# Novel Biological Catalysts: Mutagenesis of RTEM ß-Lactamase to Alter Substrate and Catalytic Specificity

Thesis by
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*To My Wife*

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To my wife Ellen, thank you for you support, patience, encouragement, and most of all your love and friendship. Without you it would have no meaning.

## Abstract

I have used the techniques of site-directed mutagenesis to study the structural requirements for substrate specificity in RTEM-1 ß-lactamase and the evolutionary relationship between the ß-lactamases and the D,D-carboxypeptidases. The D-Ala-D-Ala carboxypeptidases/transpeptidases (penicillin-binding proteins, PBPs) share considerable structural homology with class A ß-lactamases. Both enzymes recognize the ß-lactam antibiotics as substrates; however, the ß-lactamases have no observable D,D-carboxypeptidase activity.

To investigate the possibility of incorporating D,D-carboxypeptidase activity into ß-lactamase a chimeric protein was prepared by replacing a 28 amino acid sequence of ß-lactamase with the corresponding sequence from PBP-5 of *E. coli*. The resulting chimera was capable of hydrolyzing the D-Ala-D-Ala dipeptide; however, it was determined that a secondary mutation had occurred which inserted a glutamic acid residue between residues 59 and 60.

In Chapter 2, I have performed site-directed mutagenesis on the gene encoding the RTEM-1 PBP-5 chimera to delete the additional residue. The resulting chimera was not thermally stable at 37°C, but is stabilized by interaction with ß-lactam compounds such as ampicillin. Despite the finding that the ß-lactamase activity was reduced by five orders of magnitude, the chimera displays approximately one percent of the D,D-carboxypeptidase activity exhibited by wild-type PBP-5.

In Chapter 3, to further investigate the proposed evolutionary relationship between the PBPs and ß-lactamase, I have designed a series of chimeras between the R61 carboxypeptidase/transpeptidase of *Streptomyces* and RTEM-1 ß-lactamase. The design of the chimeras involved building a

chimeric substrate binding cavity within the RTEM framework. One chimera (Asp131Asn, Asn132Phe) was thermally stable and exhibited altered cell morphology in *E. coli* harboring the chimeric gene.

In Chapter 4, I describe the detailed kinetic analysis of the Asn132Phe, single-mutant chimera, and the Asp131Asn/Asn132Phe, double-mutant chimera. The double mutant shows tremendously altered catalytic activity, degrading benzyl penicillin to phenylacetylglycine and producing the corresponding transpeptidase product, phenylacetylglycyl-glycine, when reacted in the presence of glycine. The single mutant, Asn132Phe, shows no such activity. Furthermore, I have shown that while the Asn132Phe mutation blocks deacylation in the chimeric proteins, when produced in conjunction with the Asp131Asn mutation, the resulting chimera is capable of carboxypeptidase/transpeptidase activity.

Finally, in Appendix I, the description of the site-saturation of RTEM-1 residue 237 is included. Two mutants, Ala237Thr and Ala237Asn, exhibited enhanced preference for cephem over penam antibiotics. The phenotypic screening of all 19 mutants is described.

## Table of Contents

## Nomenclature and Abbreviations

Mutants are specified as Asn132Phe or N132F, if asparagine at 132 has been changed to phenylalanine. 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. All remaining abbreviations are described where applicable.

# Chapter 1

## Introduction

## INTRODUCTION

Through the process of evolution, enzymes have developed into highly efficient catalysts enabling an organism to survive and prosper in the presence of some external pressure. Exactly how the amino acid polymer facilitates the catalysis of a reaction is highly dependent on the final three-dimensional structure of the resulting protein. Thus, to understand how enzymes function, one must recognize the importance of their spatial arrangements. Recent advances in recombinant DNA research have made it possible to create mutant enzymes in which specific alteration, replacement, or hybridization of genetic material allows the production of "engineered" enzymes. Through these studies we can begin to gain a better understanding of the basic requirements for protein stability and catalytic activity by examining the effects of the structural changes on protein function.

The primary structure of any protein is its amino acid sequence. Synthesis of the linear peptide *in vitro*, however, does not guarantee the formation of a functional protein. Unless the proper three-dimensional conformation is achieved, the protein is not capable of functioning in catalysis.

Historically, the study of structure-function relationships in proteins was limited to biophysical techniques such as x-ray diffraction, nuclear magnetic resonance, circular dichroism, and electron-spin resonance in coordination with kinetic studies or phenotypic screening. These biophysical techniques, in conjunction with kinetics and phenotypic screening, are effective in elucidating specific protein interactions, but were limited to the study of native proteins or randomly generated mutants. By studying the interaction of amino acids in these proteins, a data base

could be generated and basic principles of protein association could be proposed. However, one cannot be assured of the accuracy of these data unless the principles are fully tested.

The development of site-directed mutagenesis allowed researchers to investigate the role of specific amino acids in protein structure and function (1,2). By generating specific mutations in a protein's amino acid sequence, one can begin to understand how a linear polypeptide chain is able to fold into a stable and active conformation capable of enzymatic catalysis. Through investigation of the roles of individual amino acids in protein structure and function, one then can extrapolate these basic principles to the creation of novel biological catalysts.

Early experiments in protein mutagenesis were limited to chemical modification of proteins, such as titration of a cysteine residue with a thiol reagent (3), or random mutagenesis by techniques such as UV irradiation or treatment with chemical mutagens (4). These techniques have proven effective in elucidating the roles of residues in protein structure and function, but are extremely limited due to the nonselective nature of these techniques. Progress in DNA cloning and automated oligonucleotide synthesis (5) have greatly advanced the study of protein structure/function relationships. Site-directed mutagenesis can be performed on cloned genes to alter the native amino acid sequence and generate any desired variant.

Presently, variations on two general methods of site-directed mutagenesis, oligonucleotide-directed mutagenesis and cassette mutagenesis, comprise state-of-the-art protein engineering. It has been argued that the use of the term "protein engineering" should be curtailed since an engineer knows what he is designing at the onset of the project, whereas the protein researcher must first perform the desired mutations, examine

the effects thereof, then rationalize the findings. While most mutations made are either silent or deleterious (6), progress in understanding the structural basis of biological catalysis is advancing at a rapid pace.

Oligonucleotide-directed mutagenesis (Figure 1) takes advantage of the single-stranded DNA phages such as M13 (7). A synthetic oligonucleotide, with one or more base pair mismatches, can be annealed to the single-stranded DNA encoding the chosen protein. A DNA polymerase, such as the Klenow fragment of DNA polymerase I, is then utilized to generate heteroduplex DNA. Prior to efficient selection methods, transformation of bacterium with the heteroduplex DNA, resulted in only 1-5% mutant progeny; this is primarily due to the existence of *in vivo* methyl-directed mismatch repair. Efficient selection methods (8, 9) have since been developed, such as those of Ekstein (10, 11) and Kunkle (12, 13), which allow for the production of >90% mutant progeny.

The use of oligonucleotide-directed mutagenesis to change specific residues was especially useful in the generation of surface histidine residues in cytochrome c (14). Mutagenesis of residue 62, in yeast cytochrome c, to a histidine allowed for the ruthenation of the protein and facilitated measurement of electron transfer rates between the surface ruthenium and the cytochrome iron center.

The second technique, cassette mutagenesis (15 ,16), is also an extremely powerful tool (Figure 2). The ease of cassette mutagenesis is a result of exploitation of restriction endonucleases which allow for the exchange of segments of a gene with synthetic double-stranded DNA cassette encoding any desired sequence. The lack of convenient restriction sites can be readily overcome by employing oligonucleotide-directed mutagenesis to create new sites through silent mutations. Cassette

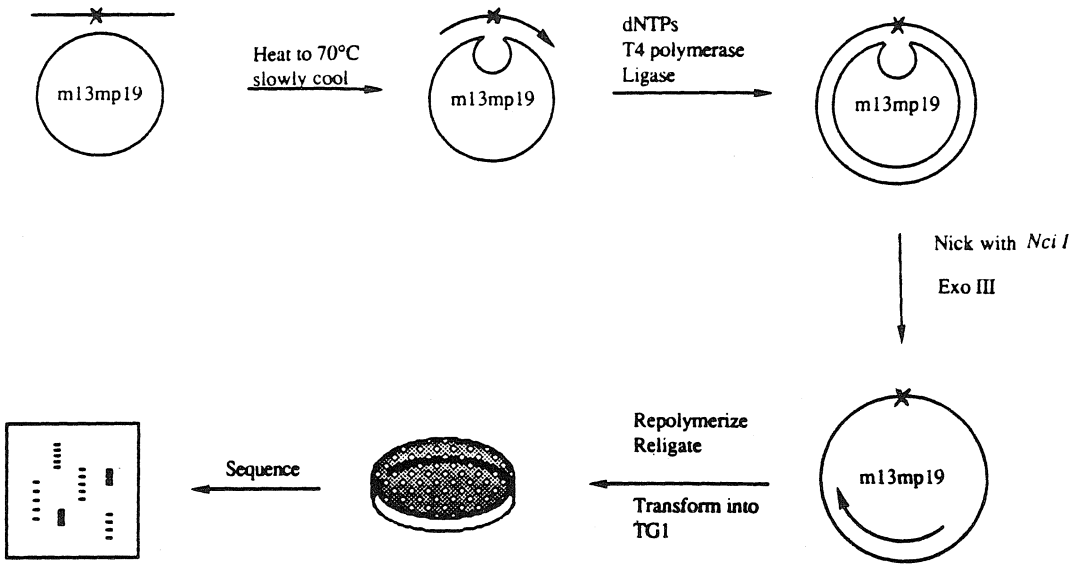



**Figure 1**- General overview of oligonucleotide-directed mutagenesis. The mutagenic primer containing the desired mutations is annealed to the single-stranded DNA. The synthetic strand is polymerized with αS-dCTP followed by nicking with Nci I. The presence of the αS-dCTP prevents endonuclease digestion of the synthetic strand. The native strand is exonucleased in the region of the mismatch, followed by repolymerization to yield the desired double-stranded DNA encoding the desired mutation.

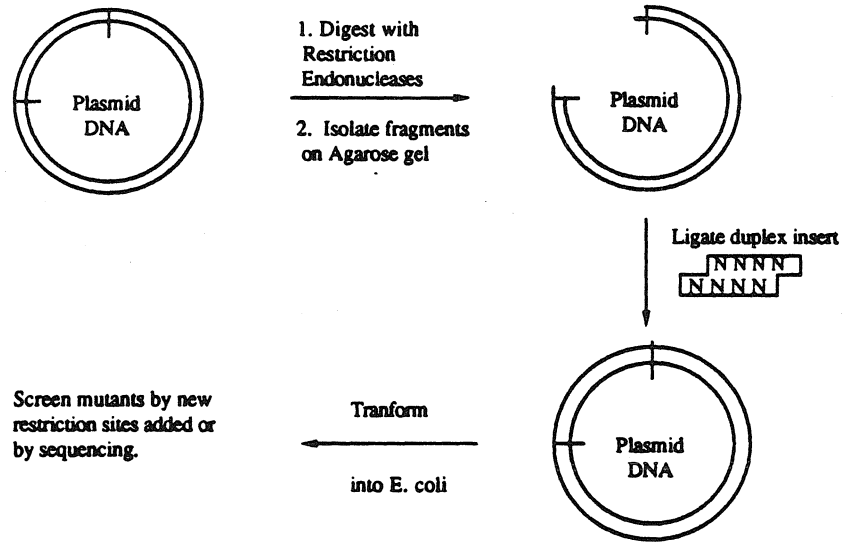



**Figure 2** - General overview of cassette mutagenesis. Plasmid DNA is digested with two restriction enzymes. A synthetic "cassette" encoding the desired mutations is made with the appropriate bas overhangs to anneal with the digested plasmid. The vector and insert are ligated to produce a mutated double-stranded plasmid.

mutagenesis has been used to facilitate the exchange of entire domains of a protein; an elegant example of this type of approach has been reported by Wharton and Ptashne (17), in which the "external" residues of an ampiphilic DNA recognition helix in the 434 repressor were exchanged with those residues present in the P22 repressor. The resulting chimera exhibited no reduction in protein stability, but acquired the P22 repressor activity. Kaiser *et al.* employed cassette mutagenesis to create an idealized hydrophobic region in the signal sequence of alkaline phosphatase. A cassette was designed to replace the native hydrophobic region with segments of either poly-leucine (18) or poly-isoleucine (19). The resulting "idealized" signal sequence was fully functional in protein secretion.

Besides the generation of specific mutations, cassette mutagenesis has been particularity useful in site-saturation and "specific" random mutagenesis. The effects of all 20 amino acids at a specific residue, or a random combination of residues in a selected region, can be investigated through the use of degenerate oligonucleotides. Work in this laboratory has proved the effectiveness of site-saturation experiments in understanding the roles of specific residues in ß-lactamase catalysis (20-23). In one such experiment we investigated of the role of residue 237 (Appendix I) in RTEM-1 ß-lactamase (24). The amide nitrogen of this residue has been proposed to be involved in the stabilization of the oxyanion intermediate (25), similar to that observed in the serine proteases (26), formed by nucleophilic attack of the active-site serine on the lactam carbonyl group. Preliminary results (27) from chemical mutagenesis of RTEM-2 had revealed a mutant, Ala 237 Thr, which showed enhanced preference for cephem over penam antibiotics. Site-saturation of this residue revealed a second mutant, Ala 237 Asn, which had an even greater

cephem hydrolysis rate. These results demonstrate two important principle of protein engineering: i) it is not a simple extrapolation from the threonine mutant to the asparagine mutant (which may have been overlooked had not all amino acid substitutions been produced) and ii) while the side-chain of residue 237 is not exposed to the substrate, subtle changes in the protein conformation, which are not necessarily limited to local conformation changes, can result in tremendously altered activity (6, 28).

Sauer *et al.* have demonstrated the usefulness of random mutagenesis of selected residues in their work on the λ repressor (29). Identification of functional mutants has clarified the roles of several residues in DNA recognition by the protein. Work by Schultz and coworkers may soon expand the replacements to unnatural amino acids (30).

The area of *de novo* protein synthesis has also been shown to be a useful technique for the study of protein structure and folding (31, 32, 33). By creating totally synthetic proteins from first principles, these experiments are by far the most stringent test of the proposed rules of protein interaction. To date, stable synthetic proteins have been created and it is not unrealistic to assume that the incorporation of function into these *de novo* proteins will soon be possible (34).

I have applied the techniques of site-directed mutagenesis to alter substrate specificity in RTEM-1 ß-lactamases and to investigate the proposed evolutionary relationship (35) between the ß-lactamases and the D-Ala-D-Ala carboxypeptidases (also known as penicillin-binding proteins or PBPs) which are responsible for the crosslinking of the bacterial cell wall peptidoglycan (36).

The D,D-carboxypeptidase/transpeptidase enzymes (36) catalyze the crosslinking of the peptidoglycan by recognition of the terminal D-Ala-D-Ala dipeptide followed by cleavage of the terminal D-alanine residue (Figure 3). This reaction is accomplished by the nucleophilic attack of an active-site serine residue (37) and results in a covalent acyl-enzyme intermediate with the penultimate D-alanine residue. This intermediate can be hydrolyzed by water, resulting in a free alanine terminus, or attacked by the ε-amino group of lysine from an adjacent strand of peptidoglycan, forming a stabilized crosslinked structure.

The PBPs have been categorized by size into two groups (36). The high molecular weight PBPs (60 to 140 kD) are generally minor components of the cell and, while not involved in D-Ala-D-Ala-carboxypeptidase activity, are necessary for cell viability. The low molecular weight PBPs (40 to 50 kD) are involved in D-Ala-D-Ala-carboxypeptidase/transpeptidase reactions and have been extensively characterized. The X-ray crystal structure of *Streptomyces* R61 has been determined to 2.0 Å resolution (38). The high molecular weight PBPs isolated from *E. coli* include 1a, 1b, 2, and 3; the low molecular weight PBPs include 4, 5, and 6.

