew at the highest level tested on the penams (2000 mg/l) and up to 100 mg/l on cephalothin at both temperatures.

Double-stranded plasmid sequencing

Plasmid DNA was prepared from seventy-five colonies from the original ligation, and ten colonies from the second (Cys/Tyr) ligation, and sequenced using a modification of the Sanger dideoxy method for denatured, double-stranded DNA[15]. Denaturation was carried out on 5 µg DNA in 20 µl 0.2 M NaOH for 5 minutes, then neutralized with 2 µl of 2 M NH_4OH, pH 4.5 and ethanol precipitated. The sequencing primer used was 5'-AACACTGCGGCCAACTTA, which is located on the sense strand 104 bases before the codon for residue 166.

The DNA pellet was suspended along with 5 pmoles of sequencing primer in 6.6 mM each of Tris-HCl, pH 7.5, NaCl, and $MgCl_2$ in a volume of 10 µl, and annealed at 37°C for 15 minutes. Chain extension reactions were performed with a SEQUENASE™ kit from United States Biochemical[16]. This kit provides premixed deoxy- and dideoxy- nucleotides, as well as sequenase™--a modified version of T7 DNA polymerase. Labelling was accomplished with [α–^{35}S]-dATP. Reactions were stopped by adding 4 µl 0.03% (w/v) bromophenol blue in deionized formamide. Samples were loaded onto 8% polyacrylamide gels, run at 1200 volts for 5 h, and autoradiographed.

Generating Asp at 166

A routine restriction digest of wild-type and several mutant plasmids with Pst I and EcoR I showed that several of the plasmids were larger than expected. Upon sequencing, it was revealed that these were the result of three degenerate inserts being ligated into the vector, as shown in Figure III-6. Although all nineteen mutants had been found, one of them (Asp) was actually part of a triple insertion that had not been detected during initial sequencing. Since no other aspartate had been discovered during sequencing, it was decided to reclaim Asp 166 from this multiple insertion rather than perform another three-fragment ligation or attempt to sequence more colonies from the initial ligation.

As outlined in Figure III-7, two inserts were removed from the triple insert containing the aspartate mutant by digesting with Sac I and then religating. For this method to be successful, cleavage had to occur at both Sac I sites; therefore, a large excess of enzyme (20 units) was used to digest 2 µg of plasmid. Following phenol extraction and ethanol precipitation, 2 units of T4 DNA ligase were added in 10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 5 mM DTT, and 1 mM ATP. After 2 hours at room temperature, DNA was transformed into competent HB101. Four of

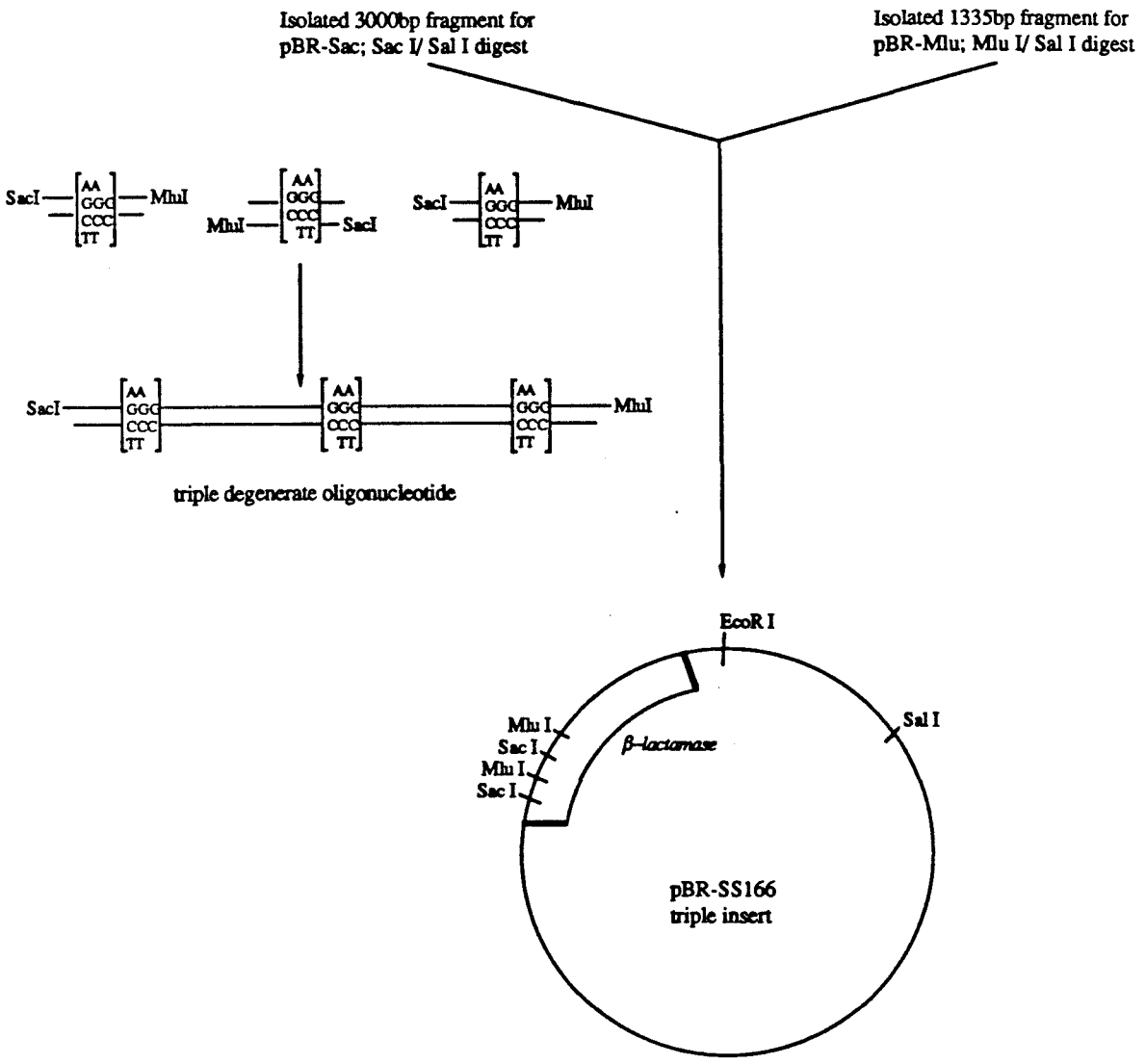


Figure III-6. Scheme for generation of a triple insertion of synthetic oligonucleotides at the Sac I and Mlu I sites of pBR322.

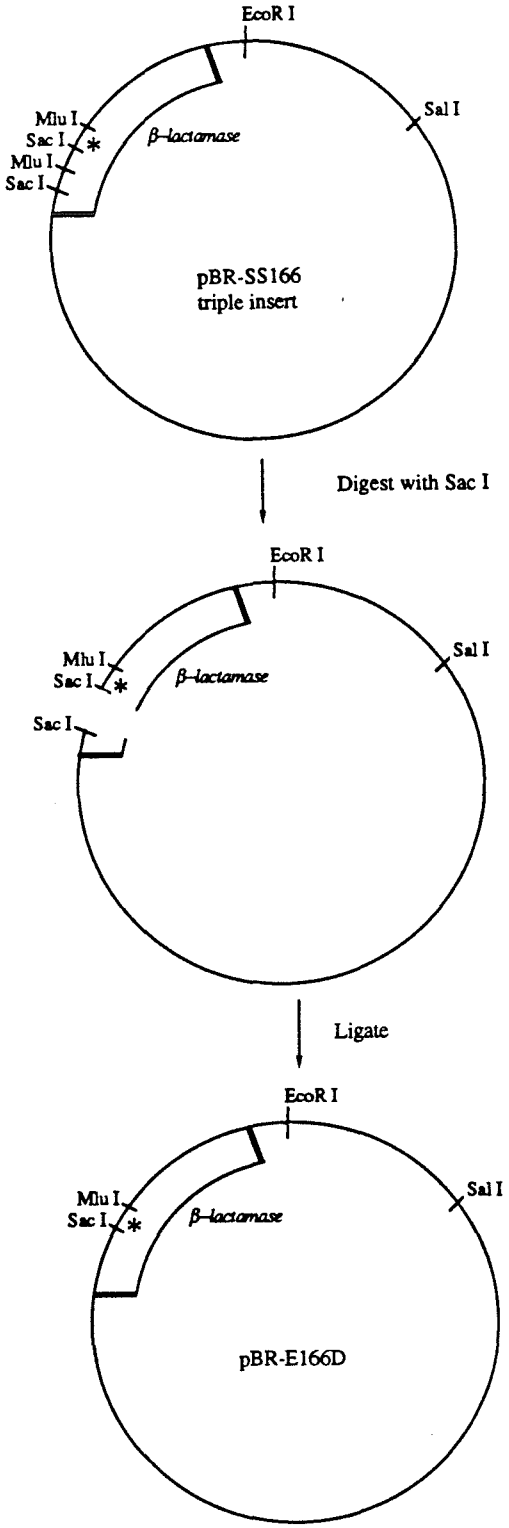


Figure III-7. Reclamation of Asp at 166, labelled with an asterisk (*) from a plasmid containing a triple insert.

the resulting colonies were chosen for sequencing to confirm the existence of Asp at residue 166 and the absence of the other two inserts.

Construction of a Double Mutant: K73E/E166K

A double mutant replacing glutamate with lysine at residue 166 and lysine with glutamate at 73 was constructed as outlined in Figure III-8. The E166K mutant was one of those constructed for site saturation at residue 166, as described above. The K73E mutant was from the site saturation at residue 73 performed previously by S. Carroll[5], in this laboratory. A Pvu I restriction was located conveniently between these two mutations in the β-lactamase gene. First, 5 μg of each mutant plasmid were digested with Sal I and Pvu I. Then, the appropriate fragments were isolated from an agarose gel and ligated. Following transformation into HB101, four colonies were chosen for sequencing to verify the presence of both mutations. The double mutant was then screened for phenotype by picking onto various antibiotic plates.

Western blots

Colonies harboring mutants were grown to late log phase (OD_{600}~1.0); a 1.5 ml sample of each was pelleted by centrifugation and resuspended in 100 μl protein sample buffer (10% v/v glycerol, 5% v/v 2-mercaptoethanol, 3% w/v sodium dodecyl sulfate, 62.5 mM Tris-HCl, pH 6.8, 1.0 mM EDTA, 0.05% w/v bromophenol blue) and heated to 95°C for 10 minutes to lyse the cells. Aliquots of 20 μl each were loaded onto a 15 cm, 12% polyacrylamide stacking gel and run at a constant current of 5 mA for 12-16 h. Protein was then transferred from the gel to DEAE nitrocellulose using a Bio-Rad Transblot cell for 6 h at 12 volts. β-lactamase was visualized following binding of rabbit anti-β-lactamase using the highly sensitive Vectastain® ABC immunoperoxidase system (Vector Laboratories)[17].

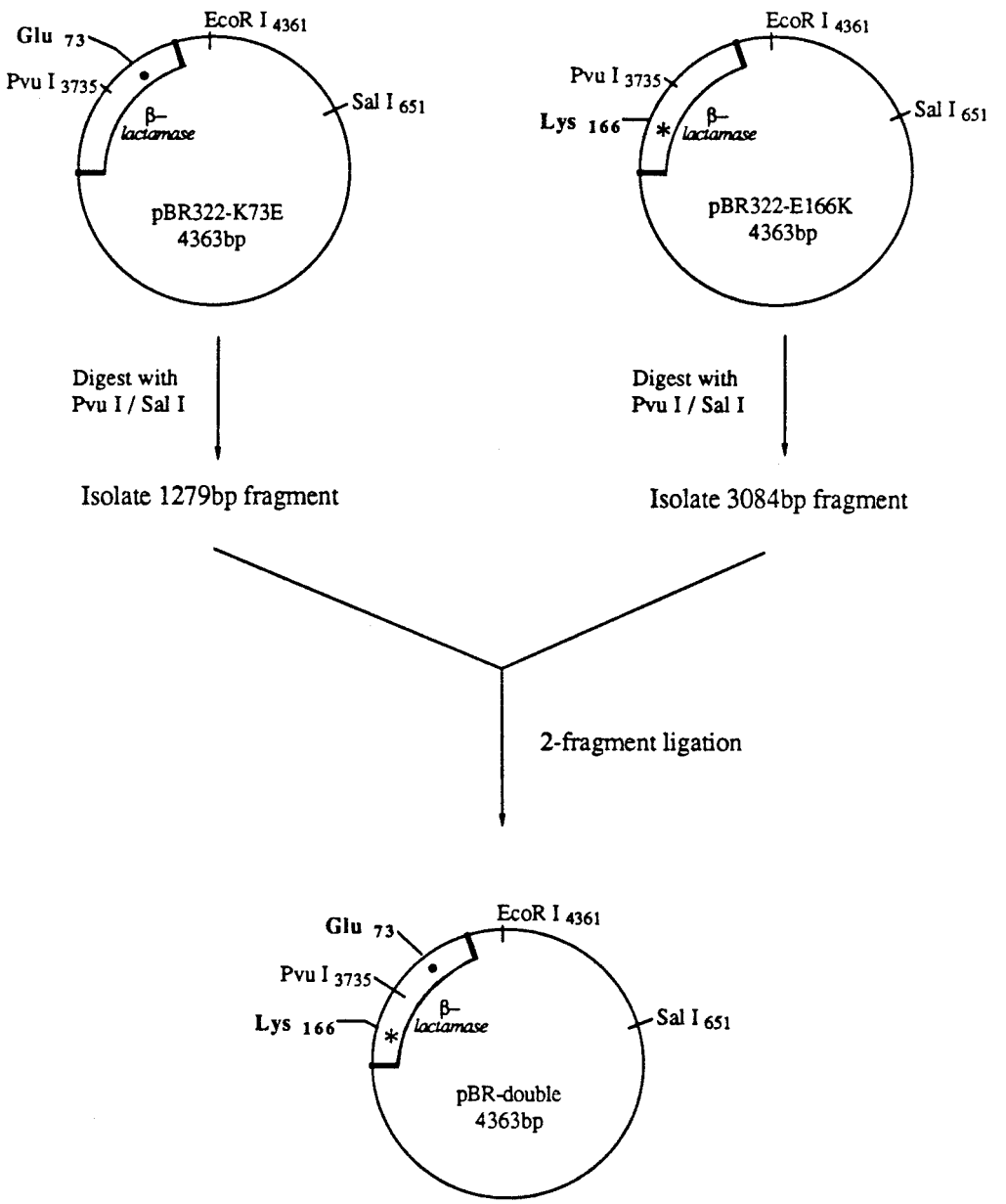


Figure III-8. Scheme for the construction of the double mutant E166K/K73E.

Expression of β-lactamase mutants in pJN

pJN is an expression vector for β-lactamase which was developed in this laboratory. It contains the β-lactamase gene under the control of the tac promoter, as well as a kanamycin gene for a selection marker (Figure III-9). Synthesis of β-lactamase is induced by the addition of IPTG. Before large quantities of mutant enzymes could be produced, their genes had to be subcloned into pJN. Mutants chosen for expression included Asp, Cys, Gln, His, Tyr, and Val, and the double mutant.

Figure III-10 outlines the scheme for transferring a mutant gene into pJN. First, both pBR322 carrying a mutation at Glu 166 and wild-type pJN (0.3 pmole each) were digested with Eco RI and BamH I. Then, to avoid isolating DNA fragments, equimolar amounts of digested pJN and mutant pBR322 were mixed and ligated. Following transformation into D1210, cells were plated onto 50 mg/l kanamycin. Resulting colonies contained either mutant β-lactamase gene, wild-type β-lactamase gene, or no β-lactamase gene; therefore, a selection scheme had to be designed to distinguish them. Those that grew after being picked onto high levels (>500 mg/l) of ampicillin contained the wild-type gene. Those that did not grow were either the targeted mutants or some other ligation product. The precise identities were determined by plasmid sequencing.