In addition to the D-Ala-D-Ala dipeptide, the D,D-carboxypeptidase/transpeptidases are able to attack the ß-lactam antibiotics (Figure 4). However, nucleophilic attack by the active site serine on the ß-lactam ring form a stable acyl-enzyme complex, eventually resulting in bacterial cell lysis (Figure 5). Tipper and Strominger initially proposed that the ß-lactam antibiotics function as structural analogues (Figure 6) to the D-Ala-D-Ala dipeptide (35) and thereby interrupt the terminal crosslinking step in peptidoglycan synthesis. They were successful in proving this hypothesis by pulse-chase experiments (39). A second group of enzymes, the

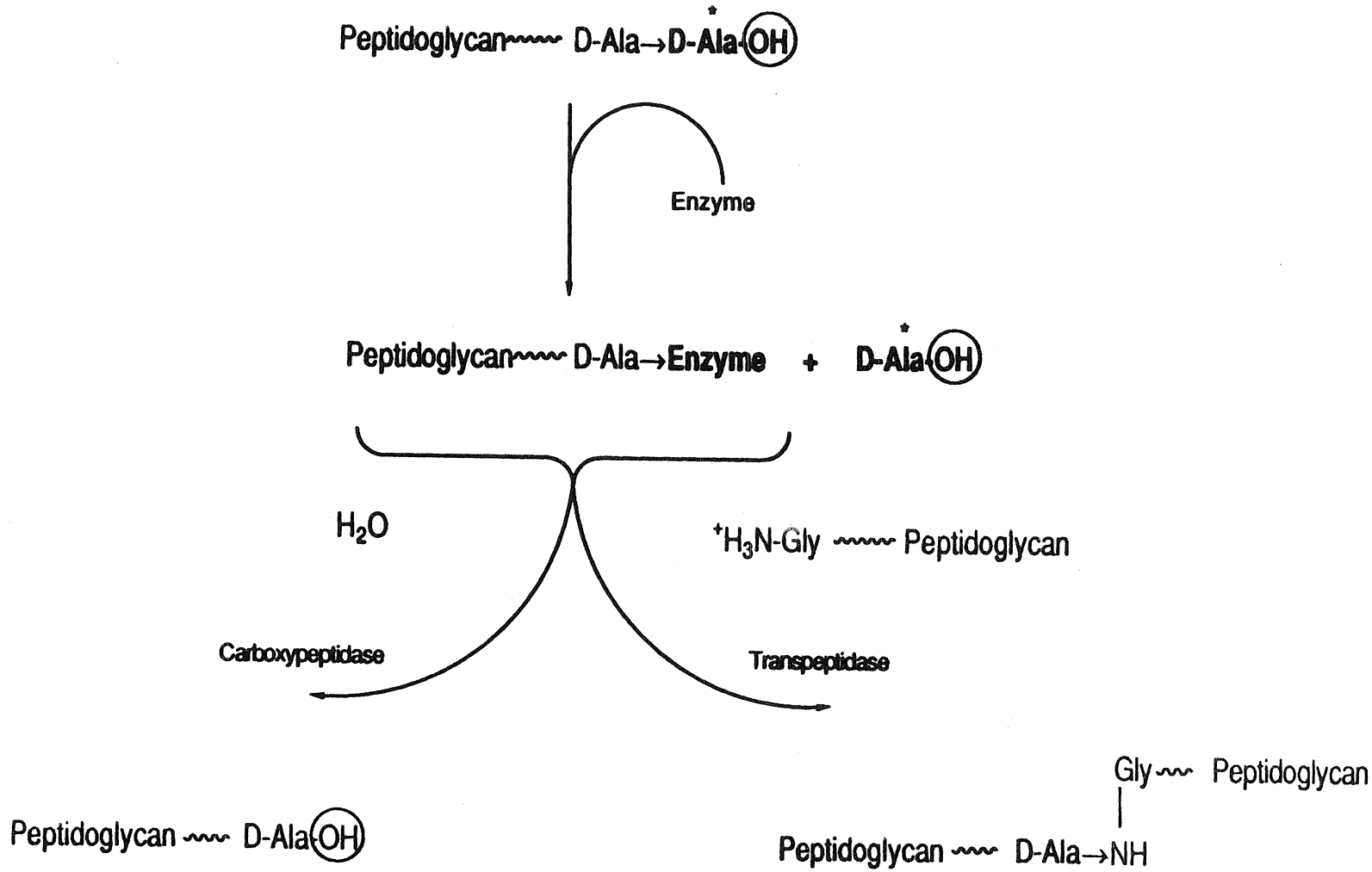
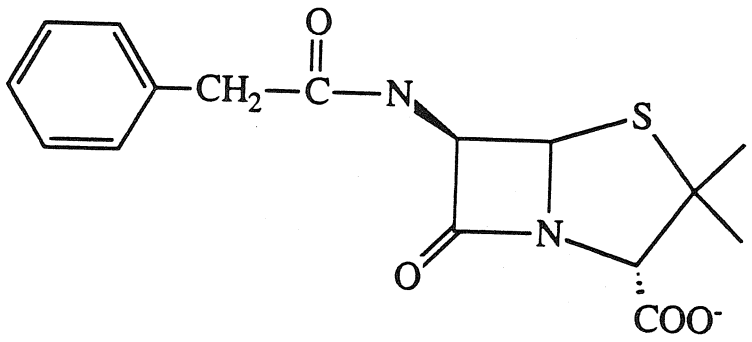



**Figure 3** - The process of bacterial cell wall biosynthesis. The strands of peptidoglycan are targeted by the D,D-carboxypeptidases, resulting in the hydrolysis of the terminal D-alanine residue. The acyl-enzyme intermediate can then be deacylated by water or an amino acceptor, the latter resulting in a stabilized crosslinked structure.

a.

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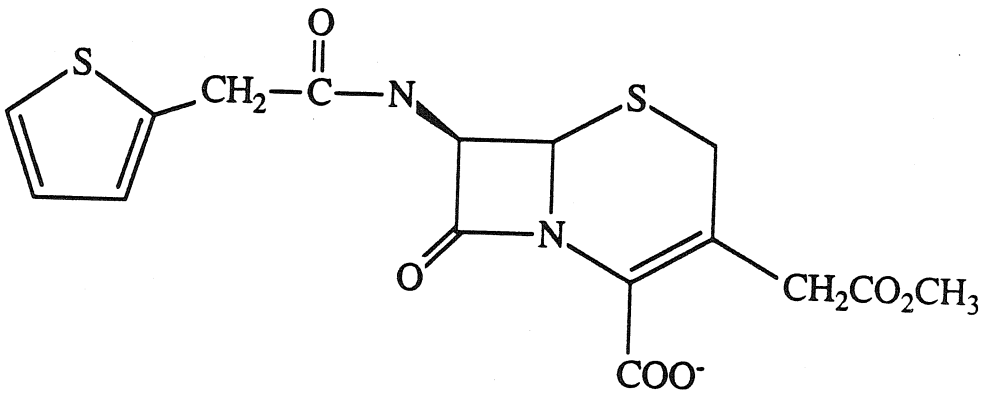


**Figure 4** - Representative ß-lactam antibiotics. a) benzyl penicillin; a penam antibiotic, as characterized by the 5,4 fused ring system, and b) cephalothin; a cephem antibiotic, as characterized by the unsaturated 6,4 fused ring system.

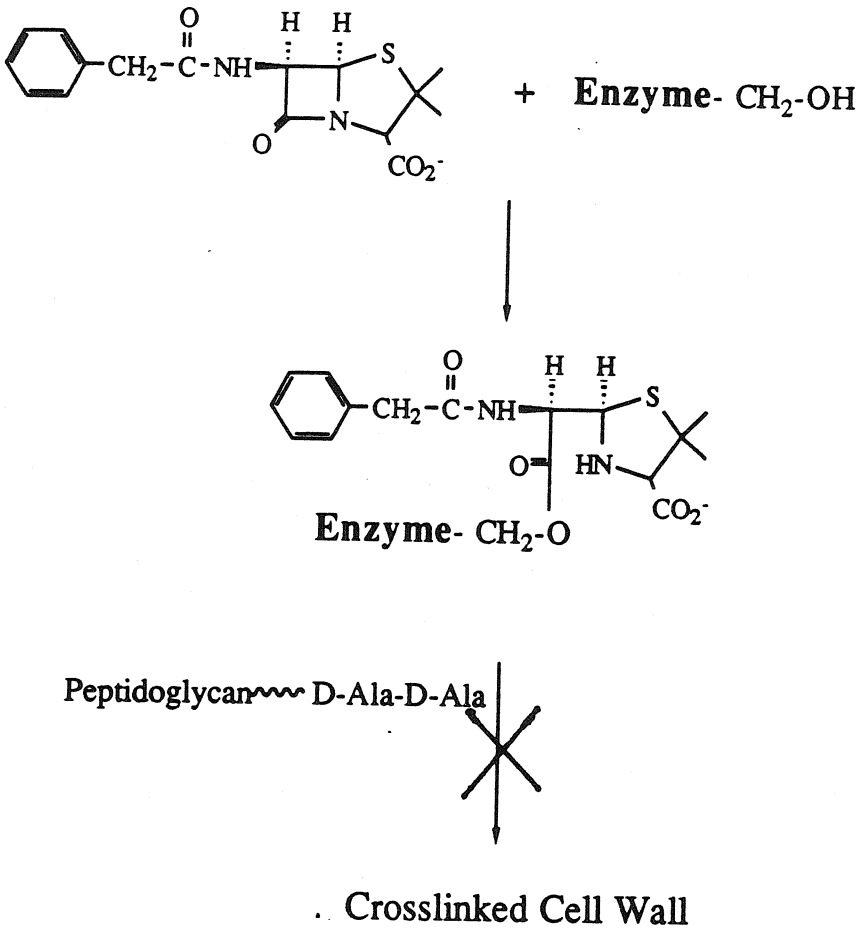


**Figure 5 -** The D-Ala-D-Ala carboxypeptidases interact with the ß-lactam antibiotics via nucleophilic attack on the lactam carbonyl by the active-site serine residue. The resulting acyl-enzyme intermediate is a stable covalent species and prevents the enzyme from crosslinking the bacterial cell wall peptidoglycan.

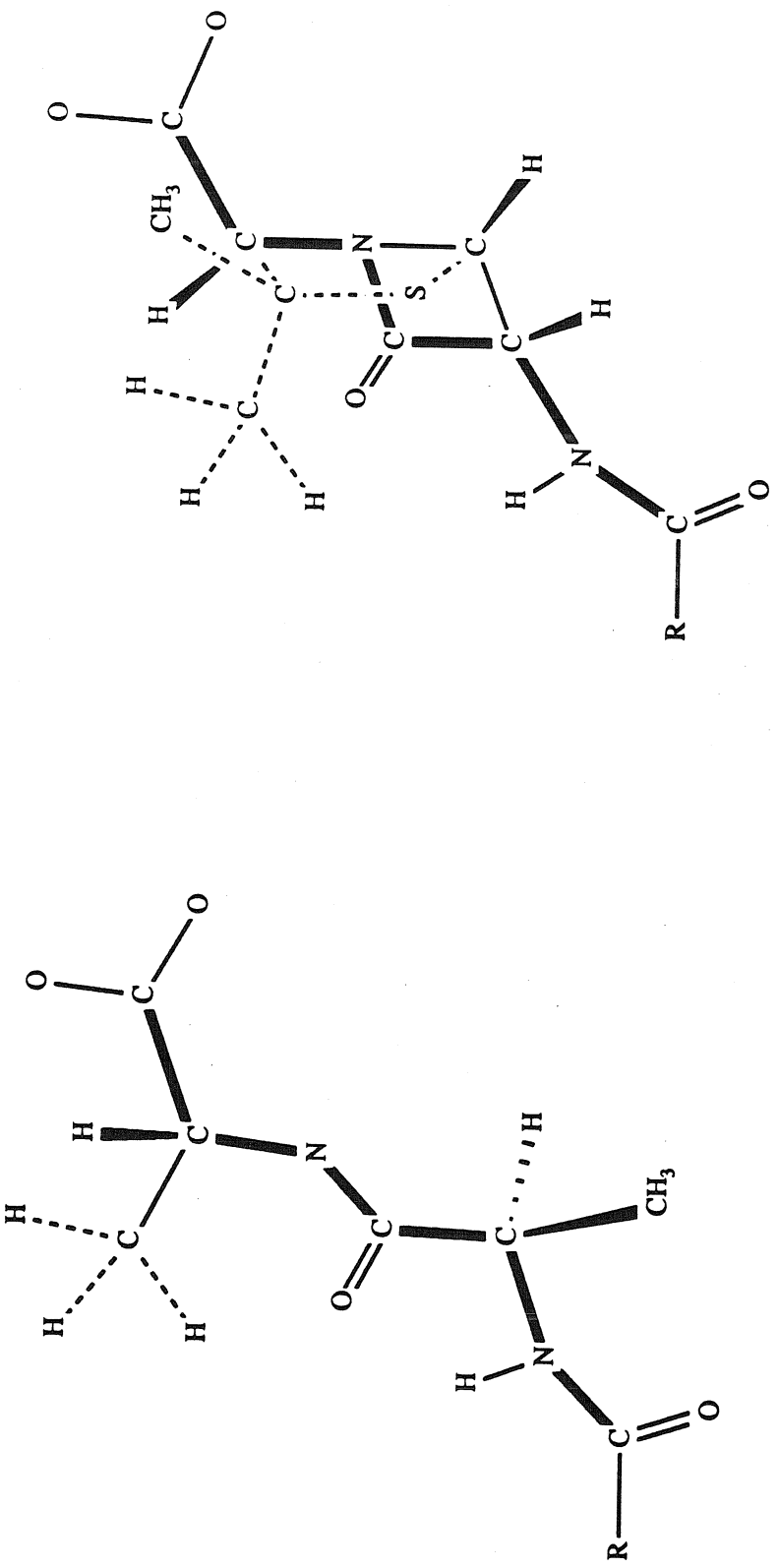

**Figure 6** - Chemical structures emphasising the close structural anology of the ß-lactam antibiotics (right) with the D-Ala-D-Ala dipeptide (left). Hydrolysis of the lactam bond leads to a conformation which is nearly superimposable with the peptide (35).

ß-lactamases, also utilize the ß-lactam antibiotics as substrates by interactionof an active-site serine with the lactam bond (40, 41, 42), but are capable of rapid deacylation of the acyl-enzyme intermediate (Figure 7). Tipper and Strominger used their findings to propose a possible evolutionary relationship between the two enzymes (35).

Over 80 ß-lactamases have been identified and divided into three categories based on sequence homology, size, and activity (43). The class A ß-lactamases include the RTEM ß-lactamases (RTEM-1, 2, & 3) of *Escherichia coli*, *Staphylococcus aureus* PC1, *Bacillis licheniformis* 749/C, and *Bacillis cereus* 569/HI. All of these enzymes exhibit considerable sequence homology (32-33%) and have a molecular weight around 30 kilodaltons. There are only two class B ß-lactamases, those of *B. cereus* and *Proteus maltophilia*. The class B ß-lactamases differ from the class A ß-lactamases in that they have molecular weights of approximately 23 kD and form a metalloenzyme complex with zinc. The class C ß-lactamases are the largest at 40 kD and include *Enterobacterium cloacae* P99 and *Escherichia coli* K12. In addition to size, the three classes also differ in target specificity. The class A ß-lactamases preferentially hydrolyze the penams whereas the other two classes target the cephem antibiotics. The X-ray crystal structures of the class A ß-lactamases from *S. aureus* PC1 (25) and *B. licheniformis* (44) have been published at 2.5 Å resolution and 2.0 Å resolution respectively. We base our model for RTEM on these closely homologous structures (Figure 8).

RTEM-1 ß-lactamase is a 28kD soluable protein isolated from E. coli. It differs from RTEM-2 ß-lactamase by one residue (residue 39) and from RTEM-3 by three residues (residues 39, 104, & 238) (45). Like the other class A ß-lactamases, RTEM preferentially hydrolyzes penam over cephem

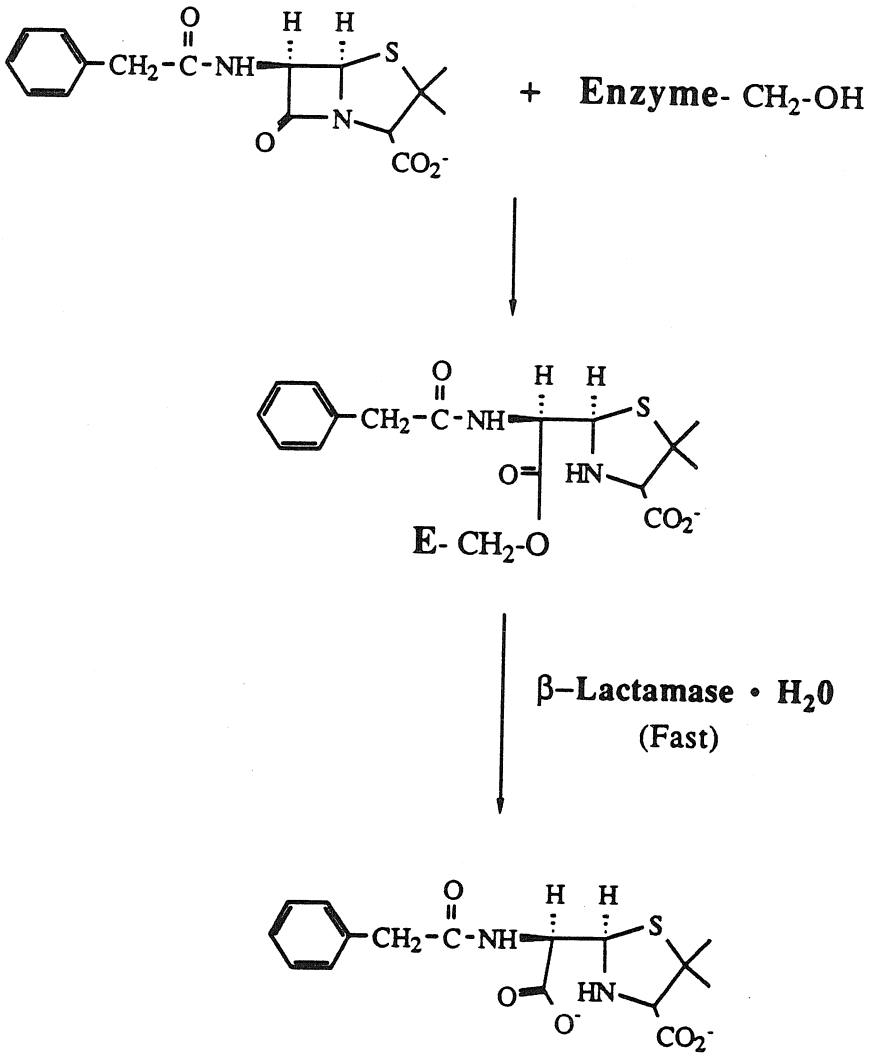


**Figure 7** - Interaction of the ß-lactamases with the ß-lactam antibiotics results in an acyl-enzyme intermediate which is rapidly hydrolyzed, resulting in an inactive antibiotic.

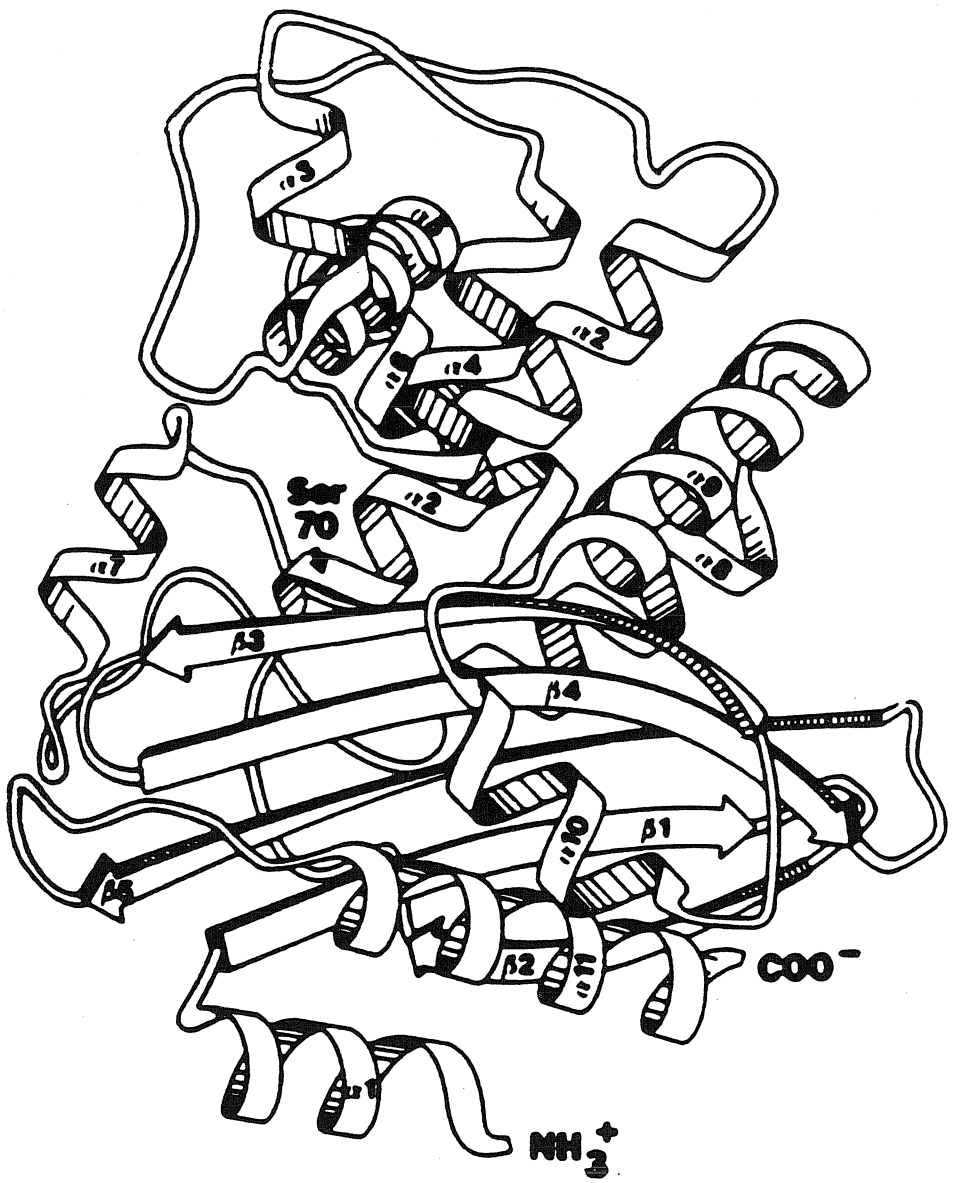


**Figure 8** - Three-dimensional structure of the class A ß-lactamase from *Staphylococcus aureus.*

antibiotics (Figure 4). This rate of penam hydrolysis has reached near evolutionary perfection with a second order rate constant ($k_{cat}/K_M \approx 10^8$) approaching diffusion control (46).

The ß-lactam substrate is subjected to nucleophilic attack (Figure 9) on the lactam carbonyl group by the active-site serine (Ser 70 in RTEM). Nucleophilic attack proceeds through a tetrahedral intermediate which is proposed to be stabilized by an oxyanion hole (25), similar to that observed in the serine proteases (26). The resulting acyl-enzyme intermediate is rapidly hydrolyzed by the interaction of Glu 166 (22), leading to an inactive antibiotic.

Much research has been performed in our laboratory to investigate the roles of several important residues in RTEM-1 ß-lactamase including site-saturation of residues Thr 71 (20), Lys 73 (21), Glu 166 (22), Lys 234 (23), and Ala 237 (24). Other research has been directed towards the generation of mutants at Ser 70 (47, 48).

While the theory that the ß-lactamases evolved from the PBPs was initially based on the substrate analogy, the PBPs and the class A enzymes have also been shown to contain homologous sequences near the active site serine (49) and have an overall secondary structural similarity (50). In both, the serine active site is incorporated into a semi-conserved triad: Ser-Thr-Xaa-Lys in ß-lactamase and Ser-Xaa-Thr-Lys in the PBPs. Yet while both groups are acylated by ß-lactam antibiotics, only the D,D-carboxypeptidases are able to catalyze the hydrolysis of the terminal D-Ala-D-Ala moiety. One notable structural difference (Figure 10) is the presence of helix G, as seen in the low resolution structure of the R61 carboxypeptidase/transpeptidase (50), which is absent in the ß-lactamases.

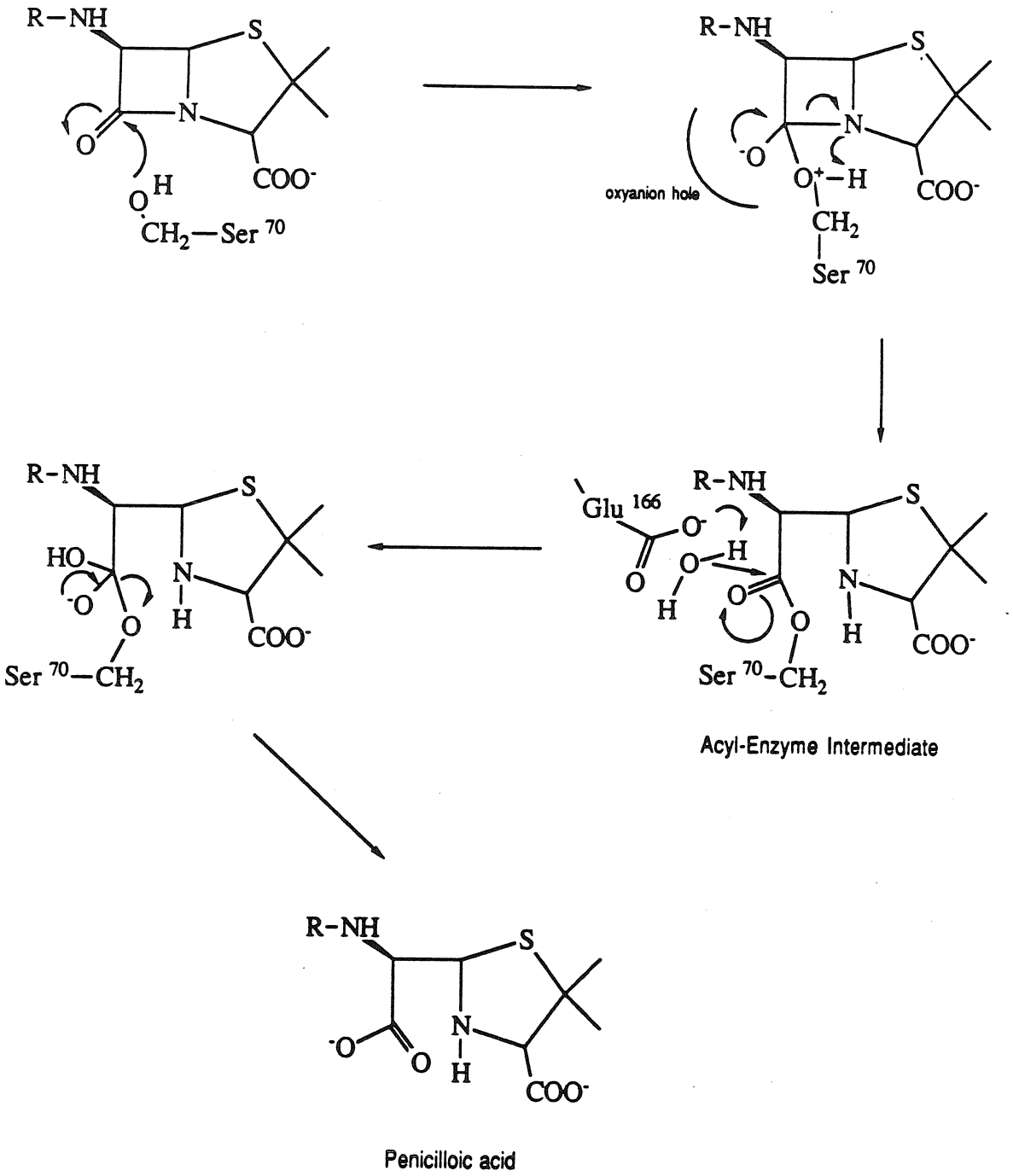


**Figure 9** - Mechanism of the hydrolysis of the ß-lactam antibiotics by the class A ß-lactamases.

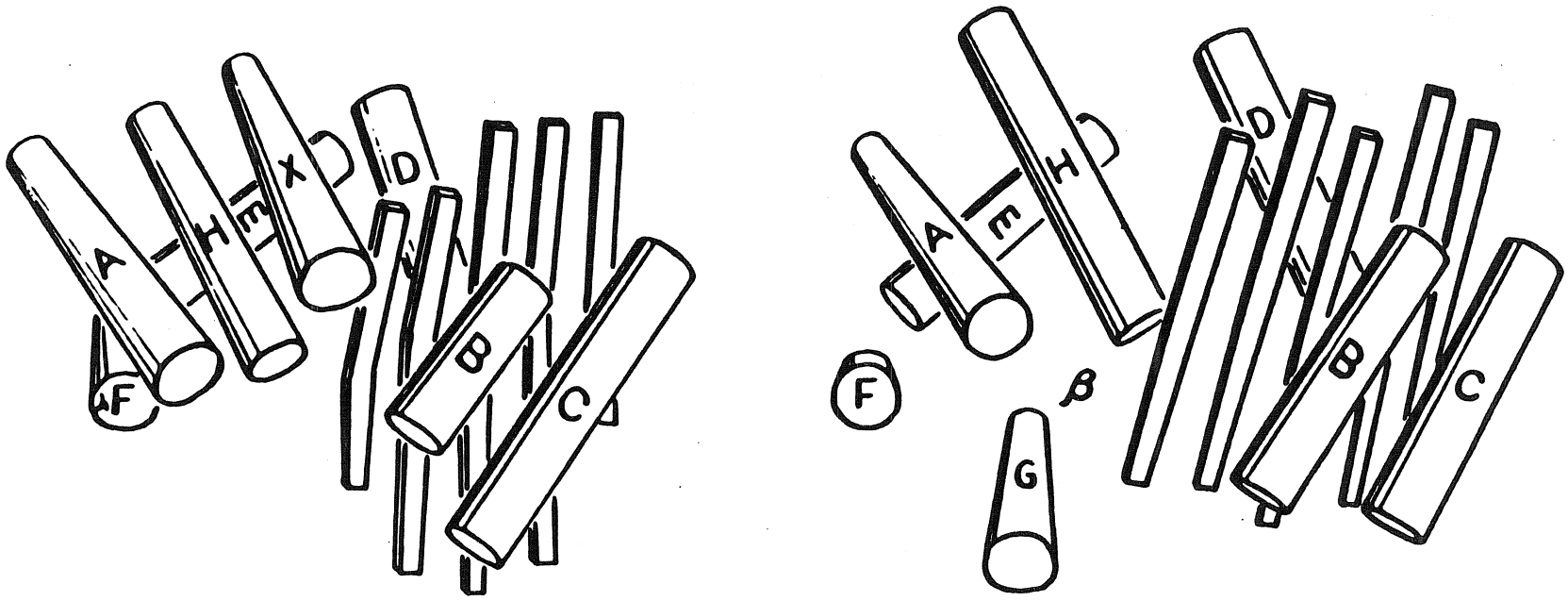


**Figure 10** - Examination of the low resolution crystal structures of the ß-lactamase from *Bacillus licheniformis* and the R61 carboxypeptidase from *Streptomyces sp.* reveals a strong conservation of secondary structures between the two classes of enzymes (50).

This helix, located in close proximity to the active site, had been proposed to be responsible for the ability of the PBPs to catalyze transpeptidase reactions. We, however, have determined that this helix is not necessary for transpeptidase activity.

There are several points of contention held by those researchers who refute the evolutionary relationship between the two enzymes. Ambler (43) has stated that the sequence homologies (20-25% overall) between the class A ß-lactamases and the PBPs is too divergent to implicate a direct relationship. A second factor is that, while both enzymes are capable of utilizing the ß-lactams as substrates, the reaction mechanism is different for each enzyme (Figures 5 & 7). Whereas the ß-lactamases rapidly deacylate the inactive penicilloic acid, the majority of PBPs fragment benzyl penicillin to phenylacetylglycine (51). And finally, several researchers question why the ß-lactamases show no activity towards the D-Ala-D-Ala dipeptide substrates.

To further investigate the evolutionary relationships between the ß-lactamases and PBPs, I have produced several chimeras which were designed to incorporate D-Ala-D-Ala carboxypeptidase activity into the RTEM-1 ß-lactamase framework. The synthesis and characterization of an RTEM-1/PBP-5 chimera is described in Chapter 2. The design and synthesis of a series of RTEM-1/R61 chimeras are described in Chapter 3. And finally, the characterization of two RTEM-1/R61 chimeras is detailed in Chapter 4. The goal of this research has been to gain a better understanding of the structural basis for biological catalysis.

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# Chapter 2

## Synthesis & Characterization of an RTEM-1 ß-Lactamase /Penicillin-Binding Protein 5 Chimera

## INTRODUCTION

The ß-lactam antibiotics are substrates of two large groups of bacterial enzymes. The first group, the penicillin binding proteins (PBPs), function as the D-Ala-D-Ala-carboxypeptidases and transpeptidases involved in the crosslinking of peptidoglycan in the final stage of cell wall biosynthesis (1). These enzymes are the natural target of the fungal antibiotics. The PBPs catalyze the hydrolysis of a C-terminal D-alanine-D-alanine dipeptide leading to a serine-ester-linked acyl-enzyme intermediate at the penultimate D-alanine residue. This step is then followed by either the hydrolysis of the intermediate to generate a free D-alanine terminus or transfer of the free amino terminus to another peptide chain. The latter reaction, known as transpeptidation, allows these enzymes to stabilize the bacterial cell wall against hypotonic lysis (Figure 1). The PBPs are also able to bind the ß-lactam antibiotics as structural analogues to the D-Ala-D-Ala dipeptide. However, reaction with these compounds, via nucleophilic attack on the lactam carbonyl group, results in a stable acyl-enzyme complex (Figure 2). This competitive inhibition, characterized by the slow rate of antibiotic deacylation, prevents the cell wall crosslinking that is necessary for cell viability.

The second group of enzymes, the ß-lactamases, are also acylated by the ß-lactams. In contrast to the the PBPs however, these enzymes rapidly deacylate the penicilloyl complex at rates nearing diffusion control (Figure 3). The second-order rate constant ($k_{cat}/K_M$) for RTEM-1 ß-lactamase utilizing benzyl penicillin as a substrate is on the order of $10^8$ $M^{-1}$ $sec^{-1}$ (2). Unlike the PBPs, the ß-lactamases show no activity towards the D-Ala-D-Ala dipeptide (1), nor is the rate of ß-lactam hydrolysis inhibited at any measurable level by the acyclic peptide substrates.