Protein Purification

One-half liter cultures of D1210 *E. coli* containing mutant plasmid in pJN were grown in L broth containing 50 mg/l kanamycin for 12-14 h at 37°C. IPTG was added to a final concentration of 0.1 mM and growth continued for 30 minutes at 37°C. Cells were centrifuged in 250 ml bottles in a GSA rotor for 10 minutes at 10,000 rpm. β-lactamase, which is located in the periplasm, was released by osmotic extrusion as follows. Pellets were resuspended in 20 ml sucrose solution

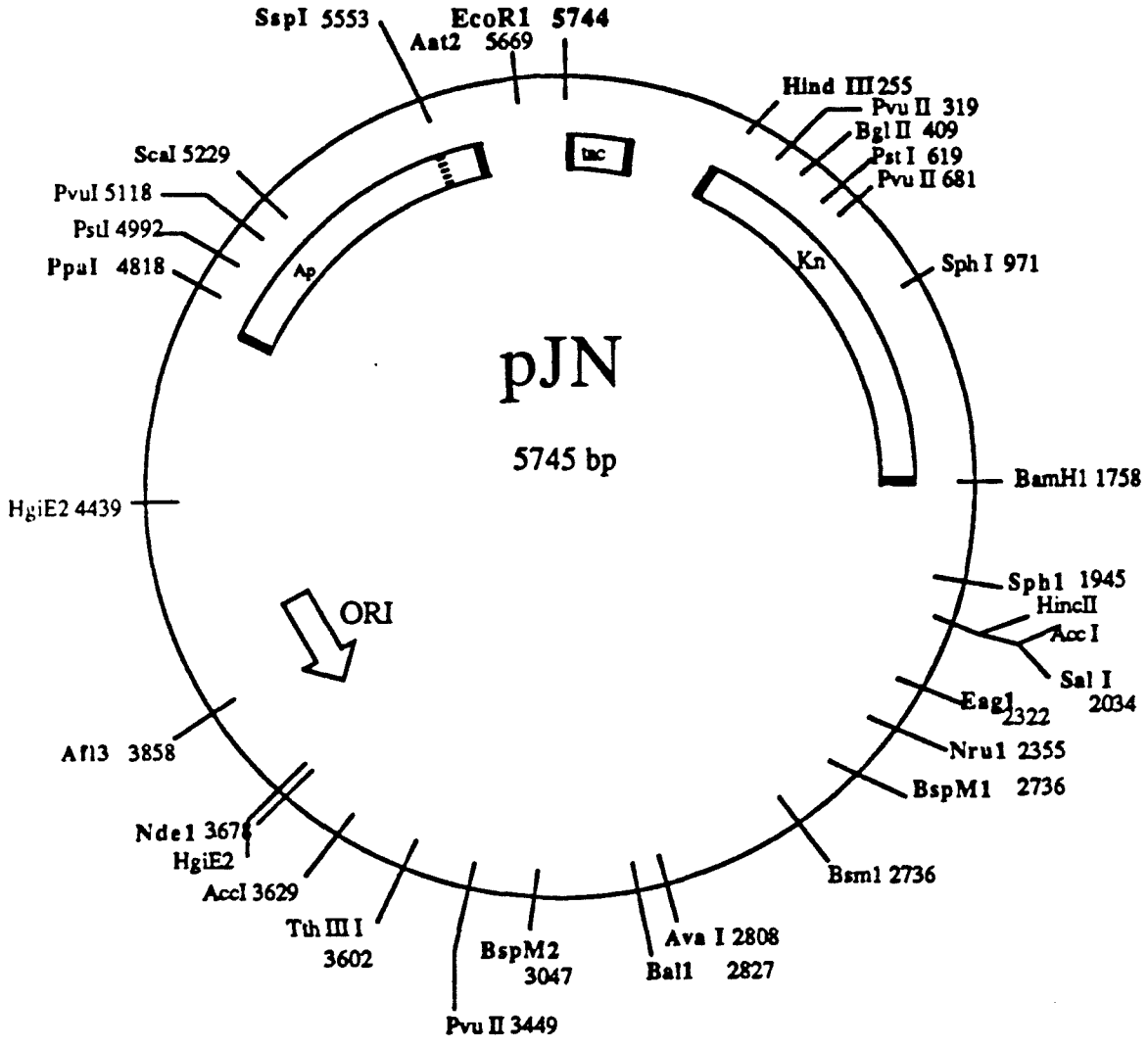


Figure III-9. Restriction map of pJN--the β-lactamase expression vector under the control of the tac promoter.

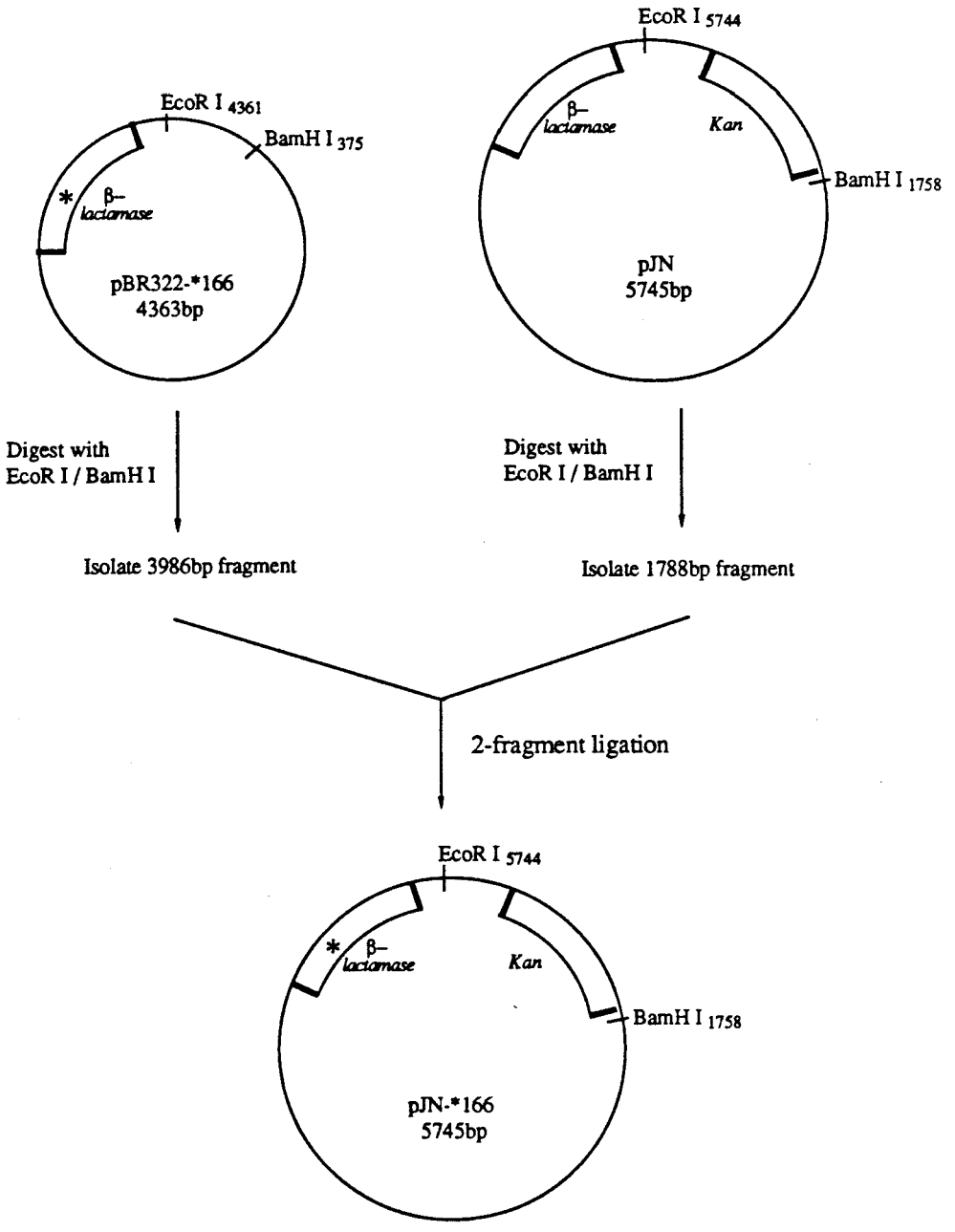


Figure III-10. Scheme for subcloning mutants at 166 into pJN.

(25 ml 1.0 M Tris-HCl, pH 7.0, 450 g sucrose, 0.5 g Na_2EDTA in 1 l) per gram of cells, and shaken at room temperature for 30 minutes. Samples were centrifuged at 10,000 rpm for 30 minutes. Supernatant was quickly removed and discarded, and cells resuspended in an equal volume of cold H_2O (4°C) and stirred in the cold room for 30 minutes. Again, samples were centrifuged at 10,000 rpm for 30 minutes. Supernatant, which contains periplasmic proteins, was saved, and the volume was reduced to 1 ml using a Diaflow cell (Amicon) under nitrogen. This sample was then filtered using a Schleicher & Schuell 0.22 μm Uniflow filter to remove any cellular debris.

Further purification was carried out using FPLC (fast protein liquid chromatography) with an anion exchange column--MonoQ™ by Pharmacia. Protein (200 μl) was loaded onto the column in 25 mM triethanolamine (TEA), pH 7.65 (solvent A) and eluted with a salt gradient using 25 mM TEA, 1 M NaCl (solvent B). The gradient used was: t=0 minutes, 100% A; t=3, 100% A; t=28, 81% A, 19% B; t=33, 100% B. The flow rate was 0.8 ml/min. Elution was monitored by A_{280}; activity was assayed with the hydrolysis of benzylpenicillin (see Kinetics, below). Peak fractions were pooled and dialyzed versus six liters of 0.1 M potassium phosphate, pH 7.0. Protein concentrations were estimated from OD_{281} using an extinction coefficient of 29,400 $M^{-1}cm^{-1}$. Samples were run on 12% polyacrylamide gels (as in Western blots) and stained with Coomassie blue to gauge purity.

Kinetics

Michaelis-Menten kinetic parameters (k_{cat}, K_M, and k_{cat}/K_M) for wild-type β-lactamase on benzylpenicillin and cephalothin were first determined using the initial velocity method. Reactions were carried out at 30°C, 0.1 M potassium phosphate, pH 7.0. All reagents were maintained at 30°C prior to beginning the

assay to avoid error from temperature flux. A Beckman DU7 spectrophotometer was used for the assays, with quartz cells of pathlength 1 cm (for penam substrates) or 1 mm (for cephem substrates). Sufficient enzyme was added such that each reaction was complete within 30 minutes, enabling an accurate measure of initial velocity (during the first 5% of the reaction) at five substrate concentrations from 50-500 μM. Rates were determined from the slope as OD/min and converted to M/sec using $\Delta\varepsilon$ of 500 $M^{-1}cm^{-1}$at 240 nm for benzylpenicillin and $\Delta\varepsilon$ of 7900 $M^{-1}cm^{-1}$ at 265 nm for cephalothin.

These parameters were also derived from the single reaction curve integration method under the same conditions as above, but the reaction was allowed to go to completion. Wavelengths used were 240 nm for benzylpenicillin and 270 nm for cephalothin. Enzyme concentration was adjusted so that completion occurred in approximately 20 minutes. For highest accuracy, substrate concentration had to be five times greater than K_M. A computer program fitting the curve of absorbance versus time to a right hyperbola used the following integrated form of the Michaelis-Menten equation

$$\frac{-\Delta t}{\Delta[S]} = \frac{1}{V_{max}} + \frac{K_M}{V_{max}} \frac{\Delta ln[S]}{\Delta[S]} ,$$

which yielded numbers identical to those from the initial rate method.

Subsequently, all kinetics runs for mutant β-lactamases were done using the integration method, because of its greater ease and accuracy. All runs were repeated at different substrate concentrations and the results averaged. The appropriate concentrations were determined by trial and error since K_M values for the mutant enzymes were unknown.

RESULTS

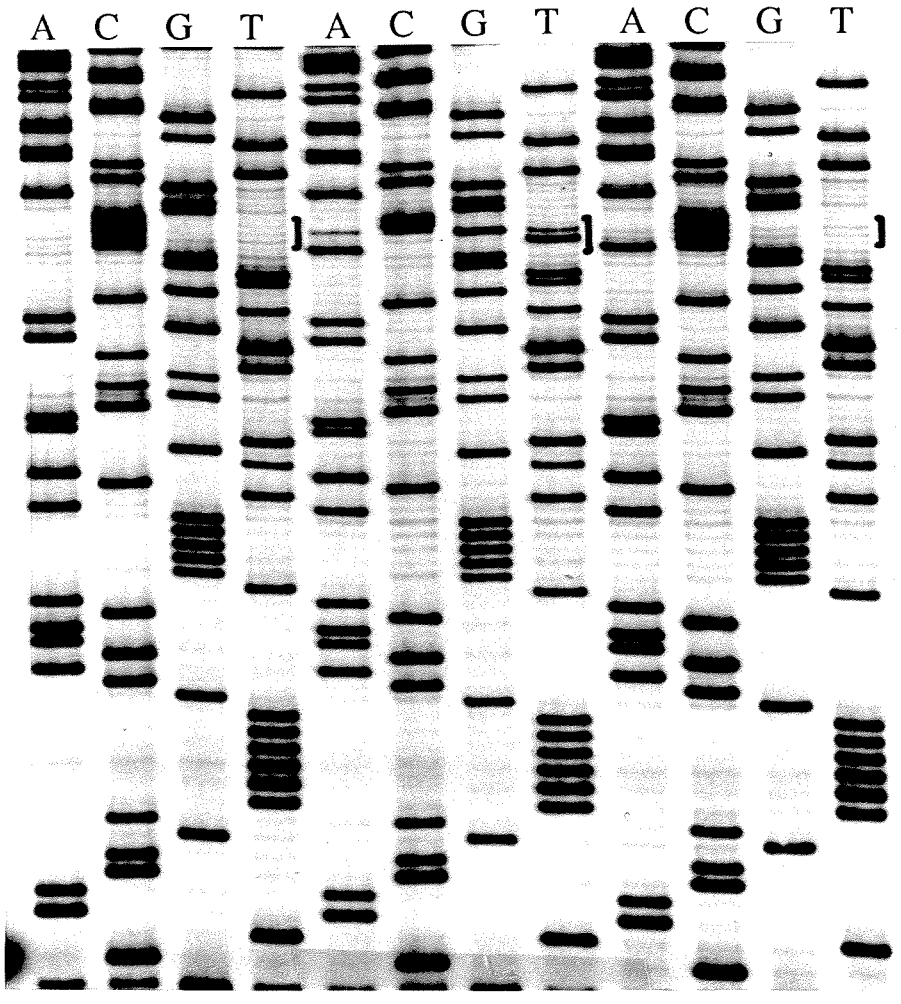
Two unique restriction sites, Mlu I and Xba I, were added to pBR322 using the Amersham *in vitro* mutagenesis kit. Because of the Eckstein selection method of this system, 80% of the plaques sequenced contained all three point mutations. With such a high success rate, ten or fewer plaques needed to be screened--much less than the thousands required for screening with hybridization when no selection process is employed. In addition, no problem was encountered in making three mutations using a single primer.

Xba I requires a *dam*- strain of *E. coli* to permit cleavage since it is sensitive to methylation at its final adenosine. [In pBR322-Mlu, the Xba I sequence (TCTAGA) is immediately followed by TC, allowing methylation at this site.] Therefore, for convenience, Mlu I was chosen as the restriction site to be used with Sac I for cassette mutagenesis. The degenerate oligonucleotides were ligated into pBR322, using these sites and Sal I in a three-fragment ligation scheme. More than 180 transformants resulted on six tetracycline plates.

One hundred seventy-seven colonies were picked onto plates of several concentrations of benzylpenicillin, ampicillin, and cephalothin for phenotypic screening at both 30°C and 37°C. Seven colonies exhibited activity equal to that of wild-type on each antibiotic. Four others were active only on low levels (100 mg/l or less) of ampicillin and cephalothin. All other colonies showed zero activity.

Sequencing was accomplished using the double-stranded DNA dideoxy method with a Sequenase™ kit. Figure III-11 shows a typical autoradiogram of a sequencing gel, with the codons for 166 bracketed. Generally, the sequences were clear and easy to read, but the high concentration of cytosine and guanine around the codon for 166 sometimes led to compression. After seventy-five plasmids were sequenced, it appeared that only cysteine and tyrosine were missing. It was thought that they would be hard to find since thymine was rarely located in the

Figure III-11. A representative sequencing gel for mutants at 166. Double-stranded plasmid dideoxy sequencing was used; labelling was with [α-^{35}S]-dATP. The codon at 166 for each mutant is bracketed.


A C G T A C G T A C G T
Pro (CCC)
Met (ATG)
Thr (ACC)

first position of the codon; Table III-I lists the frequencies of bases at each position. Overall, 27 out of the thirty-two possible codons were found in seventy sequences (see Table III-II). Therefore, a second ligation was performed to produce Cys and Tyr at position 166. Ten colonies from this ligation were then sequenced: four were Tyr and six were Cys.

A routine Pst I/EcoRI digestion of several of the mutant plasmids showed that about one-fourth were larger than expected, as seen in altered mobility on a 1.5 % agarose gel (Figure III-12). Five of nineteen mutants tested had a small Pst I/EcoR I fragment that was larger than that of the wild-type. Although several of these plasmids had already been sequenced, this increased size had gone undetected. Fortunately, eighteen of the nineteen Glu 166 mutants were of the expected size. Only Asp 166 was part of what was assumed to be a triple insertion of degenerate oligos. This Asp 166 plasmid was resequenced (Figure III-12) to identify the insertion. The bracketed section of the sequencing gel actually represents more than thirty bases. Because of the proximity of identical palindromic restriction sites in the triple insert, a major compression of the sequence had occurred. Enough of the gel was readable to verify that the sequence (Figure III-14) was indeed that of three synthetic fragments coming together in a five-fragment ligation. The problem in this instance was that the oligonucleotides were kinased prior to ligation, allowing them to ligate with other synthetic DNA, as well as with the pBR322 fragments.