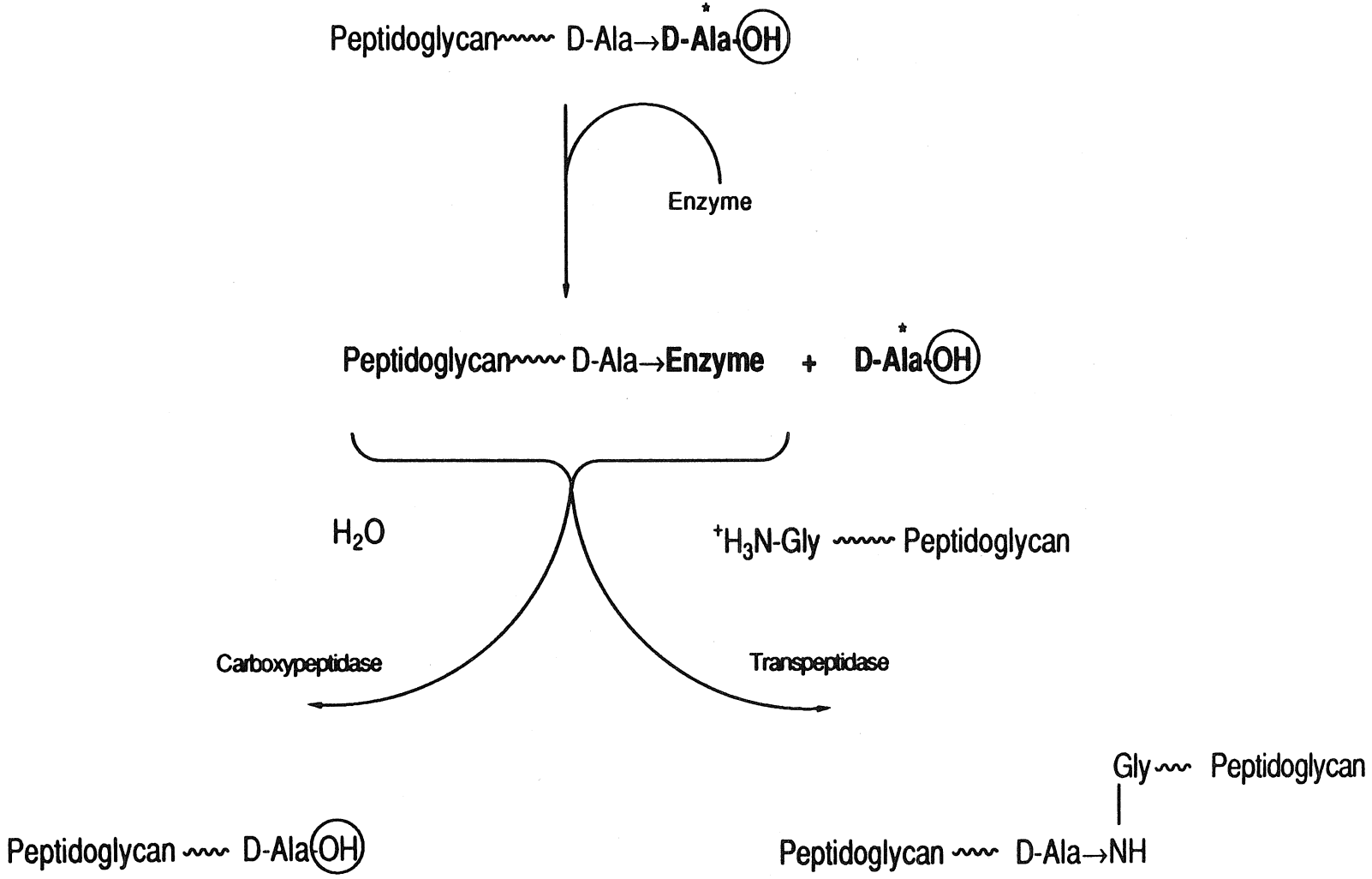



**Figure 1** - The process of bacterial cell wall biosynthesis. The strands of peptidoglycan are targeted by the D,D-carboxypeptidases, resulting in the hydrolysis of the terminal D-alanine residue. The acyl-enzyme intermediate can then be deacylated by water or an amino acceptor, the latter resulting in a stabilized crosslinked structure.

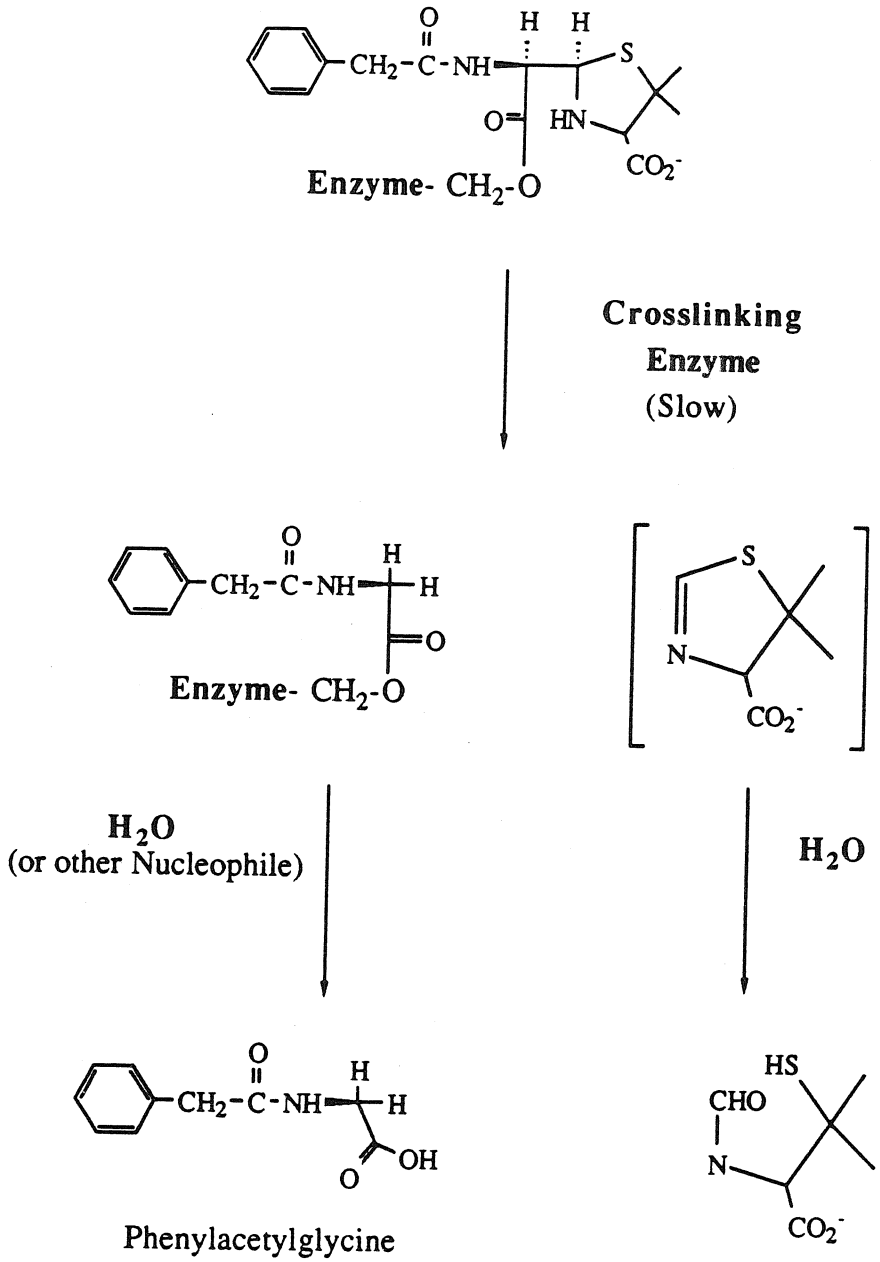



**Figure 2** - Attack of the lactam bond by the active-site serine in the D,D-carboxypeptidases results in the acyl-enzyme intermediate. The D,D-carboxypeptidases are unable to rapidly deacylate the complex, thereby the antibiotic inhibits cell wall crosslinking.

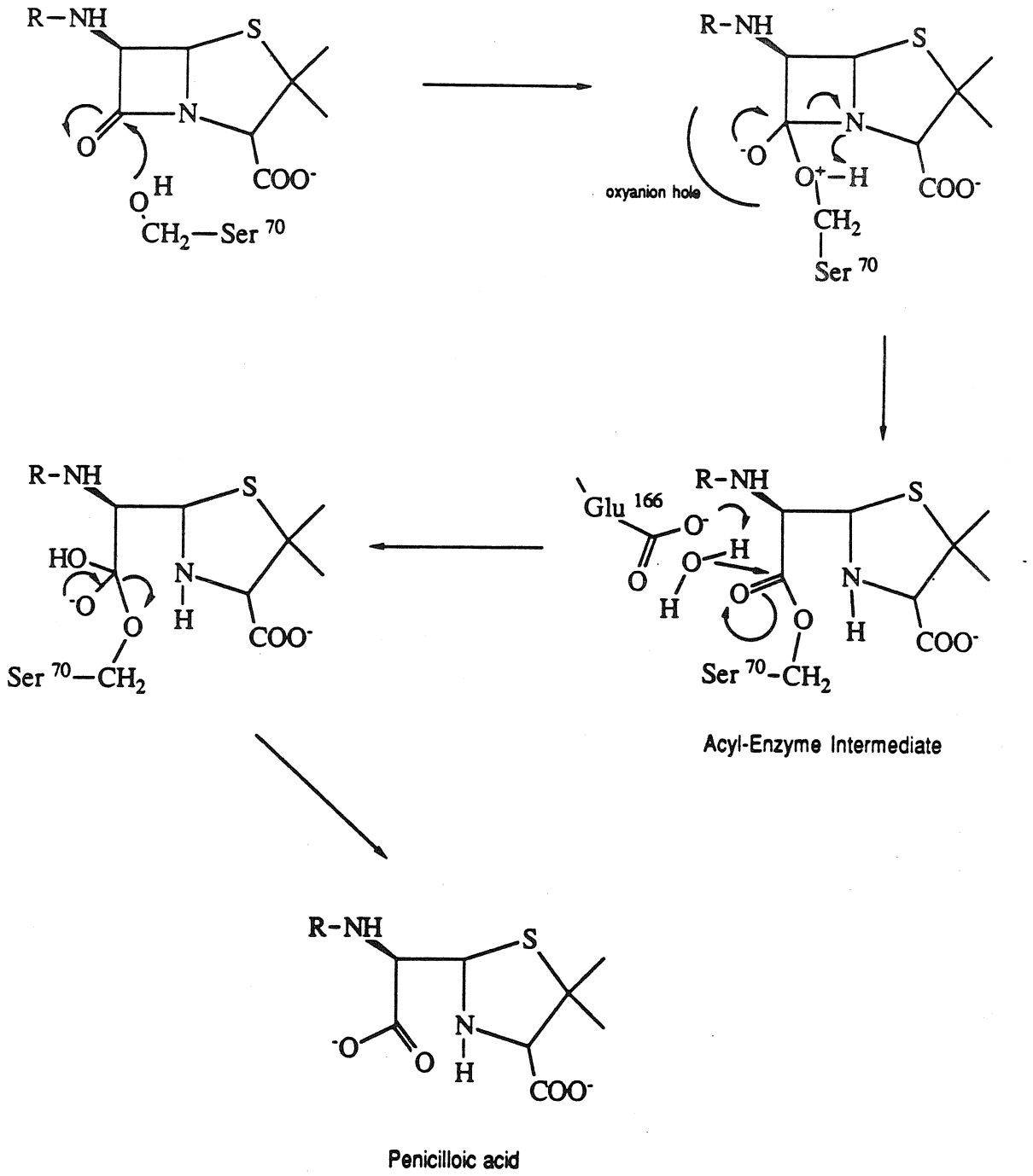



**Figure 3** - A mechanism for the hydrolysis of the ß-lactam antibiotics by ß-lacamase. The active-site serine forms an acyl-enzyme intermediate with the antibiotic which is subsequently hydrolyzed by the the transfer of a water molecule from glutamate 166.

Tipper and Strominger (3) were the first to propose a possible evolutionary relationship between the two families of enzymes. The basis of their hypothesis was the observation that the D-Ala-D-Ala dipeptide and the penam antibiotic molecular structure are surprisingly analogous (Figure 4). It is reasonable to assume that a mutation in the carboxypeptidase which would allow deacylation of the penicilloyl complex would have a genetic advantage that would surely be propagated. Examination of low resolution structures of the two enzymes reveals a conservation of secondary structural elements (4) (Figure 5). Further studies have shown that the amino acid sequences around the active-site serine in ß-lactamases exhibit significant homology (5,6) to those present in the PBPs (Table 1). In both enzymes, the active-site serine is incorporated into a semiconserved triad: Ser-Thr-Xaa-Lys in the ß-lactamases and Ser-Xaa-Thr-Lys in the PBPs. These factors combine to strengthen the initial hypothesis that the ß-lactamases have evolved to enable the bacterial host to survive the selective pressure imposed by the ß-lactam antibiotics.

A key argument against the proposed relationship is that the amino acid homology between the two enzymes is in fact low (>20%) (7). However, recent examination has revealed that the sequence homologies between individual class A ß-lactamases are also relatively low, with an average homology of ~41% homology (8). Thus it would appear that a more macroscopic effect, secondary rather than primary structure, is dominant in the proteins' function.

To further examine this relationship, research in the Richard's Laboratory by Y. H. Chang (9,10) was performed to create a chimeric enzyme consisting of the active-site sequence from PBP-5 of *E. coli*, cloned into RTEM-1 ß-lactamase with the eventual goal of recruiting

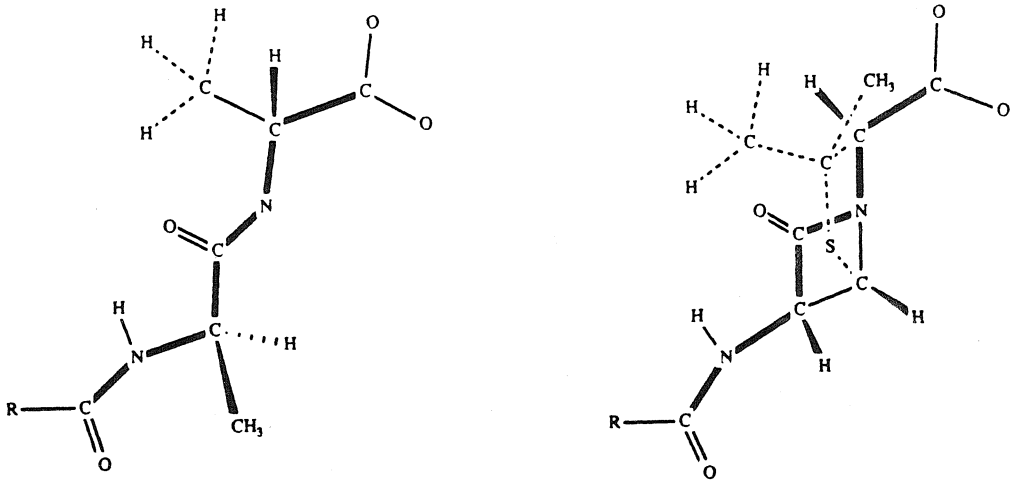


**Figure 4** - Chemical structures emphasising the close structural analogy of the ß-lactam antibiotics (right) with the D-Ala-D-Ala dipeptide (left). Hydrolysis of the lactam bond leads to a conformation which is nearly superimposable with the peptide (3).

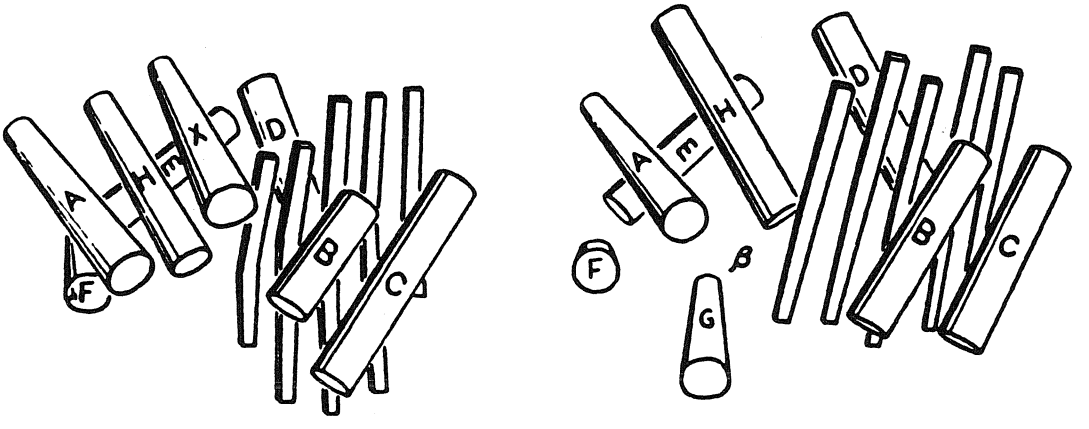



**Figure 5** - Examination of the low resolution crystal structures of the ß-lactamase from *Bacillus licheniformis* and the R61 carboxypeptidase from *Streptomyces sp.* reveals a strong conservation of secondary structures between the two classes of enzymes (4).

**Table I** - The amino acid sequences, centered around the active-site serine, of the class A ß-lactamases compared with the R61 enzyme and the low molecular weight PBPs of *E. coli* (6). The residues are numbered by the Ambler convention for ß-lactamases (7).

| | 70 | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| RTEM-1 | R | F | P | M | M | ***S*** | ***T*** | F | ***K*** | V | L | L |
| B. licheniformis | R | F | A | F | A | ***S*** | ***T*** | I | ***K*** | A | L | T |
| B. cereus | R | F | A | F | A | ***S*** | ***T*** | Y | ***K*** | A | L | A |
| S. aureus | R | F | A | Y | A | ***S*** | ***T*** | S | ***K*** | A | I | N |
| • • • | | | | | | | | | | | | |
| Streptomyces R61 | R | F | R | V | G | ***S*** | V | I | ***K*** | S | F | S |
| PBP 5 | R | R | D | P | A | ***S*** | L | T | ***K*** | P | M | V |
| PBP 6 | R | L | P | I | A | ***S*** | M | T | ***K*** | M | M | T |

D,D-carboxypeptidase activity into ß-lactamase. At the onset of this project there was no high resolution crystal structure available for a class A ß-lactamase and thus the exact spatial arrangement of the amino acids was not known. To select the specific amino acids which would be exchanged, sequence homology was examined. Among the PBPs whose amino acid sequences are known, PBP-5 of *E.coli* contains the most homologous (27%) amino acid sequence incorporating the active site serine, when compared with the class A ß-lactamases (5, 11,12). The capacity to inactivate ß-lactams is not absent in all PBPs; several can complete deacylation of the penicilloyl complex, thus displaying ineffectual levels of ß-lactamase activity. However, PBPs tend to degrade the penicilloyl moiety into phenylacetylglycine (1, 13, 14). This is not true for PBP-5, which deacylates to form penicilloic acid at a higher rate than the majority of PBPs. This finding could be a possible implication of a more synchronous evolutionary relationship with the class A ß-lactamases (11). Chang's chimeric gene synthesis was successful in creating an enzyme capable of utilizing both sets of substrates. However, this result was marred by the existence of a secondary mutation--the insertion of an additional glutamic acid residue between residues 59 and 60 (ß-lactamase numbering) (Figure 6).