To remove two of the synthetic inserts, the triple-insert plasmid containing Asp at 166 was digested with an excess of Sac I (see Figure III-7). Ligation favored closure of the long fragment with a Sac I site at both ends rather than a two-fragment ligation to regenerate the triple insert. Four colonies were picked and sequenced. All were found to contain Asp at 166 with no other inserts, completing the series of all mutants at residue 166.

Table III-I Distribution of bases at each position of the codon at position 166.

	1st position	2nd position	3rd position
A	28%	32%	- -
C	23%	20%	47%
G	37%	25%	53%
T	12%	23%	- -

Table III-II Frequency of codons sequenced during site saturation at 166.

amino acid	codon	frequency	amino acid	codon	frequency
Asparagine	AAC	1	Aspartate	GAC	2
Lysine	AAG	1	Glutamate	GAG	6
Threonine	ACC	3	Alanine	GCC	2
Threonine	ACG	1	Alanine	GCG	1
Serine	AGC	4	Glycine	GGC	4
Arginine	AGG	1	Glycine	GGG	3
Isoleucine	ATC	3	Valine	GTC	1
Methionine	ATG	3	Valine	GTG	3
Histidine	CAC	3	Tyrosine	TAC	0
Glutamine	CAG	6	STOP	TAG	0
Proline	CCC	1	Serine	TCC	1
Proline	CCG	2	Serine	TCG	1
Arginine	CGC	0	Cysteine	TGC	0
Arginine	CGG	1	Tryptophan	TGG	2
Leucine	CTC	1	Phenylalanine	TTC	2
Leucine	CTG	0	Leucine	TTG	1

Figure III-12. A 1.5% agarose gel of Pst I/EcoR I digestions of wild-type (lane 1) and nineteen mutants. Five of the mutants (lanes 2, 7, 9, 10, and 14) show decreased mobility of the small fragment because of the presence of a triple insert.

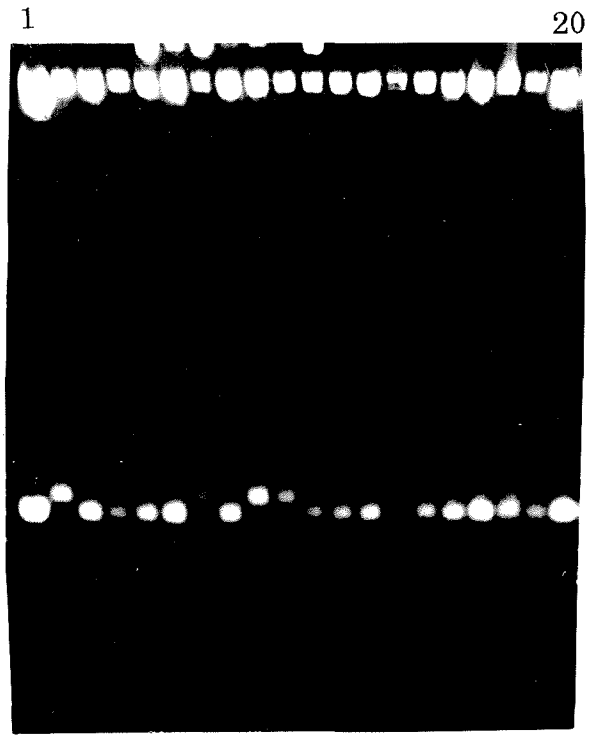
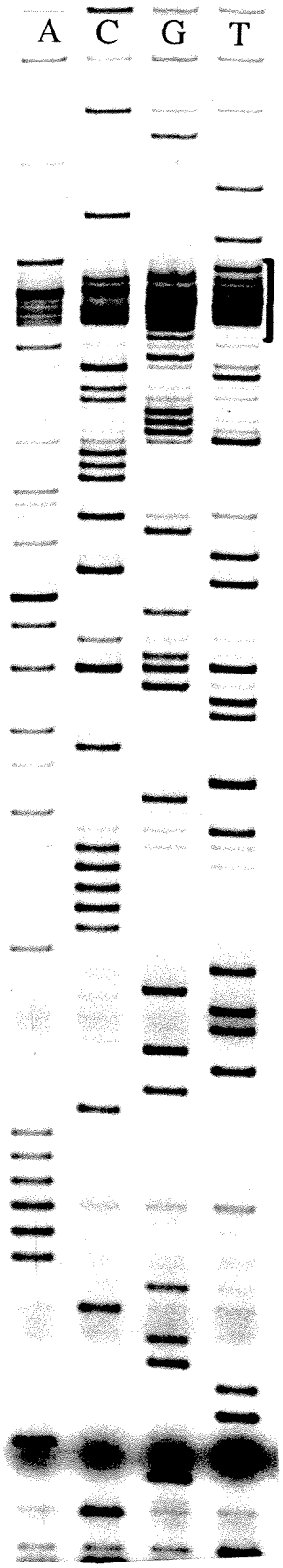

1
20

Figure III-13. Portion of a gel containing the sequence of a plasmid with Asp (GAC) at its first insert as well as two other inserts (labeled with a bracket). The compression of more than bands is due to the close proximity of identical palindromic restriction sites.


A
C
G
T

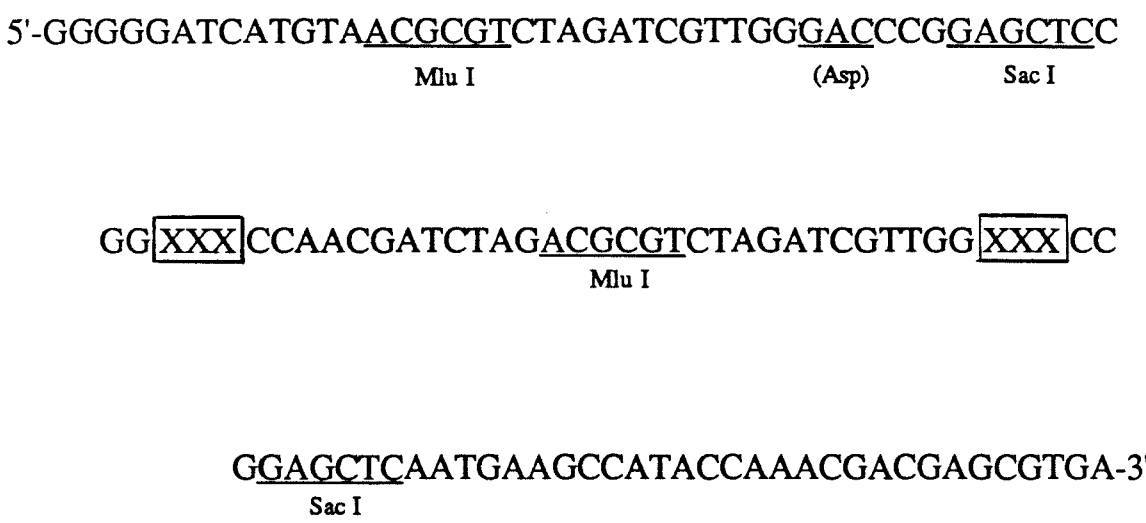


Figure III-14. Sequence of the triple insert ligated into pBR 322 in between Sac I and Mlu I. The first insert contains the codon for Asp; the second and third are unknown.

These mutants, with wild-type, were again screened on several antibiotics. As Table III-III shows, three mutants had significant activity on ampicillin (growing at 100 mg/l) and benzylpenicillin (50 mg/l). Only Asp grew on cephalothin (25 mg/l). All other mutants were inactive.

This inactivity could be due to a protein stability problem. Western blots of all β-lactamase mutants at position 166 (Figure III-15) showed that all but two--lysine and arginine--were stable at 37°C. Faint bands were seen for both of these mutants, but the rest were much darker and equal in amount to wild-type. Western blots were also run at 30°C with the same results (not shown).

Since Lys 166 is one of two unstable mutants (both of which are positively charged) the stability problem could be due to a repulsive interaction with Lys 73. To correct this, and possibly to regain activity by rebuilding the salt bridge, a double mutant was constructed from this Lys at 166 and a glutamate at residue 73 (from the site stauration performed by S. Carroll), using a two fragment ligation with Pvu I and Sal I. The presence of both mutations in one plasmid was confirmed by sequencing. A Western blot of this mutant, along with E166K, was run (Figure III-16) and showed that stability was regained by adding the second site mutation. Phenotypic screening of this double mutant, however, showed that it was completely inactive.

Several mutants that were chosen for further study were then subcloned into the expression vector pJN. These included Asp, Cys, Gln, His, Tyr, and the double mutant. Wild-type and mutant β-lactamases were isolated using a procedure adapted from J. Neitzel. Final purification was on the FPLC using conditions worked out by D. Long and S. Wolfe (personal communication). β-lactamase was eluted from an anion exchange column using a NaCl gradient (see Figure III-17). Although approximately 1 mg of pure wild-type β-lactamase resulted from this procedure, mutant enzymes which were prepared were

Table III-III Maximum resistance of mutants of Glu 166 to β-lactam antibiotics.

Amino Acid	Codon	Ampicillin (mg/l)	Cephalothin (mg/l)	Penicillin G (mg/l)
Glutamate (wt)	GAG	2000+	100	2000+
Alanine	GCG	0	0	0
Arginine	AGG	0	0	0
Asparagine	AAC	0	0	0
Aspartate	GAC	100	25	50
Cysteine	TGC	0	0	0
Glutamine	CAG	0	0	0
Glycine	GGG	0	0	0
Histidine	CAC	100	25	50
Isoleucine	ATC	0	0	0
Leucine	CTC	0	0	0
Lysine	AAG	0	0	0
Methionine	ATG	0	0	0
Phenylalanine	TTC	0	0	0
Proline	CTC	0	0	0
Serine	TCG	0	0	0
Threonine	ACC	0	0	0
Tryptophan	TGG	0	0	0
Tyrosine	TAC	100	0	50
Valine	GTG	0	0	0

Figure III-15. Western blots of all residues at position 166 of β-lactamase. The enzyme was visualized following binding of rabbit anti-β-lactamase, using the highly sensitive Vectastain ABC immunoperoxidase system. A background lane of *E. coli* protein that cross-reacts with the antibody confirms that the same number of cells was loaded in each lane. Two gels were run separately and then combined for this figure.

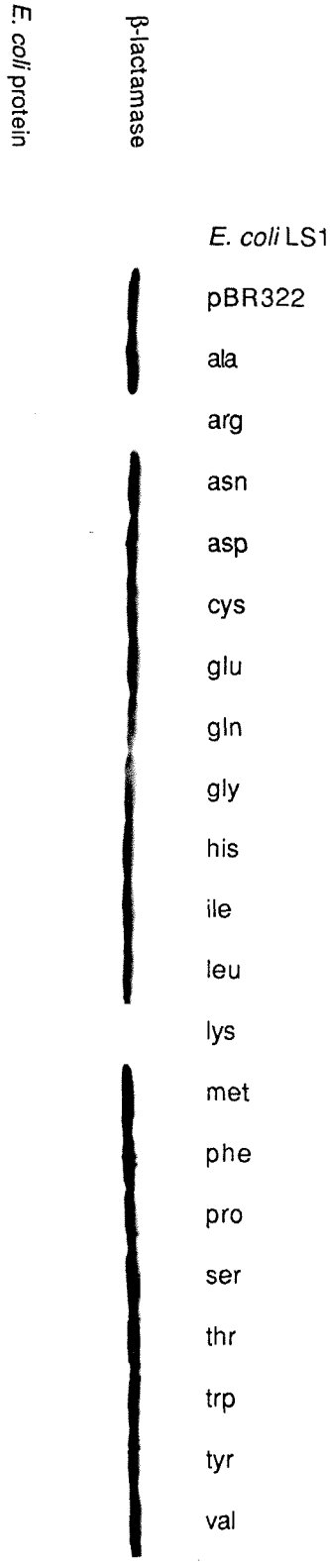
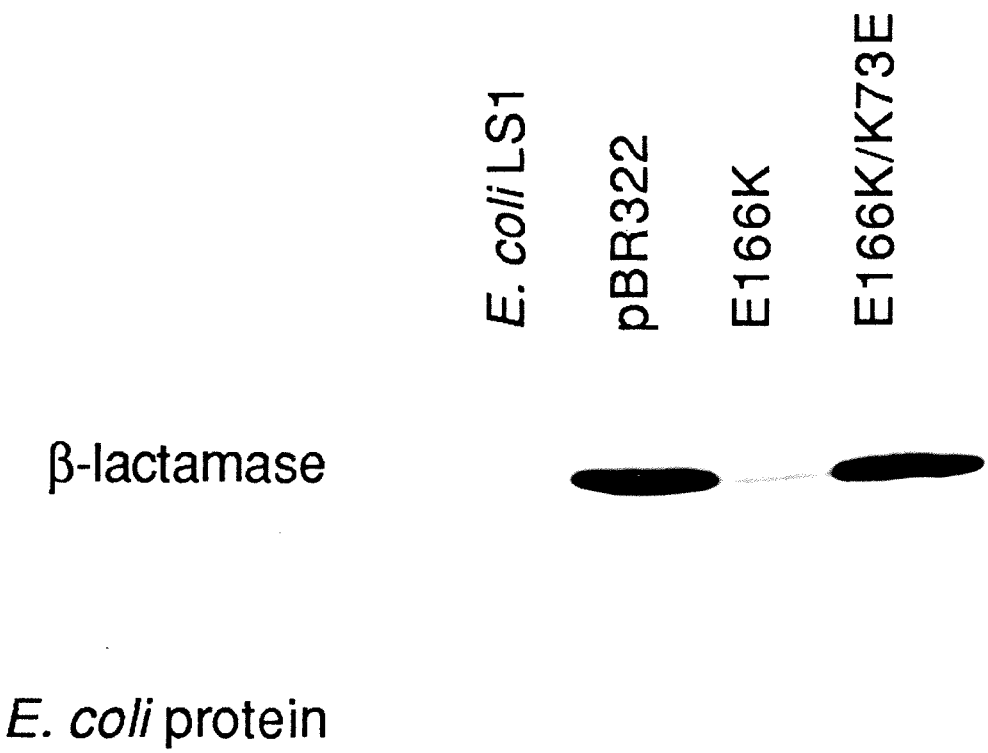

E. coli protein
β-lactamase
E. coli LS1
pBR322
ala
arg
asn
asp
cys
glu
gln
gly
his
ile
leu
lys
met
phe
pro
ser
thr
trp
tyr
val

Figure III-16. Western blot, run as in Figure III-15. This Western shows the return to full stability of the Lys at 166 mutant following a second mutation of Lys to Glu at postion 73.