The goal of my research in this area has been to remove this residue, and then to fully characterize the resulting chimeric enzyme. I detail here the synthesis and characterization of a RTEM/PBP-5 chimera which exhibits approximately 1% of the PBP-5 carboxypeptidase activity.

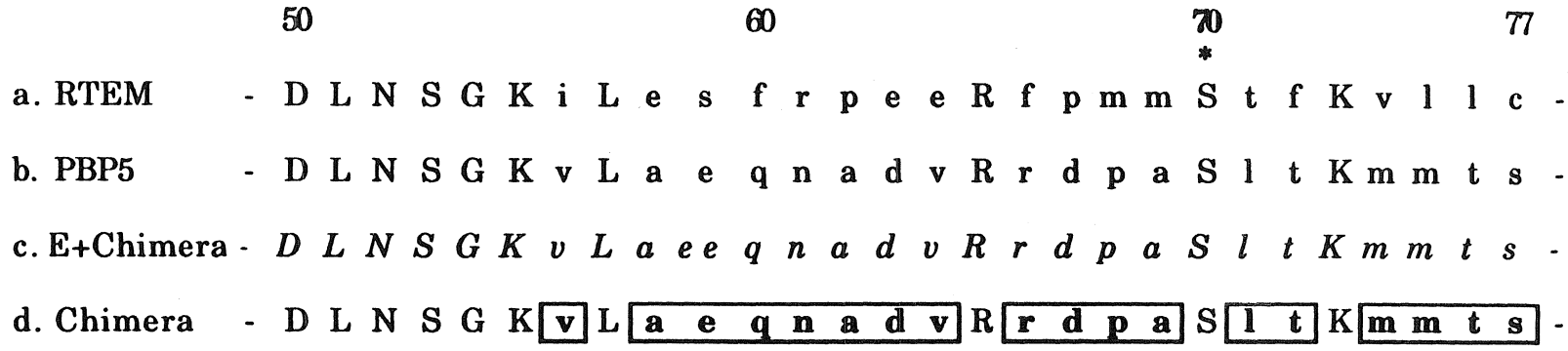



**Figure 6** - (a.) Amino acid sequence of RTEM-1 ß-lactamase which was replaced, (b.) the corresponding sequence from PBP-5 of *E. coli*, (c.) the original PBP-5 chimera which contains an additional glutamic acid between residues 59 and 60, and (d.) the corrected PBP-5/RTEM-1 chimera.

## MATERIALS AND METHODS

### Enzymes and Chemicals

Restriction enzyme Bsm I was purchased from New England Biolabs (NEBL). All other restriction enzymes, T4 DNA ligase, and T4 polynucleotide kinase were purchased from Boehringer Mannheim Biochemicals (BMB). Kanamycin sulfate was purchased from BMB; all other antibiotics were supplied by Sigma Chemical Company. [$\alpha$-$^{35}$S]-dATP, [$^{14}$C]-benzyl penicillin, and [$^{3}$H]-glycine were supplied by Amersham. [$^{35}$S]-Benzyl penicillin was obtained from Dupont/New England Nuclear. Isopropyl-β-D-thiogalactoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal), 10x ligase buffer, and molecular biology grade agarose were purchased from International Biotechnologies, Inc. (IBI). Phenol and chloroform were purchased from Fisher Scientific, Inc. DE-52 chromatography media was purchased from Whatman, Inc; Sephadex G-25 and G-100 were purchased from Sigma Chemical Company.

### Bacterial Strains

pBR322 plasmid DNA was harbored in *Escherichia coli* strain HB101. The pJN plasmids (15), which were utilized as expression vectors, were harbored in *E. coli* strain D1210. The D1210 strain contains a *lac* repressor (lac i$^{Q}$) necessary for the control of the *tac* promotor (16) present in pJN. Bacteriophage were propagated in *E. coli* strain TG1 obtained from Amersham. Culture medium was L broth (10 g tryptone, 5 g yeast extract, 5 g NaCl in 1 liter; add 15 g bacto-agar to make L plates) for plasmid preparations and 2 x YT broth (16 g tryptone, 10 g yeast extract, 5 g NaCl in 1 liter; add 15 g bacto-agar to make 2xYT plates) for bacteriophage preparations.

Transformation and Plating

Cells were made competent for transformation of plasmid DNA using a process adapted from Hanahan (17). A 250 ml Erlenmyer flask containing 25 ml of SOB media (20 g tryptone, 5 g yeast extract, 0.6 g NaCl, 0.2 g KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ in 1 liter) was inoculated with *E. coli* and incubated with shaking at 37°C to $OD_{550}$~0.3. The cells were then centrifuged (3,000 rpm for 5 minutes) at 4°C; the 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 $CaCl_2 \cdot H_2O$, 150g glycerol, 30 ml of 1M KOAc, pH 7.5 in 1 liter. Adjust to pH 5.8 with 0.2M acetic acid.). The cells were incubated on ice for 15 minutes followed by centrifugation (3,000 rpm for 5 minutes). The resulting pellet was resuspended in 4 ml transformation buffer 2 (1.2 g RbCl, 11 g $CaCl_2 \cdot H_2O$, 150 g glycerol, 20 ml of 0.5 M MOPS, pH 6.8 in 1 liter). Cells were transformed within two hours of preparation or frozen in liquid nitrogen. The frozen cells could then be used by thawing on ice for 15 minutes. Comparable efficiencies were observed with frozen cells used within two months.

DNA was added to 300 µl of competent cells in a 1.5 ml eppendorf tube with gentle mixing. These were left on ice for 40 minutes, followed by heat-shock at 42°C for 90 seconds. For plasmid DNA, 800 µl SOC media (20 g tryptone, 5 g yeast extract, 0.6 g NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 10 mM glucose in 1 liter) was added and the tubes were incubated for 1 hour at 37 °C and then plated onto L agar containing the appropriate antibiotic. For single-stranded DNA, the heat-shocked sample was added to 200µl log phase *E. coli* TG1 cells, 3 ml of melted (45°C) top agar (10g bactotryptone, 8g NaCl, 8g bacto-agar in 1 liter), mixed by inverting gently 3-4 times, and poured onto preheated (37°C) H plates (10g bactotryptone, 8g NaCl, 10g bacto

agar in 1 liter). Plates were left at room temperature for 10-15 minutes before inverting.

DNA

All DNA concentrations were estimated from absorbance at 260 nm and purity estimated by $A_{260}/A_{280}$. Ethanol precipitation of DNA was accomplished by adding 3 M sodium acetate (0.1 volume), absolute ethanol (2.5 volumes) and precipitating on dry ice for 15 minutes. The sample was centrifuged (14,000 rpm for 15 minutes) at room temperature. The resulting pellet was then washed with 100 µl of 70% ethanol, and dried *in vacuo*. The DNA is then resuspended in TE.

Wild-type plasmid pBR322 and bacteriophage M13 mp19 replicative form (RF) DNA were purchased from Bethesda Research Laboratories (BRL). Mutant plasmids and RF phage were purified from *E. coli* by the alkaline lysis method (18). Mini preps (2 ml) were performed by growing the cultures to saturation. The cells were pelleted and resuspended in Solution I (100 µl ; 50 mM glucose, 25 mM Tris (pH 8.0), 10 mM EDTA, 1 mg/ml lysozyme) and allowed to react for five minutes at room temperature. Solution II was added (200 µl ; 0.2 N NaOH, 1 % SDS) followed by incubation on ice for five minutes. The pH was neutralized with cold (4°C) Solution III (150 µl ; 60 ml 5M KOAc, 11.5 ml glacial AcOH in 100 ml), and the solution was centrifuged to remove the precipitated cellular debris. A single phenol/chloroform extraction (100 µl each) was performed followed by ethanol precipitation. This DNA was then suitable for sequencing or reaction with restriction endonucleases. Large scale preparations were further purified using ultracentrifugation in cesium chloride (0.95g/ml) / ethidium bromide (75µl/ml) gradients (single spin: 20 h, 45,000 rpm) (19) to

remove the large amounts of contaminating RNA and protein. Single-stranded phage DNA was prepared from phage supernatant by precipitation with 20% polyethylene glycol-6000 / 2.5 M NaCl (20) followed by phenol/chloroform extraction and ethanol precipitation.

Synthetic oligonucleotides (0.2 μmol) were synthesized by the Caltech Microchemical Facility using phosphoramidite chemistry on an Applied Biosystems automated DNA synthesizer, Model 380A. Oligonucleotides (1/3 of synthesis) were resuspended in 25 μl TE and 25 μl Maxam-Gilbert loading buffer (80% (w/v) deionized formamide, 50 mM Tris-borate (pH 8.3), 1 mM EDTA (pH 8.0), 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue.), and purified by preparative polyacrylamide gel electrophoresis (15-20%, 0.1 x 20 x 40 cm gel; 500 volts; 12 hours) in TBE buffer (10.8g Tris base, 5.5g boric acid, 4 ml 0.5M EDTA, pH 8.0, in 1 liter.). DNA was visualized with UV light reflected from a fluorescent indicator coated silica TLC plate. The appropriate bands were excised, the gel crushed, and then suspended in 1 ml of 2 M NaCl overnight at 37°C. The samples were desalted using two sequential G-25 Sephadex spin columns (10 minutes @ 2,500 rpm).

Restriction Digests

Restriction digests typically were performed on 5 μg plasmid DNA with 5-20 units of restriction enzyme and 3 μl 10X digest buffer (as suggested by the supplier) in 30 μl at 37°C for 1-2 hours. DNA restriction fragments were separated on 1.2% agarose gels in TAE buffer (4.8g Tris base, 1.14 ml glacial acetic acid, 2.0 ml 0.5 M EDTA, pH 8.0, in 1 liter.). The agarose gels contained 0.005% ethidium bromide (v/v), which allowed for fragment visualization by UV irradiation. DNA was isolated from the

excised gel fragment using either a UEA electroeluter (IBI) (23) or an Elutrap™ (Schleicher & Schuell) (24).

Kinasing and Annealing Synthetic Oligonucleotides

The purified oligonucleotides (100 pmol each) were added to 3 µl 1M Tris (8.0), 1 µl 100mM DTT, 3 µl 10mM ATP/100mM $MgCl_2$, and 2-5 units of T4 polynucleotide kinase in a total volume of 30 µl. The reaction was incubated for 30 minutes at 37°C, followed by 10 minutes at 65°C to inactivate the enzyme.

Complementary oligonucleotides (100 pmol each) were annealed with 10 µl 10x medium salt buffer (10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT) in a total volume of 100 µl, by heating to 95°C and allowing the temperature to cool to room temperature slowly. It is best to use a larger vessel (2-4 liters) for the annealing so that the temperature drop is gradual. No further purification was necessary for ligation.

Oligonucleotide-directed Mutagenesis

The Eco RI/Sal I 3712 base-pair fragment from the pBR322 plasmid containing the Chang ($E^+$) chimeric gene was subcloned into Eco RI/Sal I digested M13 mp19 RF DNA. The fragments (10 pmol each) were ligated using 2 units T4 DNA ligase and 5 µl 10x IBI ligase buffer, in a total volume of 50 µl. The reaction incubated at 25°C for 2.5 hours. Competent *E. coli* TG1 cells were transformed with aliquots of 1 and 5 µl of the ligation mixture. The transformed cells were plated on top agar H plates with X-gal and IPTG. Clear plaques on a lawn of TG1 represented successful clones containing the pBR322-Chimera$E^+$ Eco RI/Sal I insert; blue plaques indicated background wild-type M13 mp19 phage.

A three base-pair deletion (Figure 7) was accomplished using the Amersham *in vitro* mutagenesis kit (20), which employs the Eckstein selection method (25). All concentrations and volumes were those specified by the manufacturer unless otherwise noted. Single-stranded phage (5 pmol) was isolated and annealed to the kinased mutagenic 19mer 5'-ACTGGCGGAGCAGAATGCG-3' (8.25 pmol). Chain extension from this oligonucleotide was accomplished with the Klenow fragment of DNA polymerase I (6 units), T4 DNA ligase (6 units), and a mixture of dATP, dGTP, dTTP, and αS-dCTP as supplied The reaction was incubated overnight at 15°C. The reaction mixture was filtered through a double nitrocellulose disk filter (Schleicher & Schuell) to remove the remaining single-stranded DNA. Double-stranded DNA was recovered from the filtrate by ethanol precipitation. The heteroduplex DNA was subsequently digested with the restriction endonuclease Nci I (16 units) for 90 minutes at 37°C. Due to the the incorporation of αS-dCTP in the synthetic strand, only the native strand is cleaved, thus resulting in nicked duplex DNA. Exonuclease III (50 units; 25 minutes at 37°C) was then employed to excise the native DNA in the region of the mismatch. It is important to limit the time of reaction with Exo III to that stated in order to maintain some native-strand DNA necessary for synthesis priming. Repolymerization was accomplished by incubating the partially digested heteroduplex with all dNTP's, DNA polymerase I (6 units), and T4 DNA ligase (6 units), yielding double-stranded DNA in which both strands carried the correct deleted sequence. Competent *E. coli* TG1 cells were transformed with the final DNA solution (5 & 20 μl). Sixteen plaques were chosen for phage isolation. Three out of eight sequenced positive for the desired mutation. These were then subcloned into pJN (Figure 8) using Eco RI and Sal I, producing pJN-

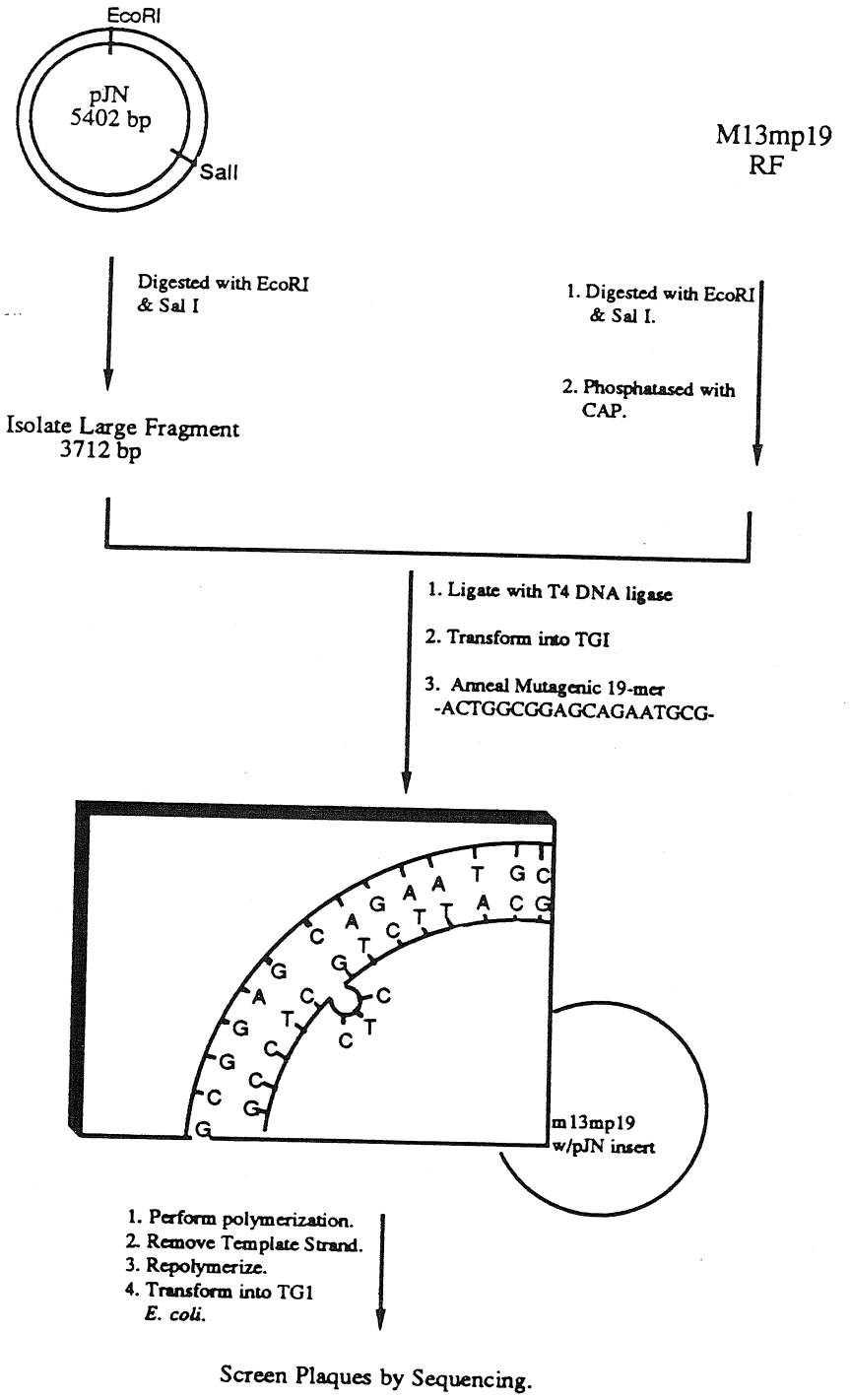



**Figure 7** - The 3712 base pair Eco RI/Sal I fragment of pJN containing the E+ chimera was subcloned into M13 mp19. Oligonucleotide-directed mutagenesis was performed to remove the extra glutamate (GAG) codon. The mutants were screened by dideoxy sequencing (29).