E. coli LS1
pBR322
E166K
E166K/K73E
β-lactamase
E. coli protein

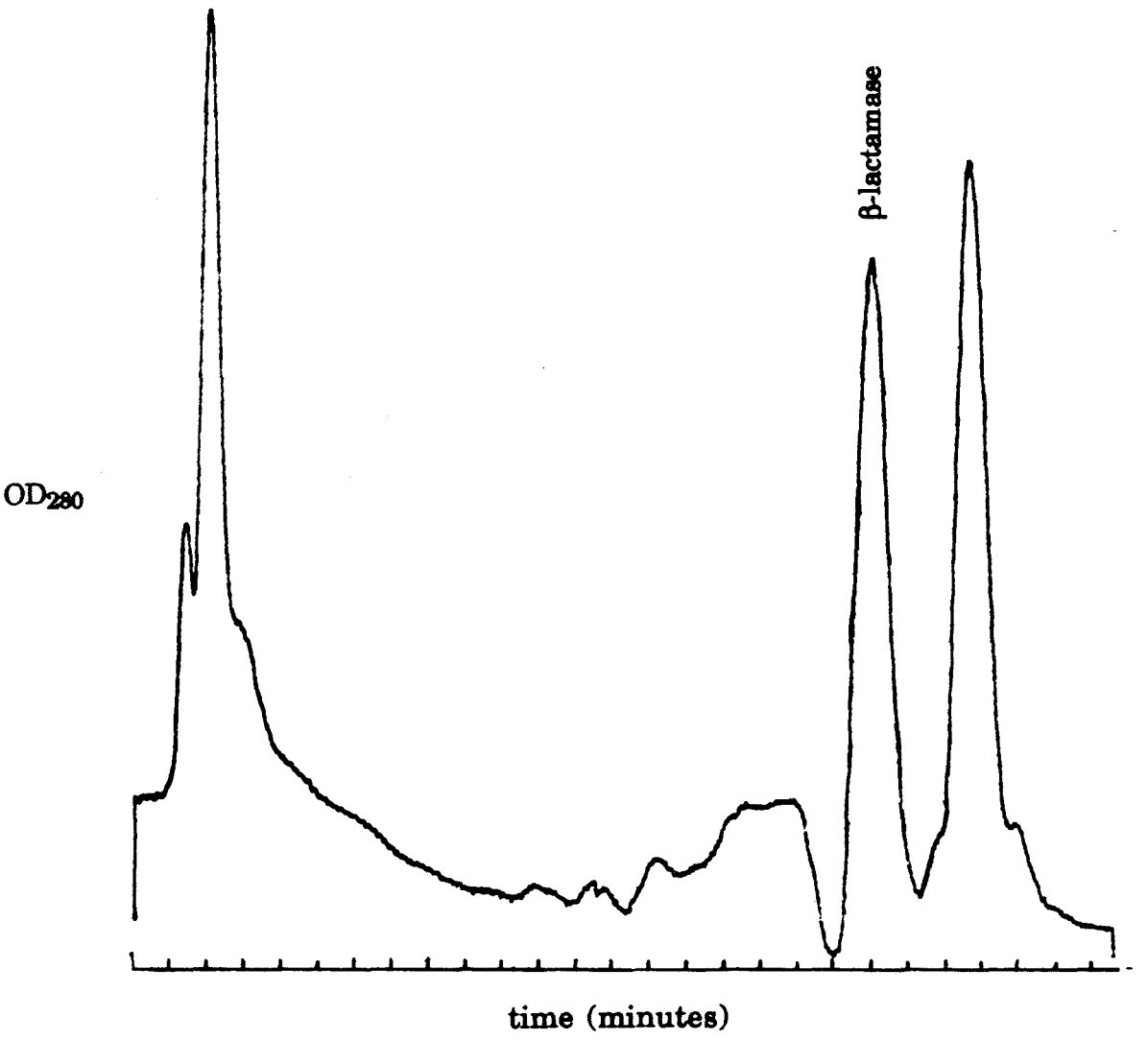


Figure III-17. FPLC trace of wild-type β-lactamase eluted with a gradient of 0-1 M NaCl. The peak for β-lactamase is at approximately 20 minutes and 13 mM NaCl.

contaminated with several other proteins as detected on a protein gel stained with Coomassie blue (not shown).

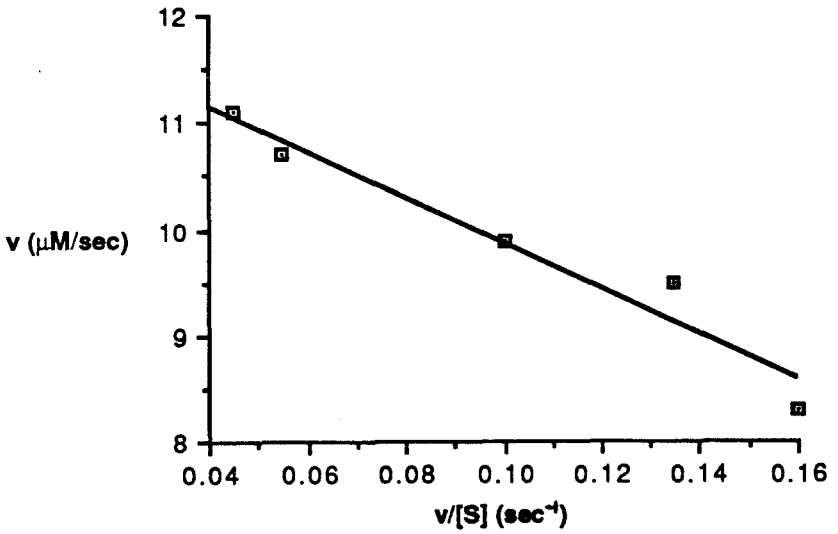
Kinetic parameters (k_{cat}, K_M, and k_{cat}/K_M) were determined on purified wild-type β-lactamase, using the initial velocity method for both benylpenicillin and cephalothin. Rates were measured during the first 5% of the reaction at five substrate concentrations. The Eadie-Hofstee linear replot method[19,20] was used to determine K_M and V_{max} (see Figure III-18 for representative plots); k_{cat} is simply V_{max} divided by the enzyme concentration.

These parameters were also determined using the single reaction curve logarithmic method[21]. This requires a substrate concentration of at least five times the K_M, and sufficient enzyme such that the reaction is complete in approximately twenty minutes. A representative curve for benylpenicillin is shown in Figure III-19. A computer program fits this curve to a right hyperbola, and then derives k_{cat} and V_{max} from the logarithmic form of the Michaelis-Menten equation. All runs were repeated and the results were reported as averages.

Results for wild-type β-lactamase and the two substrates derived from both methods are listed in Table III-IV. The initial velocity and logarithmic methods yield almost the same numbers for all parameters, and both are very close to the literature values. Because of the low number of reactions required and the ease of data handling, the logarithmic method was chosen for future kinetics runs.

The four mutants β-lactamases that were purified--Asp, Gln, His, and Tyr--were found to be contaminated with other protein. A single peak had been collected for each, but it had had an altered elution profile on the FPLC from that of wild-type β-lactamase. Apparently, it had merged with one or more other peaks that were inadvertently collected. Further purification is possible, but was not performed. Prior to the discovery of this contamination, kinetic assays had been run on all mutants using benzylpenicillin as substrate. The values for k_{cat} are

A.



B.

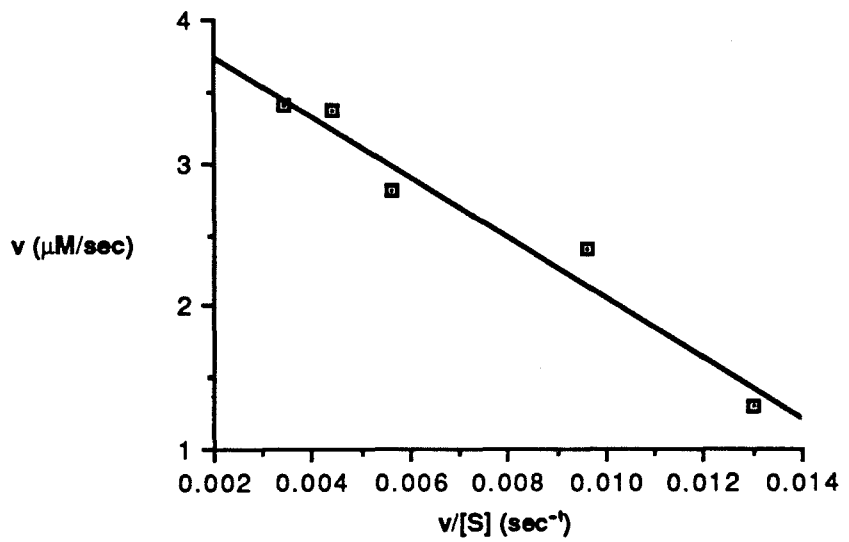


Figure III-18. Linear replot of initial rate versus substrate concentration using the Eadie-Hofstee equation for β-lactamase with a) benzylpenicillin (K_M=21.5 μM) and b) cephalothin (K_M=211 μM).

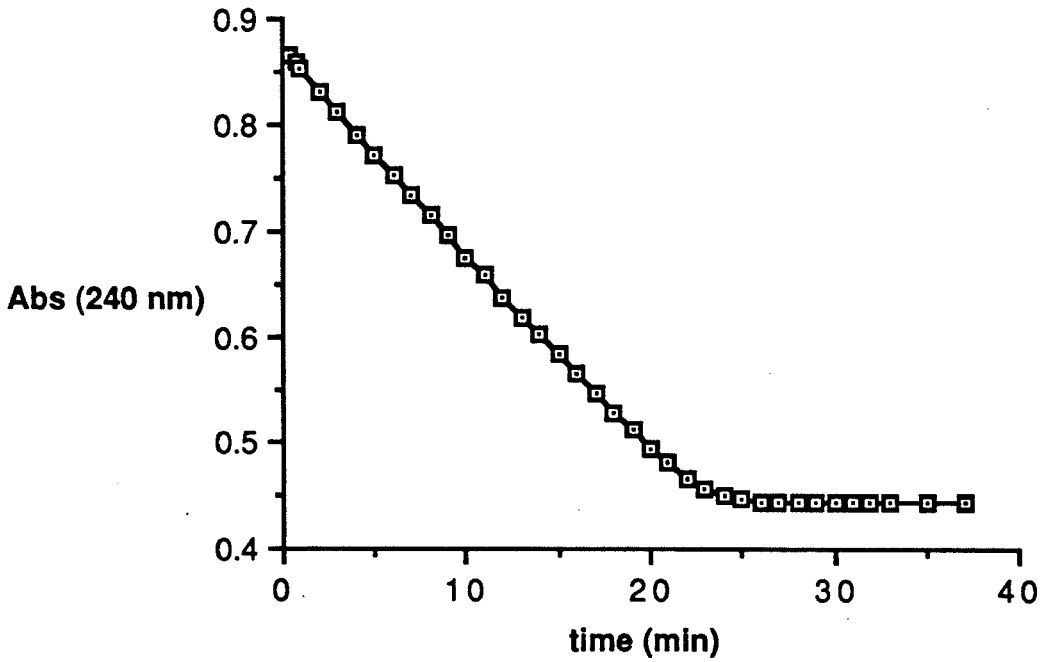


Figure III-19. Whole reaction curve of time versus OD_{240} for wild-type β-lactamase and 100 μM benzylpenicillin.

Table III-IV Michaelis-Menten parameters for wild-type β-lactamase with benzylpenicillin and cephalothin, using two methods.

Benzylpenicillin

method	K_M (μM)	k_{cat} (sec^{-1})	k_{cat}/K_M ($M^{-1}sec^{-1}$)
initial velocity	21.5	2108	9.8×10^7
logarithmic	23	2224	9.5×10^7
literature value	20	2000	1.0×10^8

Cephalothin

method	K_M (μM)	k_{cat} (sec^{-1})	k_{cat}/K_M ($M^{-1}sec^{-1}$)
initial velocity	211	123	5.8×10^5
logarithmic	207	128	6.1×10^5
literature value	200	120	6.0×10^5

Table III-V K_M of wild-type β-lactamase and three mutants for benzylpenicillin.

enzyme	K_M (μM)
wild-type	20
Asp 166	20 ±1
His 166	18 ±1
Gln 166	18 ±2

inaccurate, but the K_M's can be used, since this parameter does not depend on enzyme concentration. (It would be affected only if the contaminating proteins interacted with the substrate.) Three mutants--Asp, Gln, and His--were found to have K_M's within the range (20 ±2 μM) of the wild-type K_M (Table IV-V). Although k_{cat}, was not measured, the phenotypic results indicate that it would be much lower than wild-type in each case. Results for the fourth mutant (Tyr) could not be determined since it did not follow Michaelis-Menten kinetics.

DISCUSSION

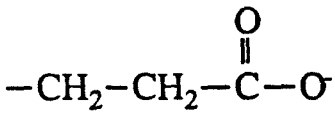
Information from the crystal structure of *S. aureus* β-lactamase was used to target glutamate 166 as a subject of site saturation. This marked the first time in this laboratory that mutagenesis had been performed based on an actual three-dimensional structure. In its location in the active site opposite the catalytic serine 70, the carboxyl side chain of Glu 166 is ideally positioned to assist a bound water molecule in the deacylation of the acyl-enzyme intermediate (see Figure III-2). In addition, Glu 166 forms a salt bridge with lysine 73 which may be critical both for local stability and for positioning the residues for catalysis. By producing multiple mutations that replace Glu 166, we hoped to dissect the catalytic process by creating an enzyme that is fully functional in the initial acylation step, but deficient in deacylation: in essence, a penicillin-binding protein[22].

Following the addition of a unique Mlu I restriction site to the β-lactamase gene, cassette mutagenesis was performed to place all codons at the site of glutamate 166. The resulting mutant colonies were phenotypically screened and then sequenced. The preliminary screening revealed that more than 90% of the mutants were inactive on β-lactam antibiotics. After sequencing seventy plasmids (using the double-stranded DNA dideoxy method), a serious flaw in the synthesis of the degenerate oligonucleotides was noticed. The percentage of thymine in the first position of the codon was extremely low (12% compared to the expected 25%). For the technique of site saturation to be effective, all bases must be added equally at each position of degeneracy. Because of this shortage of thymine, neither cysteine nor tyrosine was discovered. Subsequently, these two mutants were produced in a separate three fragment ligation using a doubly degenerate oligonucleotide insert; each was found soon after this. Although it was not difficult to produce these remaining mutants, the poor quality of the oligonucleotides undermined the power of site saturation. In situations where

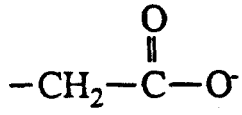
multiple sites are saturated (producing too many variants to sequence all of them), the DNA synthesis must be reliable to guarantee that a complete set of mutants has been created.

Prior to the initial three-fragment ligation, the oligonucleotides were kinased to increase the yield of mutant colonies, even though this was not essential for ligation. Kinased inserts have the potential to ligate to other inserts, as well as to vector DNA. That this had happened became noticed following restriction digests of the mutant plasmids with Pst I and EcoR I; 25% showed decreased mobility on an agarose gel. Sequencing revealed that these plasmids contained not one but three synthetic inserts. Because of the presence of two closely spaced Sac I sites, major band compression within this insert occurred on sequencing gels, obscuring the identities of two of the codons for residue 166. Aspartate at 166 was the only mutant found exclusively in one of these triple-insert plasmids. Digestion of this plasmid with Sac I followed by ligation deleted two of the three inserts, leaving a plasmid of normal size containing Asp 166 in the proper orientation.

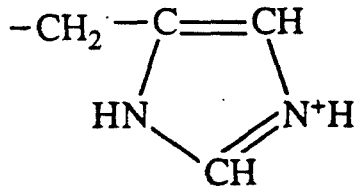
With all nineteen mutants sequenced, phenotypic activity was again tested. Sixteen were inactive; only aspartate, histidine, and tyrosine were able to grow on low levels of penam antibiotics (100 mg/l or less). Since wild-type β-lactamase confers resistance to greater than 2000 mg/l of these drugs, it is obvious that these mutations severely impair enzyme function. However, any activity is unexpected, given that glutamate 166 is conserved among class A β-lactamases[23]. Aspartate is the most obvious alternative since it also has a carboxyl side chain (see Figure III-20), but histidine and tyrosine are very surprising substitutions. Glutamine, which has an amide instead of a carboxyl group, is inactive. There is no obvious pattern relating activity to structure. If hydrogen-bonding capability to water were the only requirement, glutamine should be allowed unless the amide produces some other unfavorable interaction.



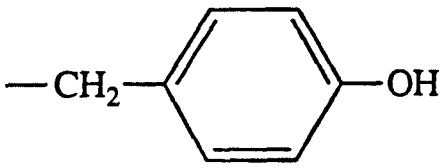
glutamate



aspartate



histidine



tyrosine

Figure III-20. Side chains of active mutants of Glu 166.

All mutants were tested for thermal stability by producing Western blots at both 30°C and 37°C. Seventeen mutants produced β-lactamase at levels comparable to wild-type. Arginine and lysine, though, were present in only small amounts at both temperatures. The presence of these positively charged side chains within the vicinity of Lys 73 is the most probable cause for this instability. All mutants remove the salt bridge between Lys 73 and Glu 166, resulting in a loss of approximately 5 kcal/mole for this favorable ionic interaction[24]. Most do this without sacrificing protein stability, but local structural perturbation at the active site is unavoidable. For the long, positive side chains, the repulsive force of the interaction with Lys 73 must cause enough structural change throughout the mutant proteins to increase susceptibility to cellular proteases. The presence of faint bands on the Westerns for these mutants indicates that β-lactamase was in fact being made for each.

To attempt to regain stability and regenerate a viable enzyme, a double mutant was constructed incorporating a mutant glutamate 73 into the lysine 166 mutant. This change could possibly restore the lost salt bridge and bring back function, although this was unlikely since neither residue could fulfill the other's hypothesized structural role in β-lactamase catalysis[1]. A Western blot of the K73E/E166K double mutant showed a dramatic increase in cellular protein level over that of the E166K single mutant. K73E had previously been shown to be as stable as wild-type β-lactamase[5], but it is unlikely that the two glutamates would be close enough to each other to cause instability. Phenotypic activity was tested for the double mutant, and it was found to be inactive. Therefore, the "surgery" repaired the problem of stability, but could not cure the loss of activity.

Several mutant β-lactamase genes were cloned into pJN, an expression vector under the control of the tac promoter. Wild-type enzyme and the Asp, Gln, His, and Tyr mutants were purified using a procedure involving osmotic extrusion

and FPLC. Although the wild-type β-lactamase was pure as detected on a protein gel, the mutants were contaminated with other protein. Further purification steps will have to be used in the future because of the altered elution of the mutants from the FPLC column.

The K_M for several of the mutants was determined even though the enzymes were not totally pure, since this parameter is not dependent on protein concentration. Asp, Gln, and His all had K_M's in the range of wild-type β-lactamase (20 μM), implying that mutants at this site do not affect substrate binding. It is important to note that even the inactive mutant (Gln) retains the capacity to bind substrate as well as wild-type, despite the fact that its catalysis is below the range of phenotypic detection (0.5%).

In summary, these results have confirmed that Glu 166 is vital to the catalytic capability of β-lactamase. Several interesting substitutions are allowed at this site, but none approaches wild-type in its ability to hydrolyze β-lactam antibiotics. The decrease in catalysis is apparently unrelated to substrate binding. The loss of the salt bridge at the active site may disturb positioning of the catalytic residues, leading to low activity, but only leads to thermal instability when lysine or arginine is substituted. Further kinetic analysis[25,26] of mutants of glutamate 166 will be required to confirm that its structural role in catalysis by β-lactamase is, in fact, assisting water in the deacylation of the covalent acyl-enzyme intermediate.

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Chapter 4

Site saturation of β-lactamase at alanine 237: Studies on A237N and A237T

INTRODUCTION

As discussed in Chapter 2, the investigation of the role of alanine 237 in the activity of RTEM-1 β-lactamase was undertaken following the discovery by Knowles of a chemically induced mutant of RTEM-2 β-lactamase, which had increased resistance to cephem antibiotics[1]. Originally identified as a change from alanine 172 to threonine, this mutant (called *h1*) was later found to be an alanine 237 to threonine mutation[2]. This increased ability to hydrolyze cephems came with a decrease in resistance to penam antibiotics.

The crystal structure of *S. aureus* β-lactamase[3]--a class A β-lactamase that is highly conserved with RTEM--shows that residue 237 is an important part of the active site (see Figure IV-1). Its amide nitrogen, along with the amide nitrogen of serine 70, forms part of an oxyanion hole, which stabilizes the tetrahedral intermediate in the hydrolysis of β-lactams. Figure IV-2 shows the proposed hydrogen bonding from the amide hydrogens to the β-lactam carbonyl oxygen, along with the oxyanion hole from a serine protease[4], for comparison. In addition, the main chain carbonyl of residue 237 apparently participates in hydrogen bonding both the amide hydrogen of the side chain of the β-lactam and the water molecule that is positioned for involvement in the deacylation of the acyl-enzyme intermediate.

Based on its position at the active site, residue 237 obviously plays an important role in the function of β-lactamase. However, since it is the amide backbone of Ala 237, which is involved directly in enzyme function, it is unknown how a mutant at that site could affect binding or catalysis. The existence of *h1* (A237T), which was generated randomly and selected for increased cephalosporin C resistance, shows that mutants at this site could have altered activity. Although a mutant at residue 237 would still possess the amide nitrogen, interactions with

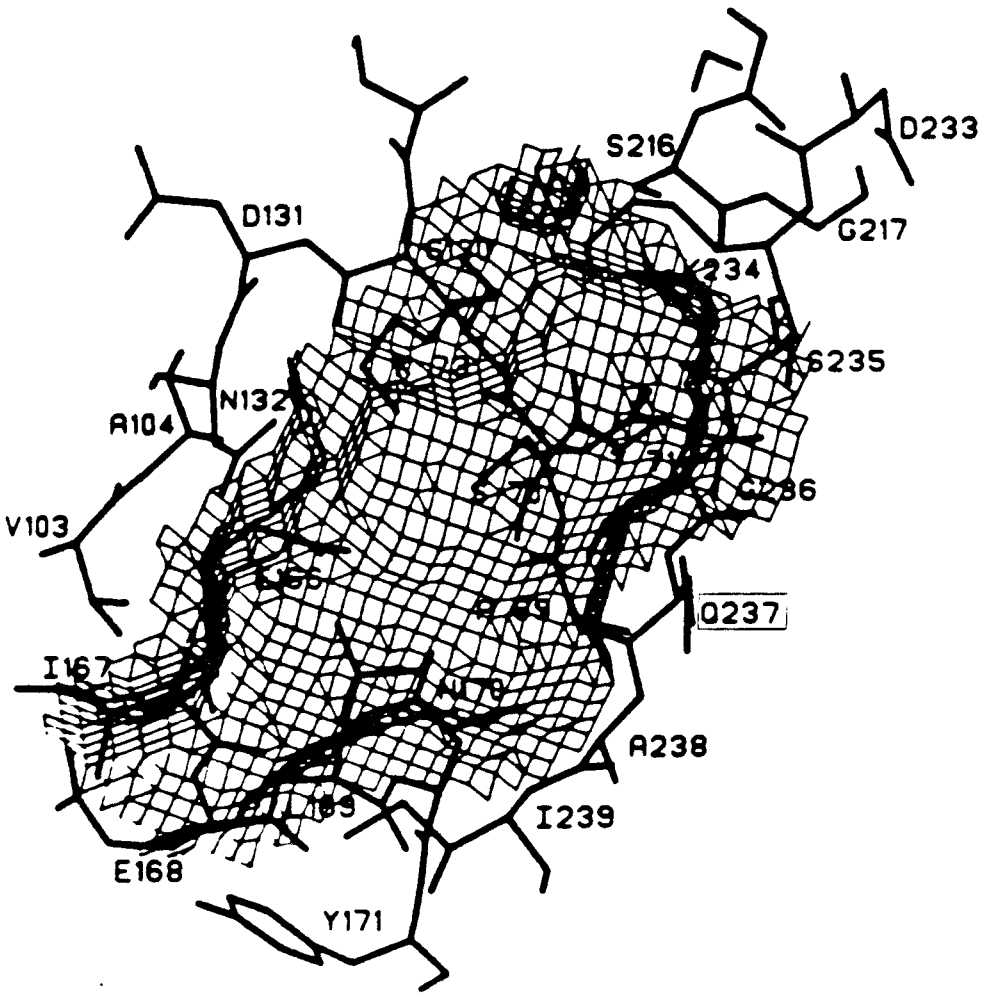


Figure IV-1. Close-up of crystal structure of *S. aureus* β-lactamase showing residue 237 at the active site substrate binding pocket.

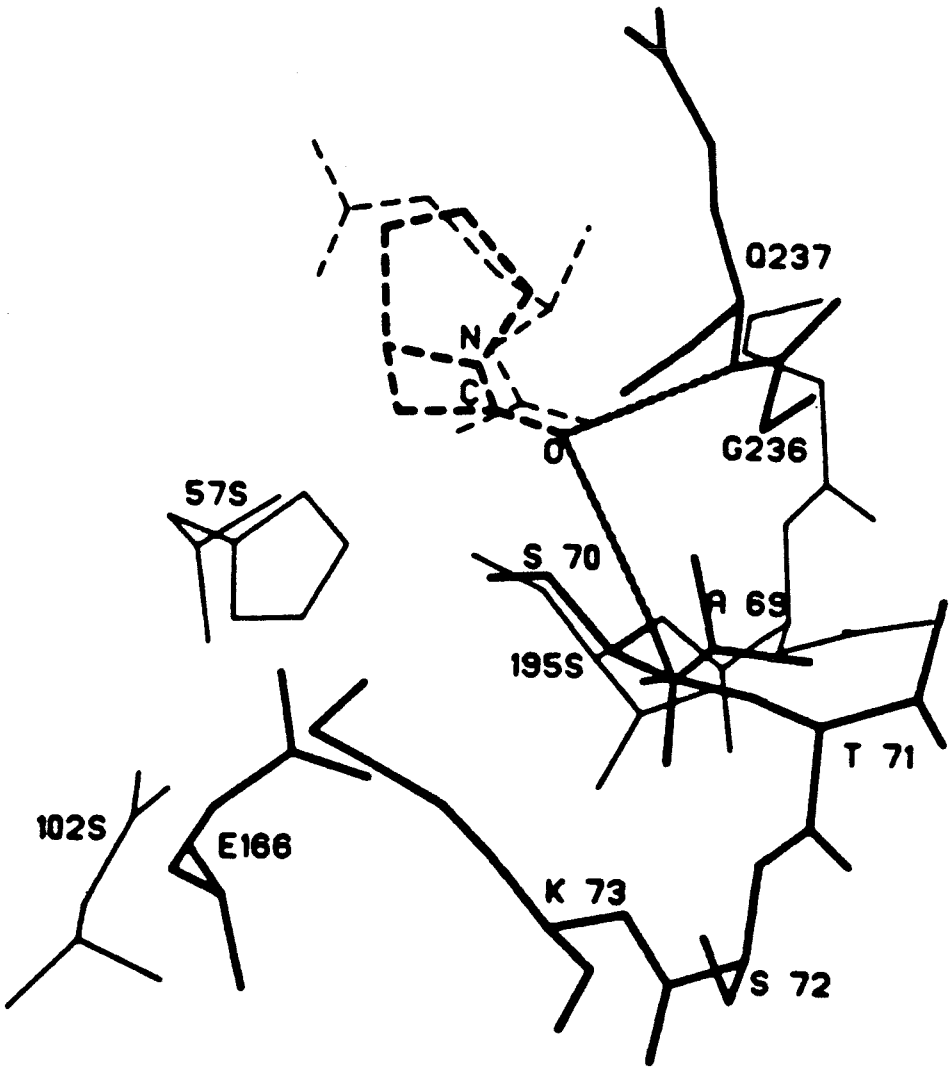


Figure IV-2. A superposition of the catalytically important residues of β-lactamase (dark lines) with bound β-lactam nucleus (thick, dashed lines) and the serine protease B from *Streptomyces griseus* (thin lines) with bound ovomucoid inhibitor (thin, dashed lines). The interaction between the β-lactam carbonyl oxygen and the amides of residues 70 and 237 are shown in thick dotted lines.

nearby amino acids must perturb the structure sufficiently to yield an altered phenotype.

Site saturation mutagenesis was successfully performed at Ala 237 in the experiments described here to search for mutants other than threonine, which have altered cephalosporin activiy. All mutants except proline retained at least some phenotypic activity, and two mutants were found that had increased cephalothin activity: the expected threonine as well as asparagine. Both of these mutants were expressed, isolated, and studied. Asparagine at 237 produced a protein that was even better at hydrolyzing cephems than the threonine mutant, which was in turn better than wild-type β-lactamase (Ala 237). Wild-type activity was higher than that of both mutants on penam antibiotics.

MATERIALS AND METHODS

Enzymes and Chemicals

All enzymes were purchased from Boehringer Mannheim Biochemicals. Antibiotics were from Sigma Chemical Company. Radioactive materials were supplied by Amersham. Isopropyl-β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal) were purchased from International Biotechnologies, Inc. (IBI). Molecular biology grade reagents agarose, phenol and chloroform were also from IBI.

Bacterial Strains

Escherichia coli were used in all experiments. Plasmid DNA was harbored in strain HB101[5]; the pJN expression vector was in strain D1210, which is lac i^Q. Bacteriophage were propagated in strain JM101[6]. Culture medium was L broth (10 g tryptone, 5 g yeast extract, 5 g NaCl in 1 l; add 15 g bacto-agar to make L plates), unless otherwise indicated.

Transformation

Cells were made competent for transformation of plasmid DNA using a process adapted from Hanahan[7]. A single colony of *E. coli* was grown in 100 ml of SOB media (20 g tryptone, 5 g yeast extract, 0.6 g NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ in 1 l) at 37°C to OD_{550}~0.3. At this point, cells were divided into two polypropylene tubes and chilled. Following centrifugation at 2500 rpm for 10 minutes at 4°C, each pellet was drained thoroughly and resuspended in 16 ml transformation buffer 1 (12 g RbCl, 9.9 g $MnCl_2 \cdot H_2O$, 1.5 g $CaCl_2 \cdot H_2O$, 30 ml of 1M KOAc, pH 7.5 in 1 l). This suspension was incubated on ice for 15 minutes, centrifuged again at 2500 rpm, and resuspended in 4 ml transformation buffer 2 (1.2 g RbCl, 11 g $CaCl_2 \cdot H_2O$, 20 ml of 0.5 M MOPS, pH 6.8 in 1 l). An aliquot of 10-20

μl of ligated DNA was added to 300 μl of these competent cells with gentle mixing. These were left on ice for 40 minutes, then heat-shocked at 42°C for 45 seconds. After the addition of 700 μl SOB, the mixtures were incubated for 1 h at 37 °C and then plated onto L agar containing an antibiotic.

DNA

Oligonucleotides were synthesized by the Caltech Microchemical Facility using phosphoramidite chemistry on the Applied Biosystems automated DNA synthesizer, Model 380A. Degenerate oligonucleotides were made equimolar in A, C, G, and T at positions 1 and 2 of the codon, and in C and G at position 3. They were then purified by preparative polyacrylamide gel electrophoresis (20%, 40 cm gel; 900 volts; 12 h); DNA was visualized with UV light reflected from a fluorescing silica plate. The appropriate bands were excised and DNA eluted in 0.2 M NaCl for 4-6 h at 55°C. Samples were then desalted using G-25 Sephadex spin columns.

Wild-type plasmid pBR322 and bacteriophage M13 mp18 replicative form (RF) DNA were purchased from Bethesda Research Laboratories. Mutant plasmids and RF phage were purified from *E. coli* by the alkaline lysis method[8]. Large scale preparations were further purified using ultracentrifugation in cesium chloride/ ethidium bromide gradients (single spin: 20 h, 45,000 rpm)[9]. Single-stranded phage DNA was prepared from phage supernatant by precipitation with 20% polyethylene glycol-6000 (in 2.5 M NaCl)[10] followed by phenol/chloroform extraction and ethanol precipitation.

Restriction digests typically used 20 μg plasmid DNA, 2-5 units of restriction enzyme, and 2 μl 10X digest buffer in 20 μl at 37°C for 1-2 h. DNA restriction fragments were run on 1.2% agarose gels, visualized with ethidium bromide, and isolated with either a UEA electroeluter (IBI) or an elutrap (Schleicher & Schuell),

according to manufacturers' instructions. Recovery was typically 40-60% following phenol/chloroform extraction and ethanol precipitation.

All DNA concentrations were estimated from absorbance at 260 nm.

Oligonucleotide-directed Mutagenesis

Before cassette mutagenesis was possible, a new Xho I restriction site had to be added at position 3436 in pBR322. First, the Sal I/Pst I 2958 base-pair fragment from pBR322 was cloned into M13 mp18 RF DNA (which had also been digested with Sal I and Pst I, then treated with alkaline phosphatase). The fragments were ligated using standard conditions and then transformed into competent JM101. Plasmid-containing cells were then plated along with saturated JM101, IPTG, and X-gal. Clear plaques on a lawn of JM101 represented successful clones containing the pBR322 Sal I to Pst I insert; blue plaques indicated wild-type phage.

Then, a single base-pair mutation was made using the Amersham oligonucleotide-directed *in vitro* mutagenesis kit[11], which employs the Eckstein method of strand-specific selection[12]. Single-standed phage was isolated and annealed to the septadecamer 5'-ATGATACCTCGAGACCC (mismatch underlined) by heating to 95°C in 10mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, and then slowly cooling to room temperature over a period of two hours. Chain extension from this oligonucleotide was accomplished with DNA polymerase I (Klenow fragment), T4 DNA ligase, and a mixture of dATP, dGTP, dTTP, and αS-dCTP. After completion, the reaction mixture was filtered to remove single-stranded DNA. Double-stranded DNA was saved and digested with the restriction endonuclease Nci I. Only the native strand is cleaved because of the presence of αS-dCTP (which interferes with cutting by Nci I) in the daughter strand. Following this, the nicked duplex DNA was digested using exonuclease III to remove the native DNA in the region of the mismatch. Repolymerization with all

dNTP's, DNA polymerase I, and T4 DNA ligase yielded double-stranded DNA in which both strands carried the correct mutated base. Because of this selection method, screening of thousands of plaques by hybridization following transformation was unnecessary. Instead, ten plaques were chosen for sequencing using the Sanger dideoxy method[13]. Nine out of ten tested positive for the desired mutation. These were then subcloned back into pBR322, again using Sal I and Pst I, to produce pBR322-Xho. The presence of an Xho I site was confirmed by restriction mapping.

Site saturation at residue 237; cassette mutagenesis

The complementary synthetic oligonucleotides containing a degenerate codon for residue 237 (Figure IV-3) were left unkinased and 50 pmole of each was annealed by heating to 95°C in 100 µl of 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, and then slowly cooled to room temperature over a period of two hours. A three-fragment ligation was then carried out, as shown in Figure IV-4. One pmole of the annealed oligonucleotides was combined with approximately 0.12 pmole each of Xho I/BamH I 3061 base pair fragment and Bgl I/BamH I 1256 base-pair fragment to give approximately an 8:1 insert to vector ratio and 25 µg/ml total DNA in a 20 µl reaction. This was incubated in a mixture of 10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, and 1 unit T4 DNA ligase for 12-14 h at 16°C; 10 µl were transformed directly into competent HB101 and plated onto 15 mg/l tetracycline plates. Five plates were prepared in order to generate sufficient numbers of colonies for sequencing. In addition, control reactions were run in the absence of insert DNA to test for contamination by wild-type pBR322.