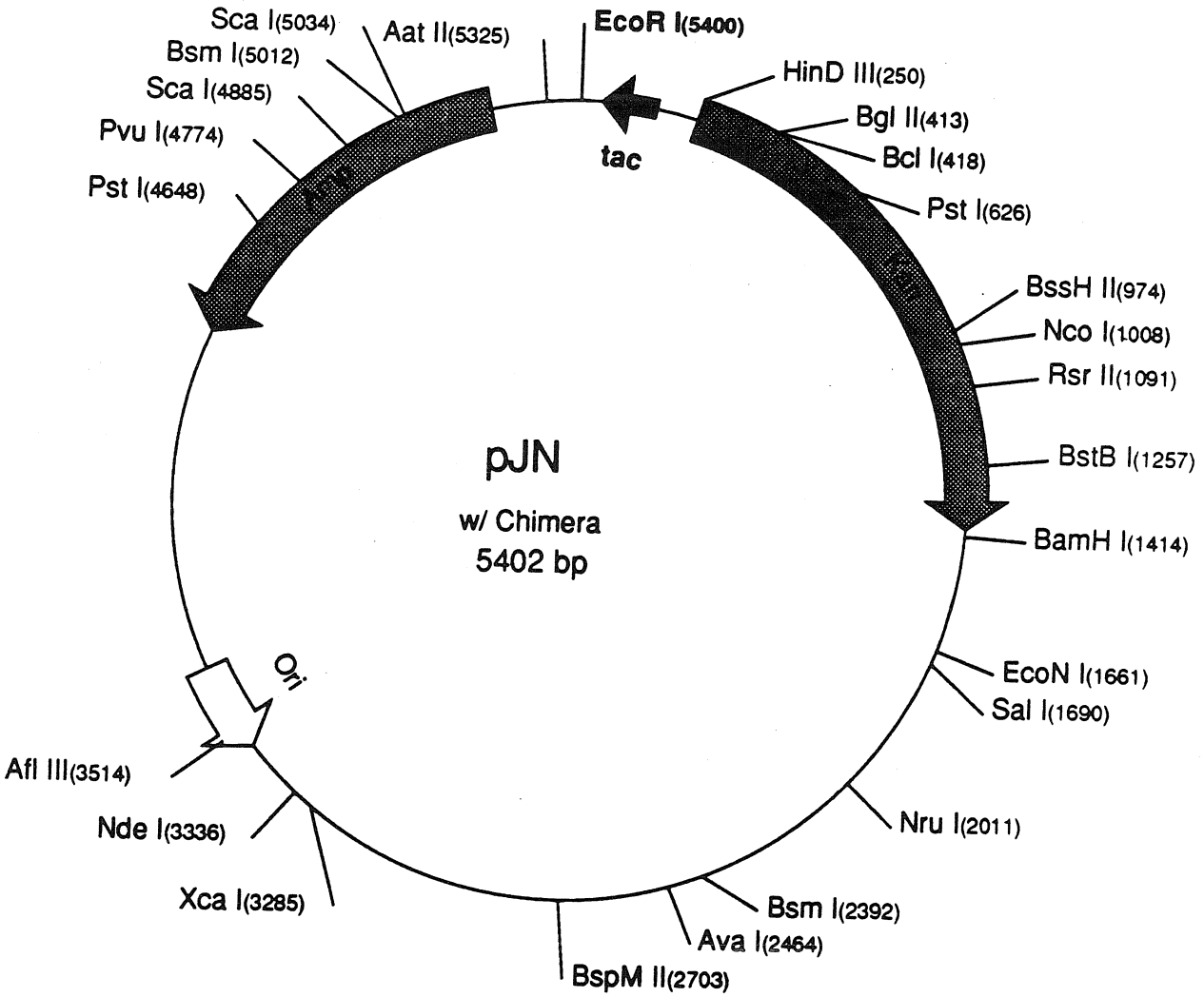



**Figure 8** - The pJN plasmid containing the PBP-5/RTEM-1 chimera under control of the *tac* promotor (16).

Chimera-45. The entire chimeric ß-lactamase gene was then sequenced to verify that no secondary mutations had occurred.

Cassette Mutagenesis

The $E^+$ chimeric gene was first subcloned from pBR322 into pJN for the three-fragment ligation (26). A synthetic cassette (27,28) was designed to replace the region in the $E^+$ chimeric ß-lactamase gene which contained the inserted glutamic acid at residue 59. The complementary synthetic oligonucleotides (100 pmol each) were left unkinased and annealed as above. A three-fragment ligation was then carried out, as outlined in Figure 9. Annealed oligonucleotides (0.4 pmol) were combined with each of the Bsm I/Nde I 1676 base-pair fragment (0.04 pmol) and the Sca I/Nde I 3726 base-pair fragment. In addition, control reactions were run in the absence of insert DNA to test for contamination by the parent plasmid. The DNA was incubated with 1 unit T4 DNA ligase and 2.5 µl IBI 10x ligation buffer in a total volume of 25 µl. The reaction was incubated for 12-14 hours at 15°C followed by transformation of competent *E. coli* D1210 with 1-5 µl of the ligation mixtures. The cells were plated onto kanamycin sulfate (50 mg/l) agar plates and incubated overnight at 37°C. The resulting colonies were picked onto kanamycin sulfate (50 mg/l) and ampicillin (100 mg/l) master plates. No colonies exhibited a resistance to ampicillin; seven out of eight sequences were verified to be the desired ligation product. This chimera was identical to that produced by oligonucleotide-directed mutagenesis under all conditions tested.

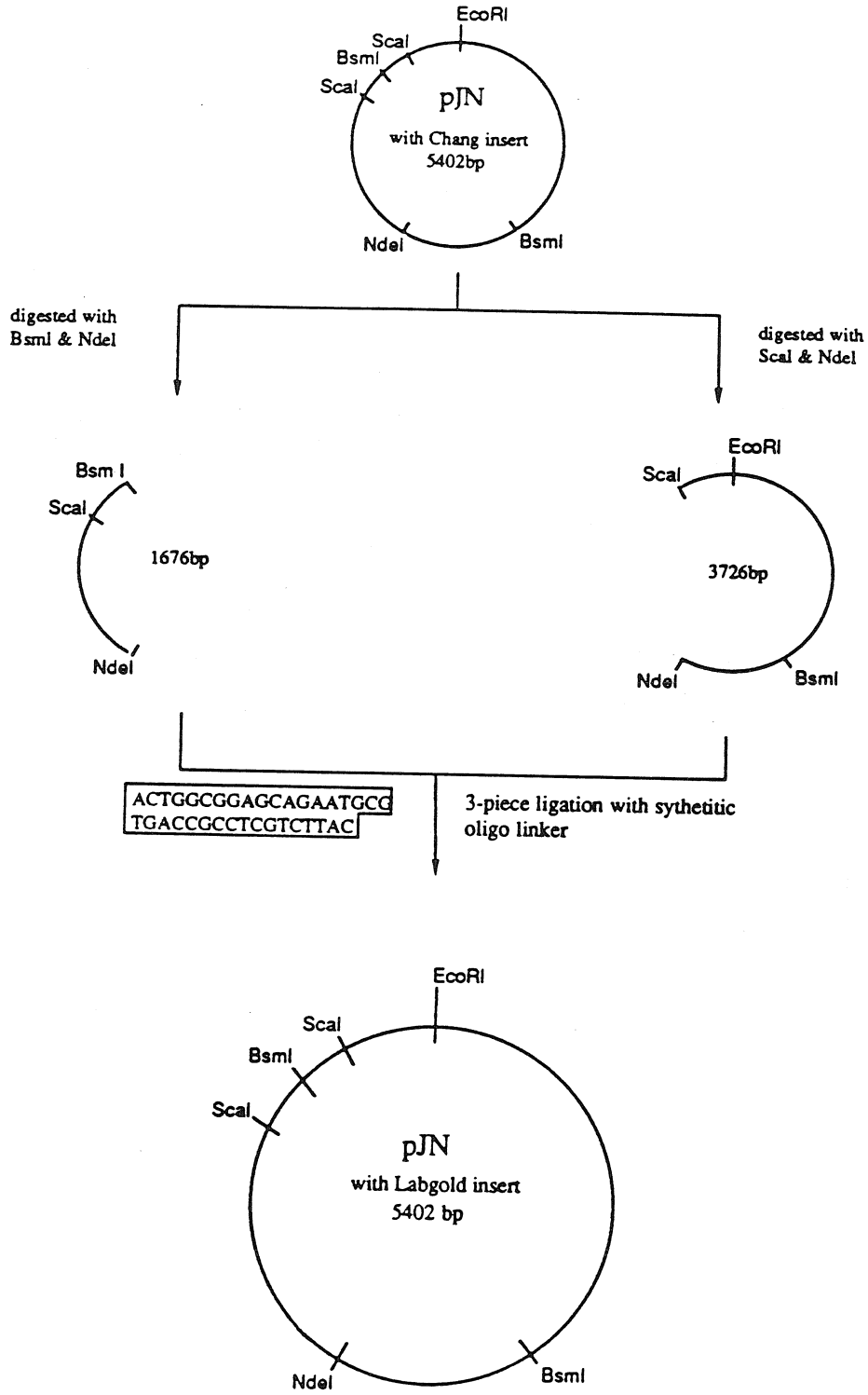



**Figure 9** - Cassette mutagenesis was employed in a three-fragment ligation to generate the PBP-5/RTEM-1 chimera.

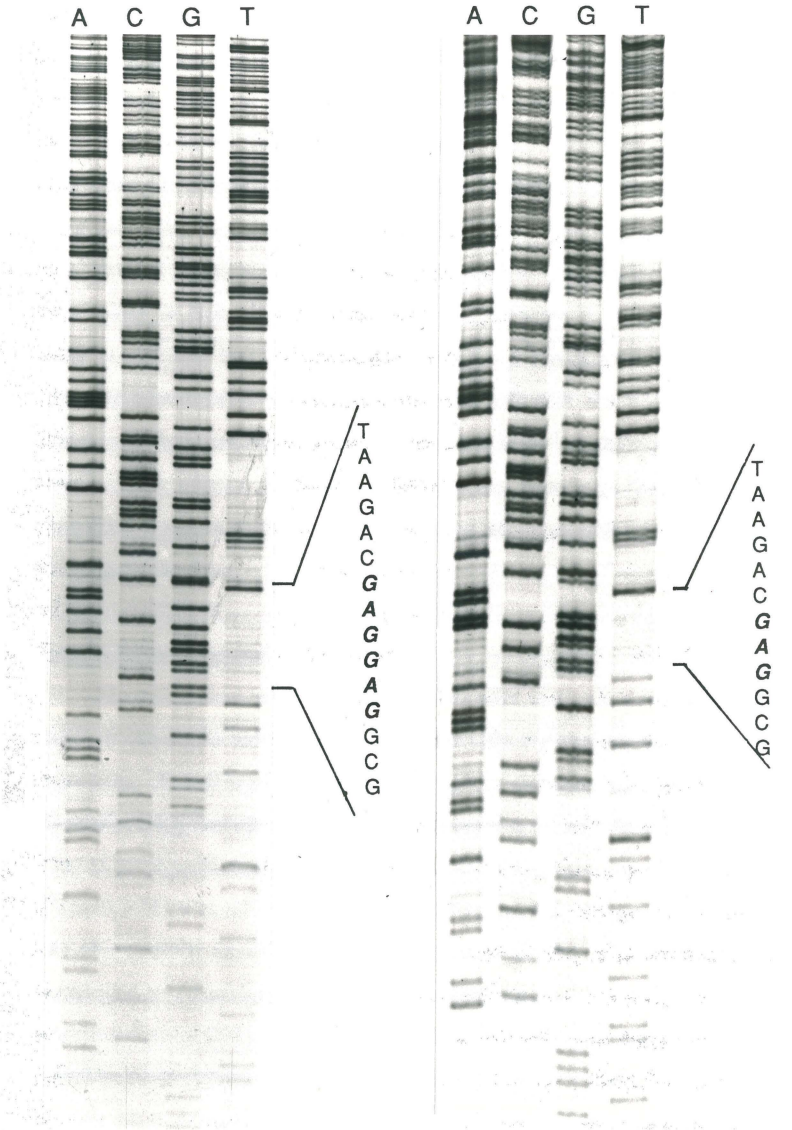
Double-Stranded Plasmid Sequencing

Plasmid DNA was sequenced by the dideoxy method (29) for denatured, double-stranded DNA (30). Plasmid DNA was denatured by treating 5 μg DNA with 20 μl 0.2 M NaOH for 5 minutes, followed by neutralization with 2 μl of 2 M $NH_4OH$, pH 4.5, ethanol precipitation and two 70% ethanol washes. The sequencing primers were synthesized to be 17-18 bases in length and lie ~100 bases upstream of the point of mutation. The DNA pellet was suspended along with 25 pmol 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 small variations on the protocol described in the SEQUENASE™ kit from United States Biochemical (31). The annealed primer-DNA complex was incubated with 0.5 μl labeling mix, 2 μl 100 mM DTT, 2 μl sequenase reaction buffer, 1 μl [α–$^{35}$S]-dATP (1000 Ci/mmol), and 2 μl diluted sequenase (1 in 8 dilution) for 3 minutes at 25 °C. The reaction mixtures were then added to the termination mixes containing the dideoxynucleotides (2.5 μl) and incubated for 10 minutes at 37°C. Reactions were stopped by adding Maxam-Gilbert (22) loading buffer (4 μl). Samples were loaded onto 5-8% polyacrylamide / TBE gels, run at 50 mA for 3 hours, and autoradiographed 12-24 hours using Kodak XAR film (Figure 10).

Western Blots

Western blots were performed to examine *in vivo* stability of the mutant enzyme. Colonies harboring the chimeric plasmid were grown to late log phase ($OD_{600}$ = 1.0): a 1.0 ml sample pelleted by centrifugation and resuspended in 100 μl protein sample buffer (10% v/v glycerol, 5% v/v 2-

**Figure 10** - Autoradiograph of a sequencing gel comparing the PBP-5 chimera produced by Y.H. Chang, containing an inserted glutamic acid, and the corresponding sequence of the PBP-5/ RTEM-1 chimera resulting from the deletion of the additional GAG codon. The mutated region displays some compression due to the high G/C content. Labeling was accomplished with [$\alpha$-$^{35}$S]-dATP.


A C G T
T A A G A C G A G G A G G C G
A C G T
T A A G A C G A G G C G

mercaptoethanol, 3% w/v SDS, 62.5 mM Tris-HCl, pH 6.8, 1.0 mM EDTA, 0.05% w/v bromophenol blue) per absorbance unit (i.e., $OD_{600}$ = 0.95 ; 95 µl buffer) and boiled for 5-10 minutes to lyse the cells. Aliquots of 15 µl each were loaded onto a 15 cm, 12% polyacrylamide stacking gel (4% stack) andrun at a constant current of 10 mA for 10-12 hours in Tris-glycine buffer (12 g Tris base, 57.6 g glycine, 40 ml 10% SDS (w/v) in 4 liters). The protein was then transferred from the acrylamide gel onto nitrocellulose (Schleicher & Schuell) using a Bio-Rad Transblot™ cell (equipped with plate electrodes) for 1.5 hours at 100 mA. ß-Lactamase was visualized following binding of rabbit anti-ß-lactamase (32) with the Vectastain™ ABC immuno-peroxidase system (33). Western blots to examine the *in vivo* stabilization effects of ß-lactam antibiotics were also performed; the procedure was the same as above with the addition of 6 mg/l ampicillin in the growth media.

Protein Expression and Purification

Mutant genes were subcloned into pJN (15), an expression vector utilizing the IPTG inducible *tac* promoter (16), for the overproduction of ß-lactamase. *E. coli* D1210 cells containing the chimeric pJN were grown in XB media (25 g Bactotryptone, 7.5 g yeast extract, 50 ml 1 M Tris (pH 7.5) per liter) at 30°C until saturated. Typical preparation volumes were 4-10 liters. IPTG (0.1 M) was added followed by cooling to 0°C. At no point after this was the protein exposed to temperatures exceeding 4°C throughout the remaining purifications. After 30 minutes the cells were harvested by centrifugation in 250 ml bottles in a GSA rotor for 10 minutes at 10,000 rpm. Periplasmic proteins, including the mutant ß-lactamase, were released by osmotic extrusion; the pellets (1 liter) were resuspended in 40 ml sucrose

solution (25 ml 1.0 M Tris-HCl, pH 7.0, 450 g sucrose, 0.5 g $Na_2EDTA$ in 1 liter) and shaken at 4°C for 30 minutes. Samples were centrifuged at 10,000 rpm for 20 minutes. The supernatant was decanted and discarded, the pellets resuspended in 40 ml of chilled $H_2O$ (4°C), and shaken at 4°C for 30 minutes. Samples were then centrifuged at 13,000 rpm for 30 minutes. The crude ß-lactamase, contained in the supernatant, was collected and filtered through a Nalgene 0.22 µm filter unit to remove insoluable cellular debris. The volume of the solution was reduced and dialysed against 25 mM triethanolamine-HCl (TEA), pH 7.25, using an Amicon ultrafiltration apparatus. The chimera was then purified on a 2.5 x 30 cm anion exchange (DE-52) column. A linear TEA gradient (25-200 mM) was used to elute the mutant. The fractions were analyzed for the presence of ß-lactamase by 12% SDS-PAGE stained with Coomassie blue (R250). The ß-lactamase containing fractions were pooled and concentrated by ultrafiltration. The semi-purified protein was then applied to a 2.5 x 60 cm gel filtration (Sephadex G-100) column, and eluted with 20 mM Tris-HCl, pH 7.0. Purity was checked by 12% SDS-PAGE stained with Coomassie blue (R250). The protein concentration was estimated by $OD_{281}$ using the extinction coefficient 29,400 $M^{-1}cm^{-1}$ (21). Purification of the chimera included 40 mg/l ampicillin in all buffers.

Attempts to purify the chimera by FPLC were unsuccessful. No active protein could be isolated from preparations purified under the conditions used for other ß-lactamase mutants; a MonoQ™ 5/5 anion exchange column was utilized. Protein (200 µl) was loaded onto the column in 25 mM 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 1.0

ml/min. This technique is efficient for purification of the wild-type and stable mutants of ß-lactamase; however, the isolation of active chimera was not possible by this process.

Kinetics

Michaelis-Menten (34) kinetic parameters ($k_{cat}$, $K_M$, and $k_{cat}/K_M$) for the chimeric β-lactamase activity were determined by Eadie-Hofstee (35) replots of initial velocity data. Measurements were obtained using a Beckman DU7 spectrophotometer with 1 cm pathlength optical quartz cells. Kinetic analyses of ß-lactamase activities were performed at 30°C, pH 7.0, 0.1 M potassium phosphate. The hydrolysis of the lactam bond was monitored at 232 nm to obtain initial velocities ($\Delta\varepsilon$ = 500 for benzyl penicillin) (44). Buffers were maintained at 30°C prior to beginning the assay to avoid error from temperature flux.

D,D-Carboxypeptidase activities were determined at 37°C, pH 7.5, 0.1 M Tris-HCl. A fluorescence assay (36,37) was employed to measure the carboxypeptidase activity utilizing diacetyl-L-Lys-D-Ala-D-Ala (38) as a substrate. Reactions (0.25 ml) were initiated at several substrate concentrations; aliquots of 50 µl were collected at intervals of 30 minutes and added to 2 mls of buffered reagent [1.5 ml *o*-phthaldialdehyde (5 mg/ml ethanol), 1.5 ml 2-mercaptoethanol (5 µl/ml ethanol), 90 ml aqueous 0.05 M sodium tetraborate, pH 9.5]. The samples were analyzed within 15 minutes of mixing on a SLM-4800 fluorescence spectrophotometer. Excitation and emission monochromators were set at 340 nm and 455 nm, respectively, with a 2 nm slit width. The fluorescence value was corrected against the time-zero value and the concentration of D-alanine determined from a calibration curve. Eadie-Hofstee replots (35) were used to determine kinetic

parameters from initial velocity data. To obtain the pH vs. activity plot, reactions were performed at 0.02 M diacetyl-L-Lys-D-Ala-D-Ala varying pH and buffer systems: 0.1 M KOAc, pH 5.0 and 6.0; 0.1 M Tris-HCl, pH 7.0, 7.5, 8.0, 8.5 and 9.0; 0.1 M $KH_2PO_4$, pH 7.0 and 8.0; 0.1 M $K_2CO_3$, pH 8.5 and 9.0. To assay the hydrolysis of the depsipeptide diacetyl-L-Lys-D-Ala-D-Lac (39), parallel reactions were initiated at a single substrate concentration. Reactions were quenched at timed intervals by snap-freezing in dry-ice/ethanol. The remaining quantity of depsipeptide starting material in the samples was determined by isolation on ABI/Kratos HPLC using a C-18 reversed phase column and linear gradient of acetonitrile/water (0.1% trifluoroacetic acid). The gradient was as follows: Buffer A=0.1% TFA in water, Buffer B= 0.1% TFA, 80% acetonitrile in water; t = 0, 100% A; t = 3, A = 100% A; t = 33, 20% B; t = 41, 100% B. The flow rate was 1 ml/min. The protein content of the samples was quantified by $OD_{214}$. A blank run (starting material only) was performed for calibration. To assay the chimeras for transpeptidase activity, depsipeptide digests (0.1 M Tris-HCl, pH 7.5, 37°C) were initiated with [$^3$H]-glycine present in the reaction mixture. The reaction was quenched at 3 hours and the mixture analyzed by HPLC for the formation of the tripeptide, diacetyl-Lys-D-Ala-Gly and the incorporation of the radiolabel.

## RESULTS AND DISCUSSION

In order to investigate the possible evolutionary relationship between the ß-lactamases and the bacterial cell wall D,D-carboxypeptidases, a chimera was designed which replaced a 28 amino acid sequence encompassing the active site region in RTEM-1 ß-lactamase (residues 50-77 in the Ambler convention (7)) with a corresponding sequence from PBP-5 (residues 24-51 in the PBP convention, which does not include residues for any leader sequence) (Figure 6). The original chimera was designed prior to the availability of high resolution crystal data. The region of mutation was chosen because it encompassed the active-site serine. Since the onset of this project, the crystal structures for the PC1 ß-lactamase (40) from *Staphylococcus aureus* and the ß-lactamase from *Bacillus licheniformis* (41) have been published. It can now be seen that the region replaced in the chimera encompasses the α-helix containing the active-site serine 70, the ß2-strand of the five-stranded anti-parallel ß-sheet and the segment of random coil which joins the two structures (Figure 11). However, in the original construction an extra glutamic acid residue was mistakenly inserted between residues 59 and 60. This chimera did, however, exhibit a measurable level of carboxypeptidase activity. My goal was then to perform site-directed mutagenesis on the existing chimeric gene in order to remove the additional residue and to characterize the resulting chimeric protein.

Two synthetic routes were designed to achieve the desired gene product, and work towards both was performed simultaneously. The desired product was achieved by both oligonucleotide-directed (20) (Figure 7) and cassette mutagenesis (27,28) (Figure 9). The resulting chimeric ß-lactamase gene was subcloned into pJN (15) (Figure 8), an expression vector that utilizes the *tac* promoter (16). In addition to the *tac* promotor,

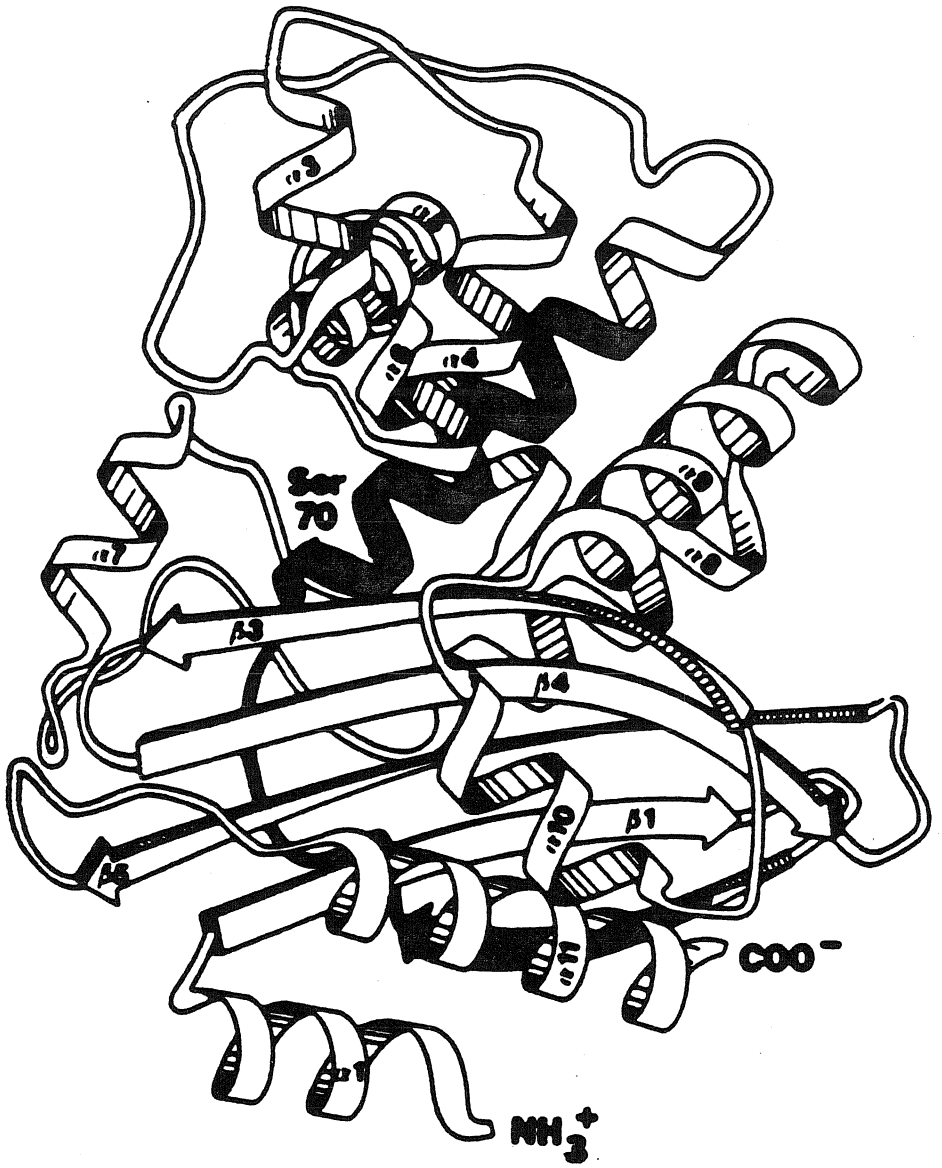



**Figure 11** - Twenty-eight amino acids were exchanged in RTEM-1 ß-lactamase with the corresponding sequence from PBP-5 of *E. coli*. The changes are shown here in the shaded regions of the α-carbon ribbon diagram of the PC1 ß-lactamase from *Staphylococcus aureus*, a closely related enzyme.

the plasmid encodes the gene for kanamycin resistance which was used as a selectable marker.

*E. coli* strain D1210 cells containing the plasmid encoding the chimera do not exhibit a resistant phenotype to penam antibiotics above the level conferred by a chromosomally encoded ß-lactamase (6 μg/ml in solution, 10 μg/ml on agar plates). The chimeric protein was isolated from the periplasm of the cells (grown in the presence of 50 μg/ml of kanamycin) by osmotic extrusion (2). The crude protein was then purified by anion exchange followed by gel filtration chromatography (9). Attempts to purify the chimera by FPLC were unsuccessful. Purity was confirmed by analysis on 12% SDS-PAGE. Protein concentration for kinetic analyses were estimated at 281 nm using the ß-lactamase extinction coefficient of 29,400 $M^{-1}cm^{-1}$ (2). Due to the instability of the chimeric protein, the entire process of protein isolation and purification was performed at 4°C (9).

Preliminary assessment of the activity of the purified chimera toward benzyl penicillin (30°C, pH 7) revealed an induction period of about 30 minutes. This phenomenon was also reported for the $E^+$ chimera (9). If the hydrolysis was then allowed to proceed to complete conversion, newly added substrate was directly hydrolyzed without such an induction period. Also, whereas incubation of the chimeric protein at 37°C for 4 hours results in complete loss of activity, the chimera retains full activity when similarly incubated in the presence of ampicillin. These results infer that the chimera is not properly folded *in vivo* and the ß-lactam antibiotics may function as a folding template. Thus it appears that the initially inactive chimeric protein undergoes a slow conformational change to an active enzyme upon binding substrate. In addition, the original $E^+$ chimera was isolated in a greater yield when low concentrations of ampicillin were

present in the preparative buffers (9). These combined results further suggest that the presence of a ß-lactam may stabilize a properly folded conformation of the chimera and thereby discourage its proteolytic degradation in the periplasm. Western blots (Figure 12) were performed to examine the effects of the ß-lactams on the *in vivo* stability of the chimera. Cells grown in the presence of sublethal levels of ampicillin show enhanced levels of chimera present in the periplasm. Thus the conformational change stabilizes the protein in addition to enhancing activity. All purifications of the chimera were performed with excess ampicillin (40 µg/ml) in all buffers in order to exploit these stabilizing effects.

Kinetic parameters (Table 2) for both ß-lactamase and carboxypeptidase activities were determined for the chimera. All kinetic determinations were performed on enzyme that had been pre-equilibrated with ampicillin to assist folding of the protein into the active conformation. With benzyl penicillin as substrate, the turnover rate is sharply reduced relative to that for the RTEM-1 ß-lactamase [~$10^{-5}$] (42,43) and somewhat increased relative to that for PBP-5 [~$10^{2}$] (5). It is important to note that the product of this hydrolysis was penicilloic acid and not phenylacetylglycine. D-Ala-D-Ala carboxypeptidase activity was assessed using diacetyl-L-lysyl-D-alanyl-D-alanine (38) and the depsipeptide analogue diacetyl-L-lysyl-D-alanyl-D-lactate (39) as substrates. Whereas RTEM ß-lactamase does not measurably catalyze the hydrolysis of either substrate (Figure 13), even with >50 µg of enzyme, the chimeric enzyme has acquired a measurable activity and shows about 1% the activity ($k_{cat}/K_M$) of the wild-type PBP-5 enzyme toward the acylated tripeptide and 0.4% toward the acylated depsipeptide. In terms of free energies of activation, this places the

**Figure 12** - Western blots of wild-type and chimeric enzymes. Lane A shows the amount of chimera present when cells were grown in the presence of sublethal levels of ampicillin; lane B shows the amount of chimera present at 37°C; and lane C shows the expression of wild-type ß-lactamase at 37°C. The increase in the intensity of the chimera band in the presence of ampicillin reflects the stabilizing effect of the antibiotic on the chimera.

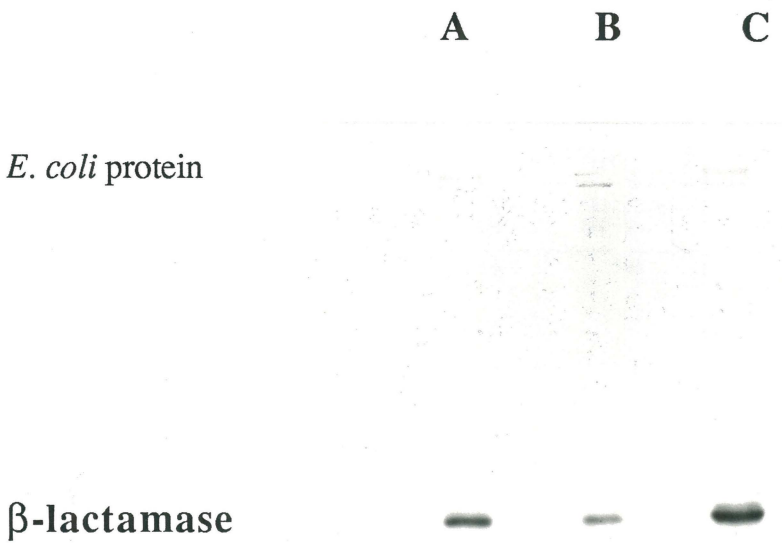

A
B
C
E. coli protein
β-lactamase

Table 2. Kinetic parameters for wild-type PBP-5 from *E. coli*, wild-type RTEM-1 β-lactamase, and the RTEM/PBP-5 chimeric enzyme.

| | diAc-Lys-D-Ala-D-Ala[d] | | | diAc-Lys-D-Ala-D-Lac[d] | | | Benzyl Penicilline | | |
|---|---|---|---|---|---|---|---|---|---|
| | Km (mM) | kcat ($sec^{-1}$) | kcat/Km ($M^{-1}sec^{-1}$) | Km (mM) | kcat ($sec^{-1}$) | kcat/Km ($M^{-1}sec^{-1}$) | Km (mM) | kcat ($sec^{-1}$) | kcat/Km ($M^{-1}sec^{-1}$) |
| PBP-5[a] | 19.2 | 0.29 | 15 | 2.4 | 1.1 | 260 | ND | $10^{-4}$–$10^{-3}$ | ND |
| RTEM-1[b] | – | – | – | – | – | – | 0.02 | 2000 | $1 \times 10^8$ |
| CHIMERA[c] | 7.7 ± 1.3 | (1.2 ± 0.17) x $10^{-3}$ | 0.16 | 3.3 ± 1 | (3.7 ± 1.5) x $10^{-3}$ | 1.1 | 0.26 ± 0.03 | (5.1 ± 0.5) x $10^{-2}$ | 196 |
| CHIMERA ($E^+$)[f] | ND | $5.8 \times 10^{-5}$ | ND | ND | ND | ND | ND | 1.3 | ND |

ND not determined
a Kinetic parameters for PBP-5 were taken from Ref. 5. Activity with benzyl penicillin is an estimate based on comparisons between PBPs involving the rates of deacylation (42, 43).
b The lack of observable activity is designated as –.
c Kinetic parameters were calculated from initial velocities.
d Assay conditions: 37°C, pH 7.5.
e Assay conditions: 30°C, pH 7.0.
f Specific activities determined from single reaction curves; $E^+$ = extra glutamate.