Figure IV-3. Synthetic insert used for site saturation at residue 237.

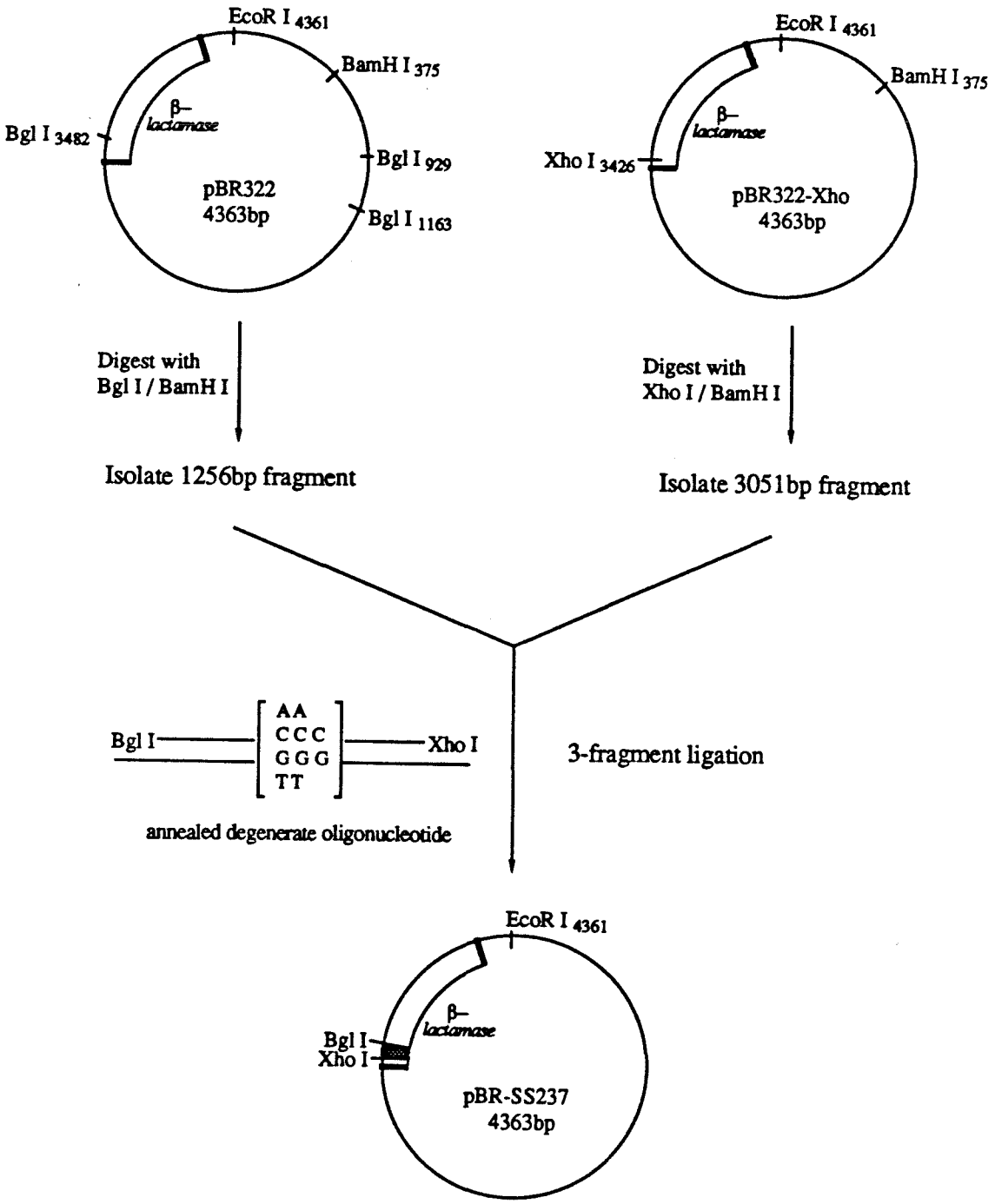


Figure IV-4. The three-fragment ligation scheme used for site saturation at 237.

Phenotypic screening

One hundred colonies from four tetracycline plates were picked, using sterile toothpicks, onto "master" tetracycline plates. These colonies were then picked onto plates containing ampicillin, benzylpenicillin, cephalothin or cephalosporin C to screen for level of resistance to penam and cephem antibiotics. Wild-type β-lactamase grew at the highest level tested on the penams (2000 mg/l) and up to 100 mg/l on both cephalothin and cephalosporin C.

Eight of the colonies grew on higher levels (500 mg/l) of cephalothin than did wild-type. To avoid the error in estimating activity that was due to the innoculum effect, activity was also tested by plating freshly grown cultures of these mutants onto plates of increasing concentrations of cephalothin. Saturated cells were diluted 1 in 10^5; 200 µl were spread onto plates containing concentrations of cephalothin ranging from 10 to 1,000 mg/l. Wild-type cells were also tested. Full growth was scored as a yield of approximately 200 or more colonies; minor growth was scored for around 20 colonies. Wild-type had full growth at 30 mg/l cephalothin, but gave one or two colonies up to 80 mg/l. The best mutant scored full growth at 225 mg/l cephalothin.

Double-stranded plasmid sequencing

Plasmid DNA was prepared from fifty colonies and sequenced using a modification of the Sanger dideoxy method for denatured, double-stranded DNA[14]. Denaturation was carried out on 5 µg DNA in 20 µl 0.2 M NaOH for 5 minutes, then neutralized with 2 µl of 2 M NH_4OH, pH 4.5 and ethanol precipitated. The sequencing primer used was 5'-AGGCGGATAAAGTTGCAG, which is located on the sense strand 74 bases before the codon for residue 237. The DNA pellet was suspended along with 5 pmoles of sequencing primer in 6.6 mM each of Tris-HCl, pH 7.5, NaCl, and $MgCl_2$ in a volume of 10 µl, and annealed at 37°C for 15 minutes.

Chain extension reactions were performed with a SEQUENASE™ kit from United States Biochemical[15]. This kit provides premixed deoxy- and dideoxy- nucleotides, as well as sequenase™, a modified version of T7 DNA polymerase. Labelling was accomplished with [α-^{35}S]-dATP. Reactions were stopped by adding 4 µl 0.03% (w/v) bromophenol blue in deionized formamide. Samples were loaded onto 8% polyacrylamide gels, run at 1200 volts for 5 h, and autoradiographed.

Western blots

Colonies harboring mutants were grown to late log phase (OD_{600}~1.0); a 1.5 ml sample of each was pelleted by centrifugation and resuspended in 100 µl protein sample buffer (10% v/v glycerol, 5% v/v 2-mercaptoethanol, 3% w/v sodium dodecyl sulfate, 62.5 mM Tris-HCl, pH 6.8, 1.0 mM EDTA, 0.05% w/v bromophenol blue) and heated to 95°C for 10 minutes to lyse the cells. Aliquots of 20 µl each were loaded onto a 15 cm, 12% polyacrylamide stacking gel and run at a constant current of 5 mA for 12-16 h. Protein was then transferred from the gel onto DEAE nitrocellulose using a Bio-Rad Transblot cell for 6 h at 12 volts. β-lactamase was visualized following binding of rabbit anti-β-lactamase[16] using the highly sensitive Vectastain® ABC immunoperoxidase system (Vector Laboratories)[17].

Expression of β-lactamase mutants in pJN

pJN is an expression vector for β-lactamase, which was developed in this laboratory. It contains the β-lactamase gene under the control of the tac promoter[18], as well as a kanamycin gene for a selection marker (Figure IV-5). Synthesis of β-lactamase is induced by the addition of IPTG. Before large quantities of mutant enzymes could be produced, their genes had to be subcloned into pJN.

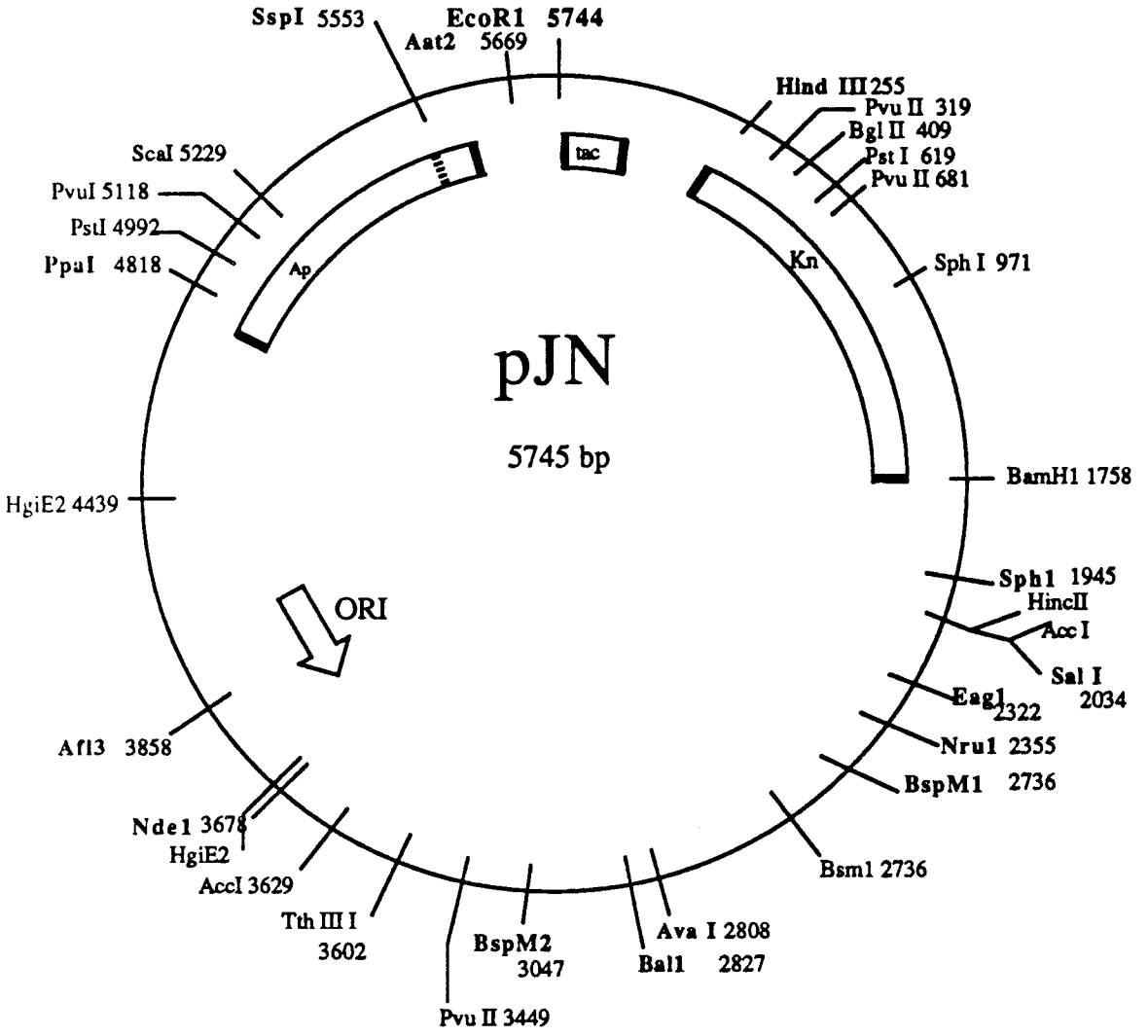


Figure IV-5. Restriction map of pJN--the β-lactamase expression vector.

Figure IV-6 outlines the scheme for transferring a mutant gene into pJN. First, both pBR322 carrying a mutation at alanine 237 and pJN (0.3 pmole each) were digested with Eco RI and Sal I. Then, to avoid isolating DNA fragments, equimolar amounts of digested pJN and mutant pBR322 were mixed and ligated. Following transformation into D1210, cells were plated onto 50 mg/l kanamycin. Resulting colonies contained either no β-lactamase gene, wild-type β-lactamase gene, or mutant β-lactamase gene; therefore, a selection scheme had to be designed to distinguish them.

To differentiate between wild-type and those mutants with high activity on cephalothin, colonies from kanamycin plates were picked onto cephalothin plates of either 25 mg/l or 500 mg/l. Those that grew on 25 but not 500 mg/l were wild-type; those that grew on 500 mg/l were the desired mutants. This assignment was verified by plasmid sequencing.

Protein Purification

One-half liter cultures of D1210 containing mutant plasmid were grown in L broth containing 50 mg/l kanamycin for 12-14 h at 37°C. IPTG was added to a final concentration of 0.1 mM and growth continued for 30 minutes at 37°C. Cells were centrifuged in 250 ml bottles in a GSA rotor for 10 minutes at 10,000 rpm. β-lactamase, which is located in the periplasm, was released by osmotic extrusion as follows. Pellets were resuspended in 20 ml sucrose solution (25 ml 1.0 M Tris-HCl, pH 7.0, 450 g sucrose, 0.5 g Na_2EDTA in 1 l) per gram of cells, and shaken at room temperature for 30 minutes. Samples were centrifuged at 10,000 rpm for 30 minutes. Supernatant was quickly removed and discarded, and cells were resuspended in an equal volume of cold H_2O (4°C) and stirred in the cold room for 30 minutes. Again, samples were centrifuged at 10,000 rpm for 30 minutes. The supernatant, which contains periplasmic proteins, was saved and the volume was

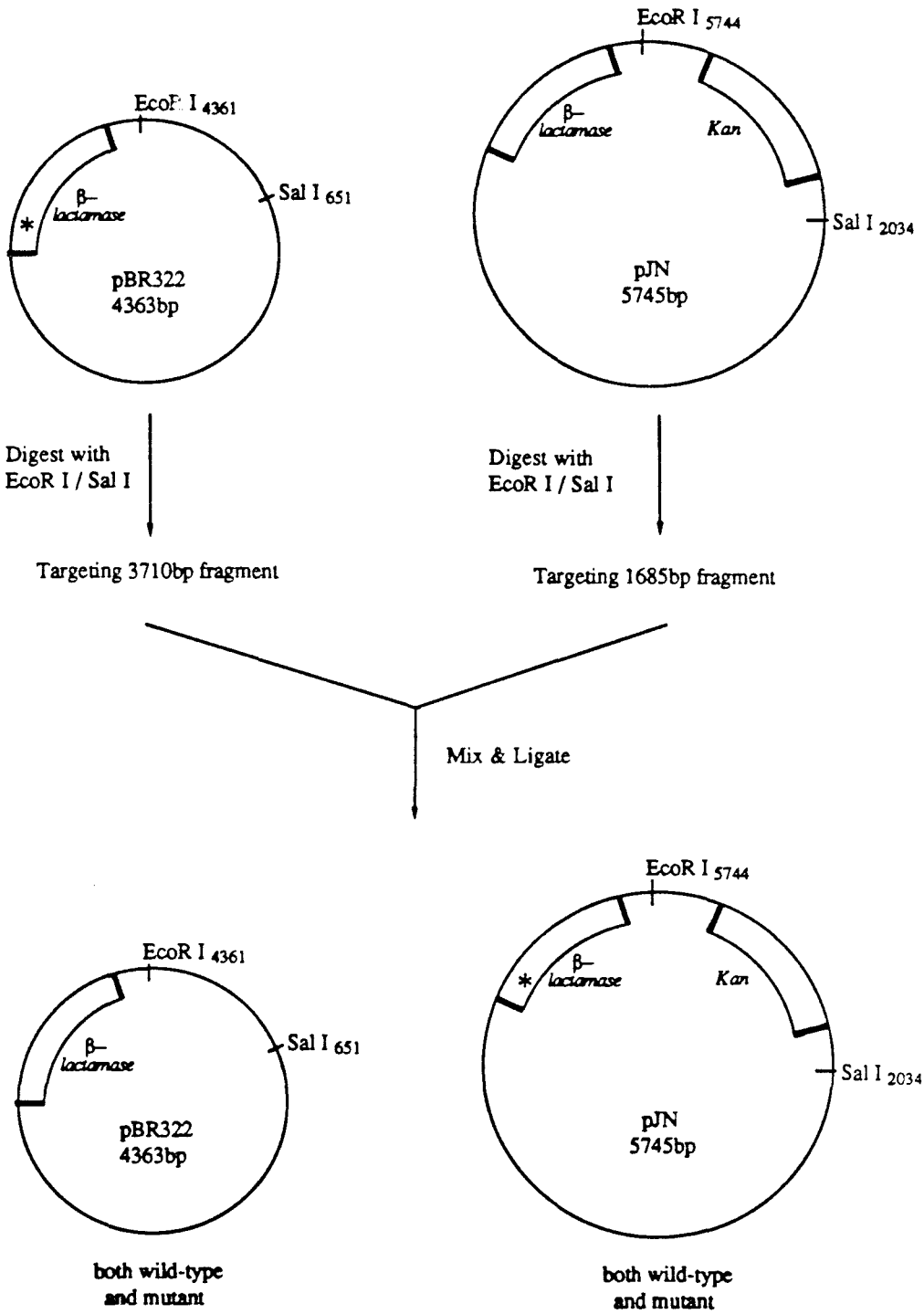


Figure IV-6. Scheme for the transfer of a mutant β-lactamase gene into pJN. An asterisk (*) denotes the site of mutation at 237. Among the mixture of ligation products that result, only those derived from pJN will be resistant to kanamycin. A phenotypic screen was used to distinguish mutant pJN from wild-type.

reduced to 1 ml using a Diaflow cell (Amicon) under nitrogen. This was then filtered using a Schleicher & Schuell 0.22 μm Uniflow filter to remove any cellular debris.

Further purification was carried out using FPLC (fast protein liquid chromatography) with an anion exchange column: MonoQ™ by Pharmacia. Protein (200 μl) was loaded onto the column in 25 mM triethanolamine (TEA), pH 7.65 (solvent A) and eluted with a salt gradient using 25 mM TEA, 1 M NaCl (solvent B). The gradient used was: t=0 minutes, 100% A; t=3, 100% A; t=28, 81% A, 19% B; t=33, 100% B. The flow rate was 0.8 ml/min. Elution was monitored by A_{280}; activity was assayed with the hydrolysis of benzylpenicillin (see Kinetics, below). Peak fractions were pooled and dialyzed versus six liters of 0.1 M potassium phosphate, pH 7.0. Protein concentrations were estimated from OD_{281} using an extinction coefficient of 29,400 $M^{-1}cm^{-1}$. Samples were run on 12% polyacrylamide gels (as in Western blots) and stained with Coomassie blue to gauge purity.

Kinetics

Michaelis-Menten kinetic parameters (k_{cat}, K_M, and k_{cat}/K_M) for both wild-type and mutant β-lactamases were determined from single, complete reaction curves using the integration method described in Chapter 3. Reactions were carried out at 30°C in 0.1 M potassium phosphate, pH 7.0. All reagents were maintained at 30°C prior to beginning the assay to avoid error from temperature flux. A Beckman DU7 spectrophotometer was used for the assays, with quartz cells of pathlength 1 cm (for penam substrates) or 1 mm (for cephem substrates). Wavelengths used were 240 nm for penams and 270 nm for cephems. The enzyme concentration was adjusted so that completion occurred in approximately 20 minutes. For highest accuracy, substrate concentration had to be five times

greater than K_M. The appropriate concentrations were determined by trial and error since K_M values for the mutant enzymes were unknown. Substrates tested included ampicillin and cephalosporin C, as well as benzylpenicillin and cephalothin.

Kinetic data were also collected on the wild-type and mutant enzymes using varying pH and reaction temperatures. To generate a pH versus k_{cat}/K_M curve, the reactions were performed on benzylpenicillin in 0.1 M potassium phosphate, but the pH was varied as follows: 6.0, 6.5, 7.0, 7.5, and 8.0. A plot of temperature versus k_{cat}/K_M was generated for each mutant on benzylpenicillin in 0.1 M potassium phosphate, pH 7.0 from 30° to 55°C in 5° intervals. Reagents were preincubated at the reaction temperature prior to initiating each assay.

RESULTS

A unique Xho I restriction site was added to pBR322 using oligonucleotide-directed mutagenesis. The Amersham *in vitro* mutagenesis kit was used to provide a selection for mutant over wild-type DNA. Because of this selection, 90% of the plaques sequenced contained the desired mutation. The resulting plasmid--pBR322-Xho--was confirmed to have an Xho I site using restriction analysis.

A three-fragment ligation scheme was used to insert a cassette of synthetic oligonucleotides containing degenerate bases at position 273 of β-lactamase. This ligation was successful, producing a total of 150 colonies on five tetracycline plates. Background ligations lacking the insert DNA gave zero colonies on three plates--indicating no contamination by wild-type pBR322. One hundred colonies were picked onto a master tetracycline plate from which activity screening was done.

Phenotypic screening was accomplished by picking colonies onto plates containing varying concentrations of 50 to 2000 mg/l of ampicillin, benzylpenicillin, and cephalothin. Seventeen mutants were phenotypically dead, having no growth on any of the plates. Most of the other colonies had activities approaching that of wild-type. However, eight grew on higher levels of cephalothin (500 mg/l) than did wild-type (100 mg/l). To gauge this increased activity more accurately, these eight mutants and wild-type were grown to saturation, diluted, and plated on multiple levels of cephalothin. In this way, the innoculum effect (wherein colony survival can depend on the number of cells on the toothpick) was avoided. With this method, wild-type β-lactamase survived on 30 mg/l cephalothin, although a few colonies were observed on plates of higher concentration. Of eight mutants that had increased activity on cephalothin as determined by picking, four grew on 140 mg/l plates and one had colonies on 225 mg/l cephalothin. This indicates a markedly increased resistance to cephem antibiotics. Of the other four, two had

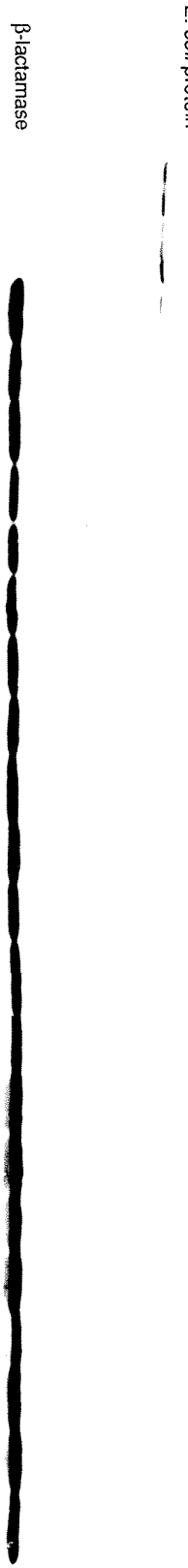
noticeably more colonies than wild-type up to 100 mg/l, while the other two were equal to wild-type.

Sequencing of these mutants was undertaken by M. Labgold using double-stranded dideoxy sequencing with the aid of the Sequenase™ kit from U.S. Biochemicals. This method allowed rapid determination of sequences at a rate of up to twenty per day. All twenty residues at position 237, and the stop codon TAG, were found within the first 42 plasmids sequenced. One sequence showed a lack of insert and yielded a dead mutant. Western blots (Figure IV-7) confirmed that all mutants were thermally stable and present in amounts equal to those of wild-type, except the amber mutant--which showed no band for β-lactamase.

Table IV-I summarizes the activity of each mutant as determined by picking colonies onto benzylpenicillin, ampicillin, cephalothin, and cephalosporin C. The number listed in the table gives the highest concentration of antibiotic at which each colony grew. The only inactive mutant is proline (the amber mutant also shows no activity). Of the rest, all grew on either 2000 mg/l (the maximum level tested) or 1000 mg/l of both benzylpenicillin and ampicillin. Growth on the cephem antibiotics was as follows: Arg, Glu, Gln, Ile, Phe, and Val mutants failed to survive on cephalothin; two of these (Arg and Gln) showed moderate activity on cephalosporin C, while the rest were inactive. Therefore, there are six mutants that show loss of activity on cephems while exhibiting close to full activity on penams. It is noteworthy that one of these, glutamine, is the wild-type residue for *S. aureus* β-lactamase, the only class A β-lactamase that has other than alanine at residue 237.

Four mutants that had wild-type activity on benzylpenicillin and ampicillin showed a significant increase in activity on cephalothin (500 mg/l): asparagine, aspartate, serine and threonine. These four were also the only mutants to show equal activity to wild-type on cephalosporin C (100 mg/l). Since picking colonies

Figure IV-7. Western blots of all residues at position 237 of β-lactamase. The enzyme was visualized following binding of rabbit anti-β-lactamase, using the highly sensitive Vectastain ABC immunoperoxidase system. A background lane of *E. coli* protein that cross-reacts with the antibody confirms that the same number of cells was loaded in each lane. Two gels were run separately and then combined for this figure.



E. coli LS1

amber 237

pBR322

ala

arg

asn

asp

cys

glu

gln

gly

his

ile

leu

lys

met

phe

pro

ser

thr

trp

tyr

val

Table IV-I. Activity of mutants on four substrates by picking colonies. (Number given is highest concentration of antibiotic--in mg/l--on which colonies grew.)

Amino acid	Ampicillin	Benzyl-penicillin	Cephalothin	Cephalosporin C
wt	2000	2000	100	100
Arg	2000	1000	0	25
Asn	2000	2000	500	100
Asp	2000	2000	500	100
Cys	2000	2000	100	50
Gln	2000	2000	0	25
Glu	2000	2000	0	0
Gly	2000	2000	75	50
His	1000	2000	25	50
Ile	2000	2000	0	0
Leu	2000	2000	25	50
Lys	1000	2000	25	50
Met	2000	2000	100	75
Phe	2000	2000	0	0
Pro	0	0	0	0
Ser	2000	2000	500	100
Thr	2000	2000	500	100
Trp	1000	1000	75	75
Tyr	2000	2000	25	25
Val	2000	2000	0	0
Amber	0	0	0	0

Table IV-II. Activity of mutants from plating onto cephalothin. (Number given is colonies counted after plating diluted cells onto varying amounts of cephalothin.)

	cephalothin (mg/l)												
	10	20	30	40	50	60	70	80	90	100	120	140	225
wt	200+	200+	200+	15	8	0	1	2	0	0	0	0	0
Thr	200+	200+	200+	200+	200+	200+	200+	200+	200+	200+	200+	200+	0
Asn	200+	200+	200+	200+	200+	200+	200+	200+	200+	200+	200+	200+	12
Asp	200+	200+	50	11	3	0	0	0	0	0	0	0	0
Ser	200+	200+	50	3	1	1	0	0	0	0	0	0	0

onto antibiotic plates is not the most accurate method of determining phenotypic activity, these mutants were also tested for activity by plating dilute cell growths onto varying concentrations of cephalothin. Table IV-II lists these results as the number of colonies growing at each concentration. By this method, wild-type β-lactamase confers resistance up to 30 mg/l cephalothin, as do the aspartate and serine mutants. However, colonies appear as high as 140 mg/l cephalothin for threonine and 225 mg/l for asparagine (intermediate concentrations were not tested).

The two mutants showing greatest resistance to cephem antibiotics--threonine and asparagine--were chosen for further study. First, their genes were transferred into the expression vector pJN, which has the β-lactamase gene under control of the inducible tac promoter. Successful subcloning was verified by sequencing. Mutant enzymes were then isolated with a simple, two-day procedure based on that of J. Neitzel[20]. Final purification was on FPLC, with the elution of protein using a NaCl gradient. β-lactamase eluted at about 20 minutes as seen by OD_{280} and confirmed by assaying on benzylpenicillin. Purity of the pooled fractions was confirmed by running a polyacrylamide gel and staining with Coomassie blue (Figure IV-8). The major protein seen is β-lactamase; some minor impurities are also present. The average protein yield was 1 mg of wild-type β-lactamase and 300 μg mutant per 500 ml growth. The yields were lower for mutants since fewer fractions were collected from the FPLC because of poor separation of peaks (compared to wild-type).

Kinetic parameters (k_{cat}, K_M, and k_{cat}/K_M) for wild-type and mutant β-lactamases were determined using the complete reaction curve logarithmic method[21], as described in Chapter 3. The substrates used for the kinetic assays were benzylpenicillin, ampicillin, cephalothin, and cephalosporin C. The results for the penams are listed in Table IV-III; results for cephems are in Table IV-IV.

Figure IV-8. Protein gel of purified mutants of β-lactamase at 237, stained with Coomassie blue. Lane **a** is a crude protein mixture from cells induced with IPTG; lane **b** is a crude protein mixture from uninduced cells; lanes **c** and **d** are the purified mutants A237N and A237T, respectively.

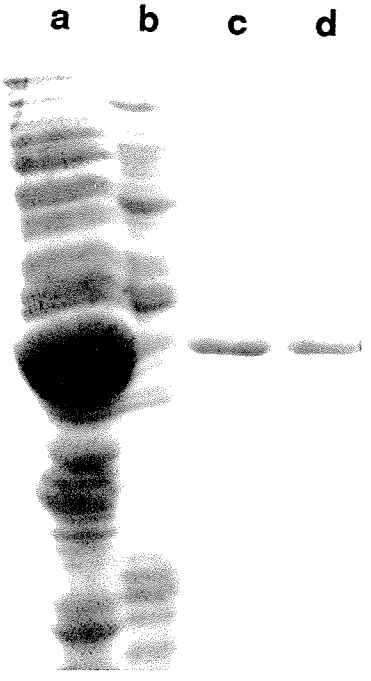

a
b
c
d
β-lactamase

Table IV-III. Kinetic parameters of mutants and wild-type enzymes on penams.

Benzylpenicillin

	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($M^{-1}sec^{-1}$)
Alanine (wt)	23 ± 2	2224 ± 120	9.5×10^7
Asparagine	144 ± 3	840 ± 30	5.8×10^6
Threonine	54 ± 1	315 ± 4	5.8×10^6

Ampicillin

	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($M^{-1}sec^{-1}$)
Alanine (wt)	65± 1	1670 ± 103	2.6×10^7
Asparagine	633 ± 5	2670 ± 86	4.3×10^6
Threonine	45 ± 1	225 ± 8	5.1×10^6

Table IV-IV. Kinetic parameters of mutants and wild-type enzymes on cephems.

	Cephalothin		
	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($M^{-1}sec^{-1}$)
Alanine (wt)	207 ± 15	128 ± 7	6.1×10^5
Asparagine	101± 2	230 ± 11	2.3×10^6
Threonine	66 ± 1	50 ± 3	7.5×10^5

	Cephalosporin C		
	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($M^{-1}sec^{-1}$)
Alanine (wt)	687 ± 6	57 ± 1	8.2×10^4
Asparagine	956 ± 28	299 ± 21	3.1×10^5
Threonine	199 ± 6	29 ± 3	1.5×10^5

K_M is an apparent dissociation constant and gives a measure of the amount of enzyme bound to a substrate[22]. Substrates with lower K_M's bind to the enzyme more readily. k_{cat} is a first-order rate constant, or the turnover number of an enzyme; thus, a higher k_{cat} implies that more molecules of substrate are converted to product per unit time. On benzylpenicillin, wild-type β-lactamase has both a lower K_M and a higher k_{cat} than either mutant. On ampicillin, A237T has a decreased K_M, but also a much reduced k_{cat}; A237N, on the other hand, increases in both K_M and k_{cat}. These combine again to make wild-type the better enzyme. On cephalothin, the opposite occurs: A237T is again reduced in both parameters, but these effects combine to make it a better catalyst than wild-type. For A237N, however, a 50% reduction in K_M , along with an 80% increase in k_{cat}, make it vastly superior to wild-type. For cephalosporin C, A237T is reduced in both parameters, as it was for cephalothin. A237N in this case shows a 50% increase in K_M, but compensates with a 420% increase in k_{cat} to make it better than both wild-type β-lactamase and the threonine mutant at hydrolyzing cephem antibiotics.

k_{cat}/K_M represents the apparent second-order rate constant; it determines the specificity of an enzyme for competing substrates[22]. For this reason, it is the most important parameter for comparing mutants. Table IV-V lists the comparative k_{cat}/K_M on the four substrates, relative to wild-type β-lactamase. From this table, it is apparent that both mutants show marked improvement over wild-type β-lactamase in catalyzing the hydrolysis of cephem antibiotics. Asparagine is even better than the previously discovered threonine mutant; it shows almost a four-fold increase in activity over wild-type. However, the wild-type β-lactamase is still more active on penam antibiotics.

Assays on benzylpenicillin at varying temperature and pH were also performed with wild-type β-lactamase and the mutant Asn and Thr at 237. Plots were made of temperature or pH versus relative activity by setting k_{cat}/K_M at pH 7.0

Table IV-V. Comparison of relative k_{cat}/K_M of mutants on four substrates.

	benzyl-penicillin	ampicillin	cephalothin	ceph. C
Alanine (wt)	1.00	1.00	1.00	1.00
Asparagine	0.06	0.17	3.77	3.78
Threonine	0.06	0.20	1.23	1.83

and 30°C equal to one. As shown in Figure IV-9, the wild-type enzyme reaches a sharp peak in activity at 40°C and then levels off at higher temperatures. It exhibits no instability to heat. In contrast, A237T peaks at 50°C and then activity declines quickly at 55°C from 160% to 40%. A237N peaks in activity at 35°C, drops gradually, and then rapidly falls to 20% activity at 55°C. Both mutants and wild-type β-lactamase show an equal response to pH (see Figure IV-10), peaking at pH 7.0 and decreasing in activity at both higher and lower values.

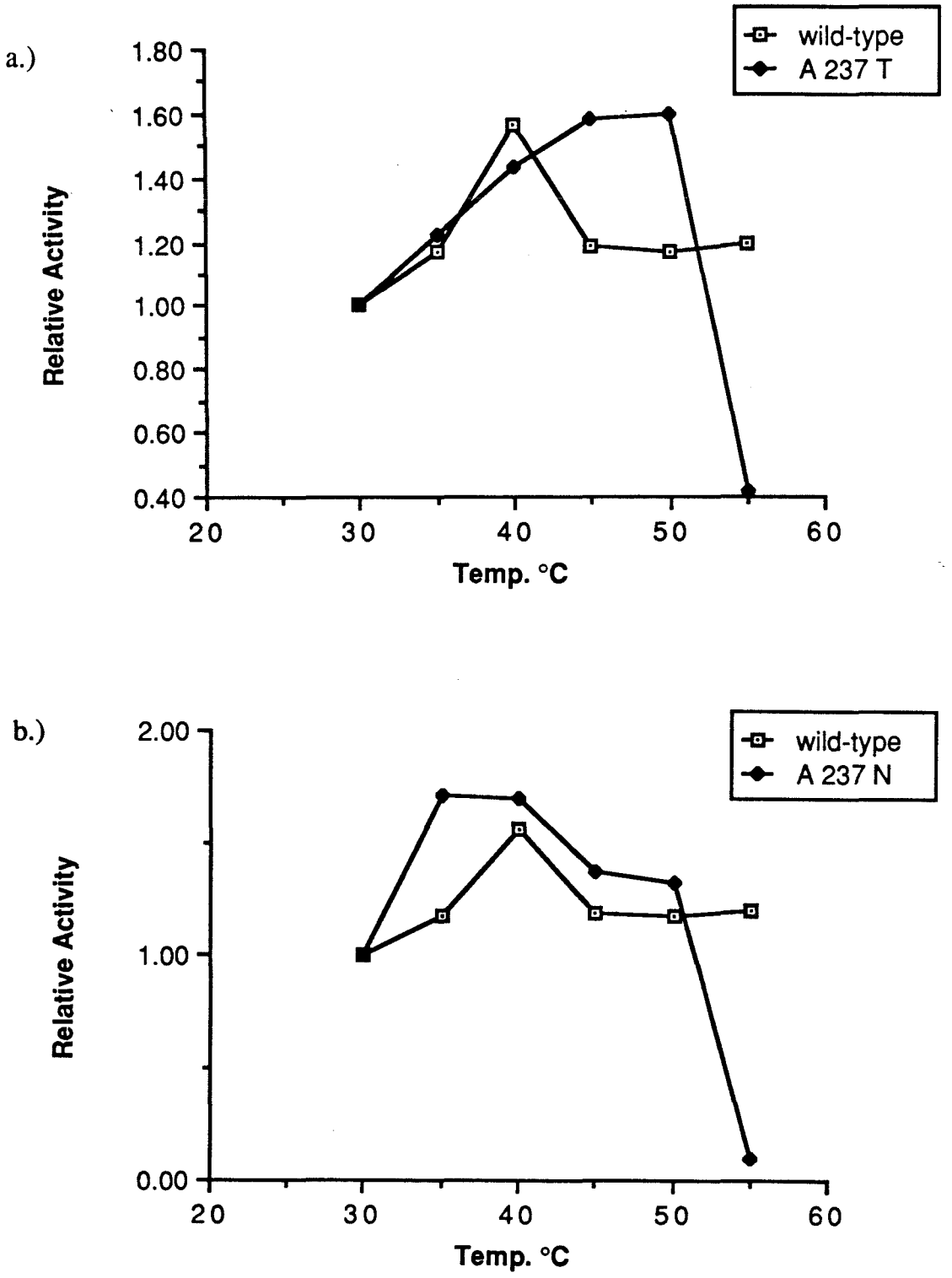


Figure IV-9. Plots of relative k_{cat}/K_M versus temperature for wild-type β-lactamase and a.) A237N or b.) A237T.

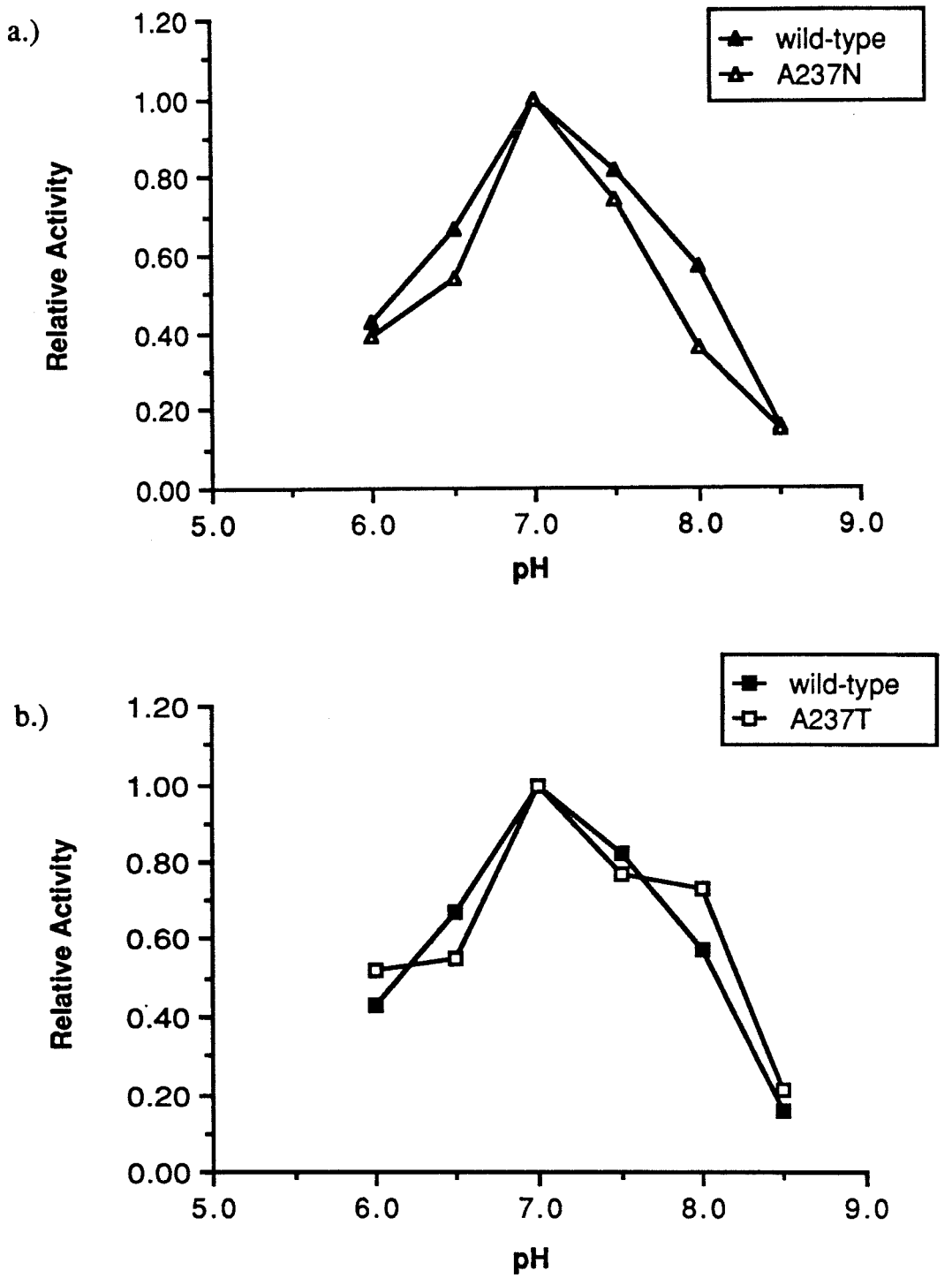


Figure IV-10. Plots of relative k_{cat}/K_M versus pH for wild-type β-lactamase and a.) A237N or b.) A237T

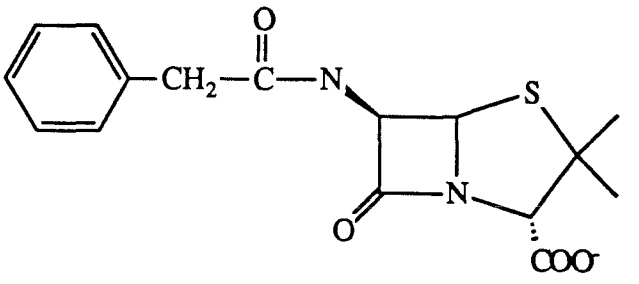
DISCUSSION

Site-saturation mutagenesis[23] was performed on alanine 237 of RTEM-1 β-lactamase based on the existence of a mutant (threonine) at that site that showed a substantially increased resistance to cephem antibiotics[1,2]. Although Ala 237 is not conserved among the class A β-lactamases[24], the crystal structure of the enzyme from *S. aureus* shows that the amide backbone of this residue forms part of the oxyanion hole, which stabilizes the acyl-enzyme intermediate. (In *S. aureus* β-lactamase, residue 237 is glutamine[25].) The goal of producing all mutations at this site was to discover whether any change besides threonine would produce an increased resistance to cephalosporins.

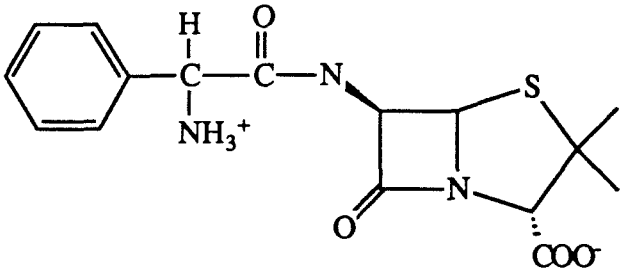
All twenty residues (and the amber mutant) were found within the first forty-two plasmids sequenced. This quick discovery of all mutants was due to a combination of good fortune and correct oligonucleotide synthesis. Activity of these mutants was determined by two methods: picking colonies onto agar plates containing different concentrations of four β-lactam antibiotics and plating dilute cultures of these colonies onto different concentrations of cephalothin. The latter method is a more accurate way of measuring phenotype. Figure IV-11 shows the structures of the four antibiotics tested.

Proline was the only completely inactive mutation at residue 237, while arginine, glutamate, glutamine, isoleucine, phenylalanine, and valine were inactive on cephalothin. Most interesting were those mutants that grew on high levels of cephalothin. Aspartate and serine were as active as wild-type on cephalothin (as seen by both methods). Threonine (as expected) and asparagine were even more active than wild-type β-lactamase. While wild-type, when plated, grew well only up to 30 mg/l cephalothin, both Thr and Asn grew at 140 mg/l. Western blots of all mutants of residue 237 showed that each was stable at 37°C and present in amounts comparable to wild-type β-lactamase--even proline.

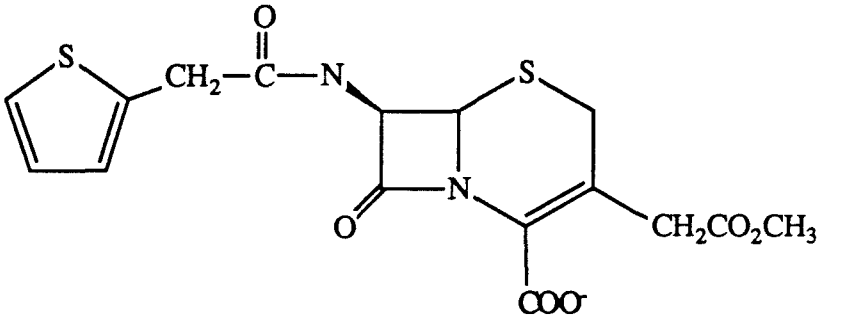
a.



b.



c.



d.

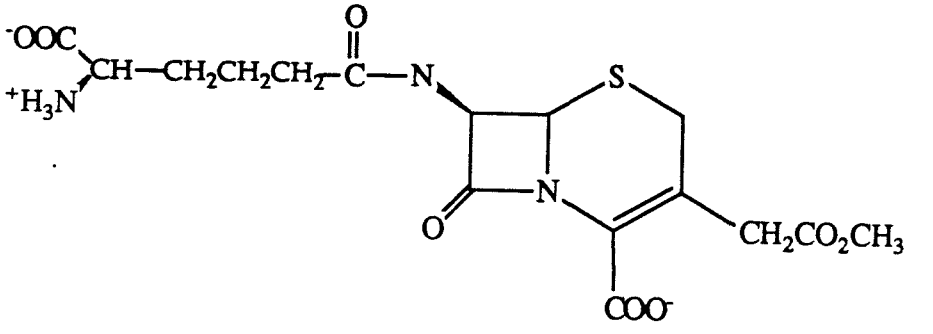


Figure IV-11. Substrates used for phenotypic screening and kinetics; penams are a.) benzylpenicillin and b.) ampicillin; cephems are c.) cephalothin and d.) cephalosporin C.

Both the asparagine and threonine mutants were expressed and purified. Unlike the mutants of Glu 166 (Chapter 3), these mutants were shown to be pure as seen on a protein gel stained with Coomassie blue. One or two additional bands are present, but they are very faint. Kinetic assays were run on the two mutants and wild-type β-lactamase with all four substrates. The kinetic results confirm those from the phenotypic screenings. Although both mutants are less active than wild-type on the penam antibiotics, they are better than wild-type on the cephems.

The most important parameter for comparing mutants is k_{cat}/K_M, which represents the apparent second-order rate constant; it determines the specificity of an enzyme for competing substrates[22]. As Table IV-V shows, asparagine 237 has 380% of the activity of wild-type on both cephalothin and cephalosporin C; threonine has 123% of wild-type activity on cephalothin and 183% on cephalosporin C. A plot of temperature versus relative k_{cat}/K_M shows that both mutants maintain a high activity at intermediate temperatures, but it falls off drastically for both at 55°C. Even though they are stable at 37°C, the structural change in each mutant is enough to cause instability at higher temperatures.

The structural basis for the marked change in substrate activity profile must be related to the role of residue 237 in catalysis. The side chain of this residue does not have a direct role in catalysis, since it is the amide backbone that forms part of the oxyanion hole. However, changes in the side chain must lead to a shift in the position of residue 237 because of altered contacts with its nearby residues. This is analogous to the structural changes observed in mutants of T4 lysozyme with high resolution crystallography[26,27]. Mutations of some residues can cause structural perturbations both in the vicinity of the change, and as far away as 20 Å. These changes in β-lactamase cause the enzyme to be structurally altered such that it reacts differently to the penam and cephem antibiotics, producing a decrease in resistance to the former and an increase in the latter.

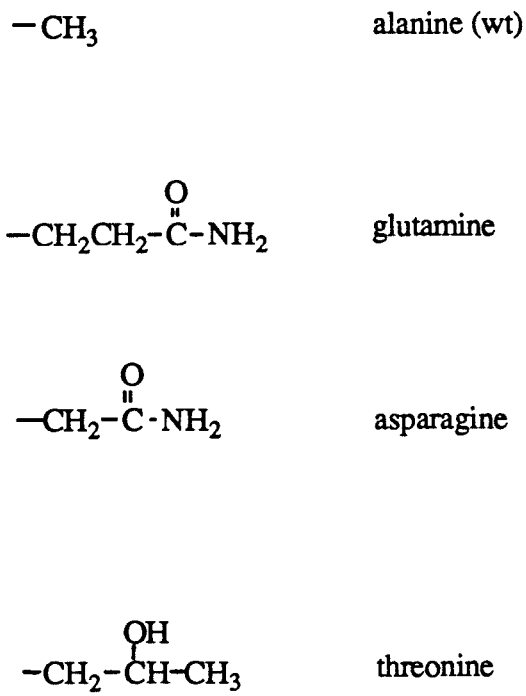


Figure IV-12. Side chains of active mutants at 237 in β-lactamase.

Proline is a special case because of its role as a structure breaker; it cannot be accommodated in the β-sheet in which alanine 237 is located. Figure IV-12 depicts the side chain structures of asparagine and threonine, as well as alanine and glutamine (residue 237 in *S. aureus*). Asn and Thr have quite similar side chains, so they are likely to interact with their surroundings in a similar manner. Both are obviously quite different from alanine. Glutamine, which is phenotypically inactive on cephalothin, differs from asparagine by a single methylene group. Therefore, the range of changes allowing increased activity on cephalothin is extremely narrow.

The results of this site saturation at alanine 237 point out the value of this method of mutagenesis. Threonine 237 was generated from alanine following random chemical mutagenesis and phenotypic selection for increased resistance to cephalosporins. The genetic cause of this mutation is a single base change (GCC→ACC). The most active mutant, asparagine, is a two-base change (AAC) that could not have been produced from alanine using random mutagenesis. It was also not an obvious choice to be produced through oligonucleotide-directed mutagenesis.

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