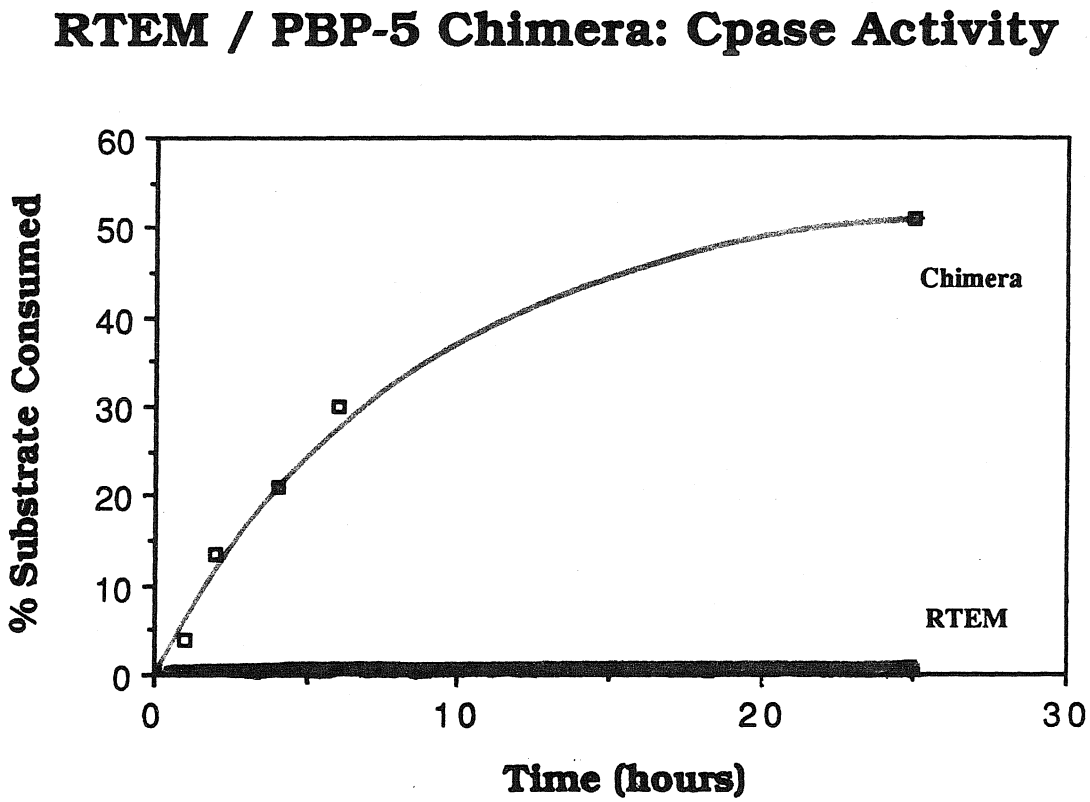



**Figure 13** - Twenty-four hour reaction profiles for the PBP-5/RTEM-1 chimera and the wild-type ß-lactamase utilizing diacetyl-L-Lys-D-Ala-D-Ala as a substrate. The RTEM enzyme shows no measurable activity toward the acyclic peptide substrate.

stabilization by the chimera of the transition state for hydrolysis of D-Ala-D-Ala dipeptides within about 2.7 kcal/mole, and for hydrolysis of the depsipeptide within 3.3 kcal/mole, of that achieved by PBP-5 itself. In both cases, the hydrolysis products are those produced by PBP-5. The non-enzymatic hydrolysis of a peptide bond in aqueous solution at neutral pH is on the order of $3 \times 10^{-9}$ sec-1 (45), thus the rate of the reaction catalyzed by the chimera is about $10^9$ that for uncatalyzed amide hydrolysis at pH 7.

Like the chimera with the additional glutamic acid, the corrected chimera exhibits a pH profile (Figure 14) for carboxypeptidase activity similar to that which we observe for the parent RTEM-1 ß-lactamase (32) on penam substrates and very different from that of the PBP-5 wild-type enzyme (46). Neither chimera displays any buffer dependency, whereas PBP-5 itself shows a marked decrease in activity in carbonate and phosphate buffers.

As explained in Chapter 1, the PBPs' primary function in the bacterial cell wall is the catalysis of the transpeptidation reaction by transferring the carboxyl group of the cleaved amide to an amine such as glycine (1,46). The RTEM-1/PBP-5 chimera does not display any such activity; no transpeptidase transfer to glycine as a potential acceptor was observed even at 100 mM glycine. This lack of transpeptidase activity was originally believed to reflect the absence in the ß-lactamase/PBP-5 chimera (29 kDa) of an α-helix likely to be present in PBP-5 (42 kDa); this additional helix (G), seen at low resolution in the R61-carboxypeptidase (Figure 5) and therefore inferred in the closely homologous PBP-5, is proximal to one end of the enzymatic cleft which contains the binding site for ß-lactam substrate. It had been presumed that this helix could contain a structural component, such as an amino acid or peptide binding site, necessary for

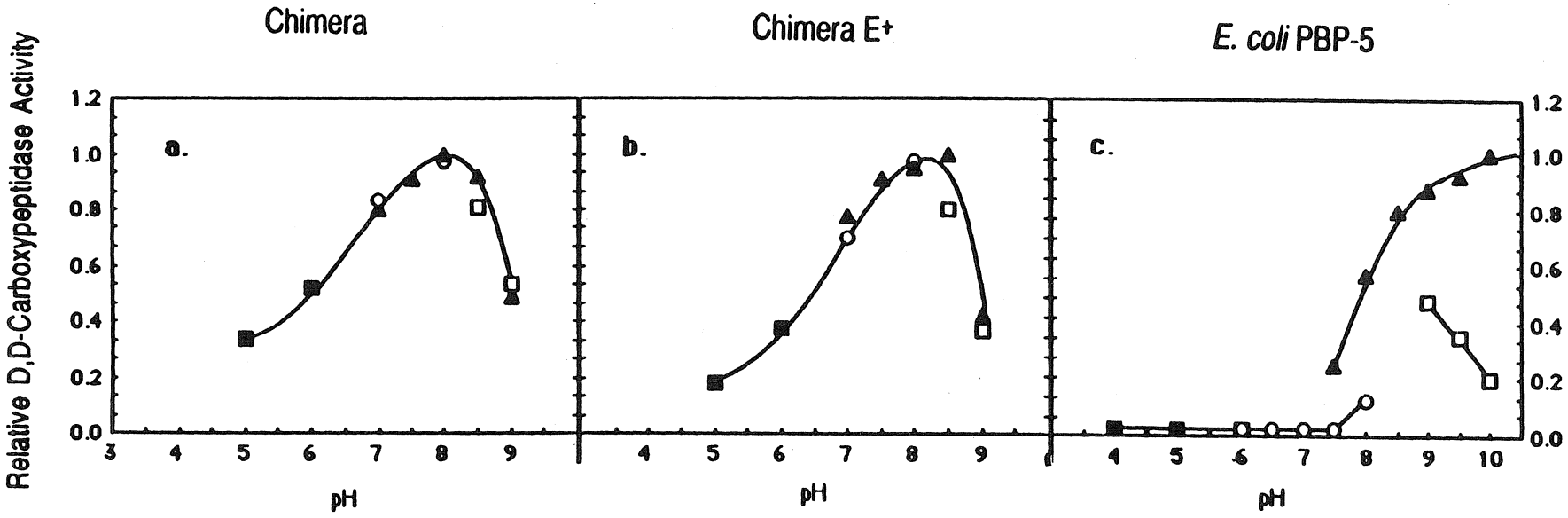



**Figure 14** - pH dependency of the D,D-carboxypeptidase activities of the PBP-5/RTEM-1 chimeras and wild-type PBP-5 (46). ■ - Potassium Acetate; o - Potassium Phosphate; ▲ - Tris - HCl; □ - Potassium Carbonate.

transpeptidase activity (6). No less important, this helix would also help to exclude water from the active site thereby encouraging transpeptidase as distinct from carboxypeptidase activity. However, while the possible importance of this helix should not be ignored, subsequent research detailed in Chapter 3 has shown that transpeptidase activity is possible without this helix.

It is important to address, especially in cases such as this where the enzymatic activities are exceedingly low, the possibility of contamination from the wild-type PBPs in chimera protein preparations. As previously stated, wild-type ß-lactamase exhibits no carboxypeptidase activities. There are several results which would refute the possibility that the D,D-carboxypeptidase activities observed are derived from contamination with PBP-5 including: (i) Proteins purified by the procedures for isolating ß-lactamase and its mutants from *E. coli* containing derivatives of pBR322 have observable D,D-carboxypeptidase activity only when the chimera is encoded in the plasmids. (ii) The two chimeras, both the original (9) and the one described herein, have markedly different levels of activities from each other and both have pH dependencies very different from those reported for PBP-5. (iii) Neither chimera catalyzes a transpeptidase reaction with glycine whereas PBP-5 does. It is therefore unlikely that the observed activites are from a contaminating source.

## CONCLUSION

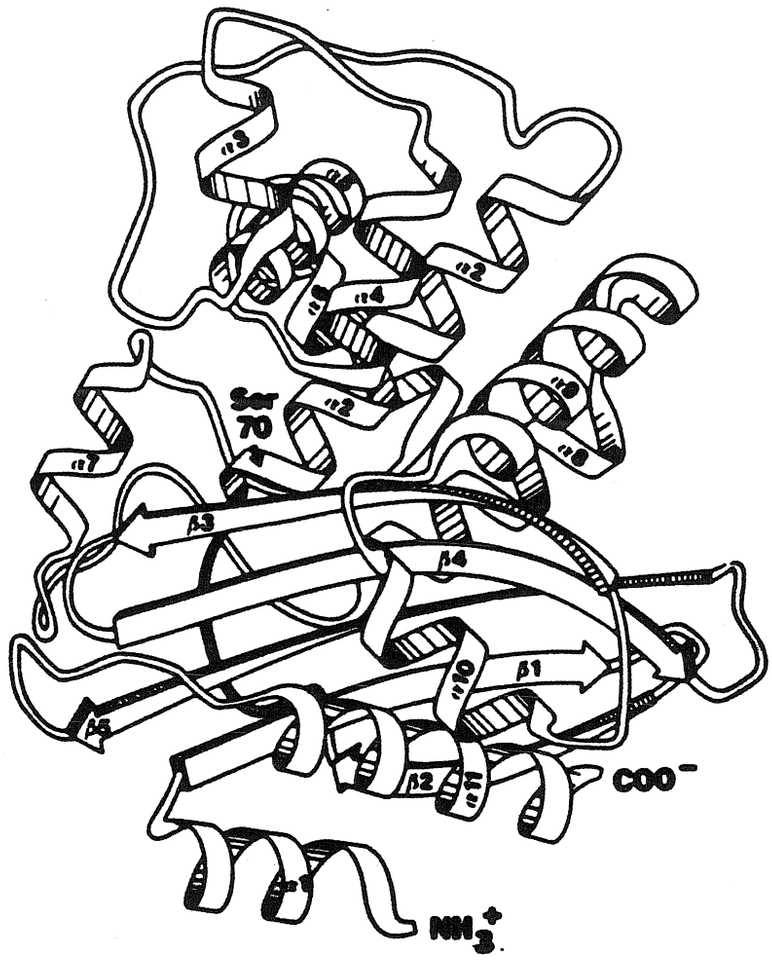
The development of the PBP-5/RTEM-1 chimeras has been a significant step in protein engineering. By producing unique restriction sites in the ß-lactamase gene by efficient oligonucleotide-directed mutagenesis, we were able to transpose a 28 amino acid segment from PBP-5 of *E. coli* into RTEM-1 ß-lactamase by cassette mutagenesis. Other work in the area of chimeric proteins has produced viable mutants with altered catalytic activity. Successful chimeras have been produced primarily by domain-swapping in systems such as phosphoglycerate kinase (47) and the aspartate and ornithine transcarbamoylases (48). However, no research to date has been reported on mutagenesis replacing a large **internalized** amino acid sequence as described here.

The construction and analysis of the PBP-5/RTEM-1 chimeras has been a very important starting point for future work investigating the evolutionary relationship of these two enzymes. We are presently unable to predict which specific residues in the chimera are responsible for the altered catalytic properties and whether catalysis results from changes in a few particular interactions between enzyme and potential substrate or from larger, more general conformational changes. Nor can it be determined, at this juncture, whether the effect observed is one of specifically changed substrate specificity or rather one of reduced specificity, therefore resulting in a promiscuous enzyme.

Ongoing work is underway to investigate which residues are involved in the altered catalysis. A chimera which replaced only those residues incorporated in the α-helix containing the active-site serine (49) exhibited no measurable carboxypeptidase activity. The extreme instability of this chimera made characterization difficult. I have proposed that the random

coil, preceeding the α-helix in linear sequence, may be responsible for the alignment of the helix in the proper geometry necessary for catalysis. I have designed a chimera (Figure 15), which is designed to replace residues 64-69 in RTEM-1 ß-lactamase with those residues present in PBP-5. Initial research (50) has yielded a chimeric enzyme which shows a temperature sensitivity similar to the previous chimera and no phenotypic resistance to ß-lactam antibiotics.

These results have been essential for the design of the chimeric mutants to be described in Chapter 3. While these findings do not establish the evolutionary relationship between the ß-lactamases and the D,D-carboxypeptidases, they provide some proof that ß-lactamases contain the elements of a dormant carboxypeptidase activity. Accordingly, the results of this work to date can be regarded as a significant first step toward the goal of designing proteins with altered and potentially novel catalytic activities.




| | |
|---|---|
| RTEM | D L N S G K i L e s f r p e e R f p m m S t f K v l l c g |
| PBP5 | D L N S G K v L a e q n a d v R r d p a S e t K m m t s y |
| Chimera 1 | D L N S G K i L e s f r p e **v R r d p a** S t f K v l l c g |

**Figure 15** - Amino acid sequence exchanged in the PBP-5/RTEM-1 loop chimera. The mutation replaces the residues in the random coil leading to the α-helix containing the active serine; by changing the orientation of the α-helix, the proper conformation for carboxypeptidase activity could be achieved.

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# Chapter 3

## Creating a Chimeric Substrate Binding Cavity: Construction of a Series of RTEM-1 ß-Lactamase / R61 Carboxypeptidase Chimeras

## INTRODUCTION

The class A ß-lactamases show tremendous similarities to a large group of enzymes collectively known as penicillin binding proteins (PBPs). These PBPs function as D-Ala-D-Ala-carboxypeptidases, cleaving the terminal alanine from one D-Ala-D-Ala dipeptide and transferring the N-terminal remainder to another polypeptide in a transpeptidase reaction (1). This crosslinking stabilizes the bacterial cell wall and maintains cell viability. These enzymes are also capable of targeting the ß-lactam antibiotics as substrates. However, nucleophilic attack by these enzymes on the lactam carbonyl group results in a stable serine-ester-linked acyl-enzyme complex (2). Thus the enzymes are inactivated and are unable to crosslink the bacterial cell wall. In contrast, the ß-lactamases show no measurable activity towards the D-Ala-D-Ala dipeptide, but are capable of a near diffusion controlled hydrolysis (~$10^8$ $M^{-1}$ $sec^{-1}$) (3) of the ß-lactam antibiotics. It has been proposed on the basis of structure (4,5), sequence (6,7), and substrate (8) similarity that the ß-lactamases have evolved from the PBPs.

To examine the possible evolutionary relationship between the PBPs and the class A ß-lactamases, a chimeric enzyme was designed and constructed (Chapter 2). The project was undertaken at a time when high resolution crystal data, from either of the two classes of enzymes, was unavailable. The available low resolution crystal structures (4,9) were only capable of discerning secondary structure, therefore the residues were selected for replacement by sequence homology centered around the active-site serine. In this original chimera, PBP-5 of *E. coli* was chosen as the carboxypeptidase donor for two basic reasons. First, PBP-5 contains the highest amino acid homology (27%) in the active site compared with the

class A ß-lactamases (7, 10, 11). Secondly, interaction of PBP-5 with penam antibiotics yields the corresponding penicilloic acid; a stable compound with no antibacterial properties. This is in contrast to the majority of PBPs which enzymatically degrade benzyl penicillin to phenylacetylglycine (1, 12, 13). These two factors combined to infer the possibility of the closer evolutionary relationship and thus a logical choice for initial attempts at chimera synthesis.

The resulting chimera was successful in catalysing the hydrolysis of the D-Ala-D-Ala dipeptide with approximately 1% the activity of the wild-type PBP-5. This seemingly modest activity is substantial since the parent RTEM-1 ß-lactamase exhibits no detectable carboxypeptidase activity. No less important was the fact that a semi-stable and viable chimera was generated, capable of utilizing both carboxypeptidase and lactamase substrates. However, while these results did not prove that the class A ß-lactamases evolved from the PBPs, they did provide evidence that the catalytic machinery necessary for carboxypeptidase activity is present in the ß-lactamases. The low level of carboxypeptidase activity in the chimera and the inability to catalyze transpeptidation raised the question of whether substrate specificity of the chimera had been adapted to recognize an explicit set of previously unusable substrates, or whether the substrate specificity had merely been reduced, generating a promiscuous enzyme.

Two high resolution crystal structures for class A ß-lactamases (14, 15) have been published since the design and construction of the PBP-5/RTEM-1 chimera which reveal that the structural changes made in the chimera encompass the α-helix containing the active-site serine, the ß2-strand of the five stranded antiparallel ß-sheet, and the random coil

connecting the two structures. Precisely which amino acid changes were responsible for the altered activity was not immediately evident.

With the insight gained from the PBP-5/RTEM-1 chimera and newly published crystal structures, I designed and constructed a series of chimeric enzymes involving RTEM-1 ß-lactamase and the R61 carboxypeptidase of *Streptomyces* (1, 16). The R61 carboxypeptidase was chosen as the carboxypeptidase donor because both crystal coordinates and extensive kinetic data are available. An additional factor which influenced this choice was that R61 has a greatly enhanced turnover rate (1) of the synthetic substrate diacetyl-L-Lys-D-Ala-D-Ala, compared to the other PBP's (3300 $min^{-1}$ for R61 vs. 174 $min^{-1}$ for PBP-5). Unlike PBP-5 of *E. coli*, the R61 enzyme is not capable of simple ß-lactam hydrolysis to the corresponding penicilloic acid, but rather fragments benzyl penicillin into phenylacetyl glycine and N-formyl-D-penicillamine (12, 13) (Figure 1). Whereas it was initially advantageous to use PBP-5 as the carboxypeptidase donor because it was capable of penicilloic acid production, use of the R61 enzyme could possibly alter the ß-lactamase catalysis in the chimera to yield the fragmented product. Isolation of this product would be additional evidence of a truly altered catalysis pathway and proof that activity had been incorporated into the chimera from the carboxypeptidase enzyme.

The mutations in the PBP-5/RTEM-1 chimera were concentrated around the active-site serine. While these residues are undoubtedly important, the goal of the R61 project has been to create a chimeric binding cavity by mutation of residues that are not adjacent to the active serine in the linear amino acid sequence in addition to the mutations in the α-helix. As in the original chimera, these additional mutations would involve replacement of a particular amino acid with that present in R61, as

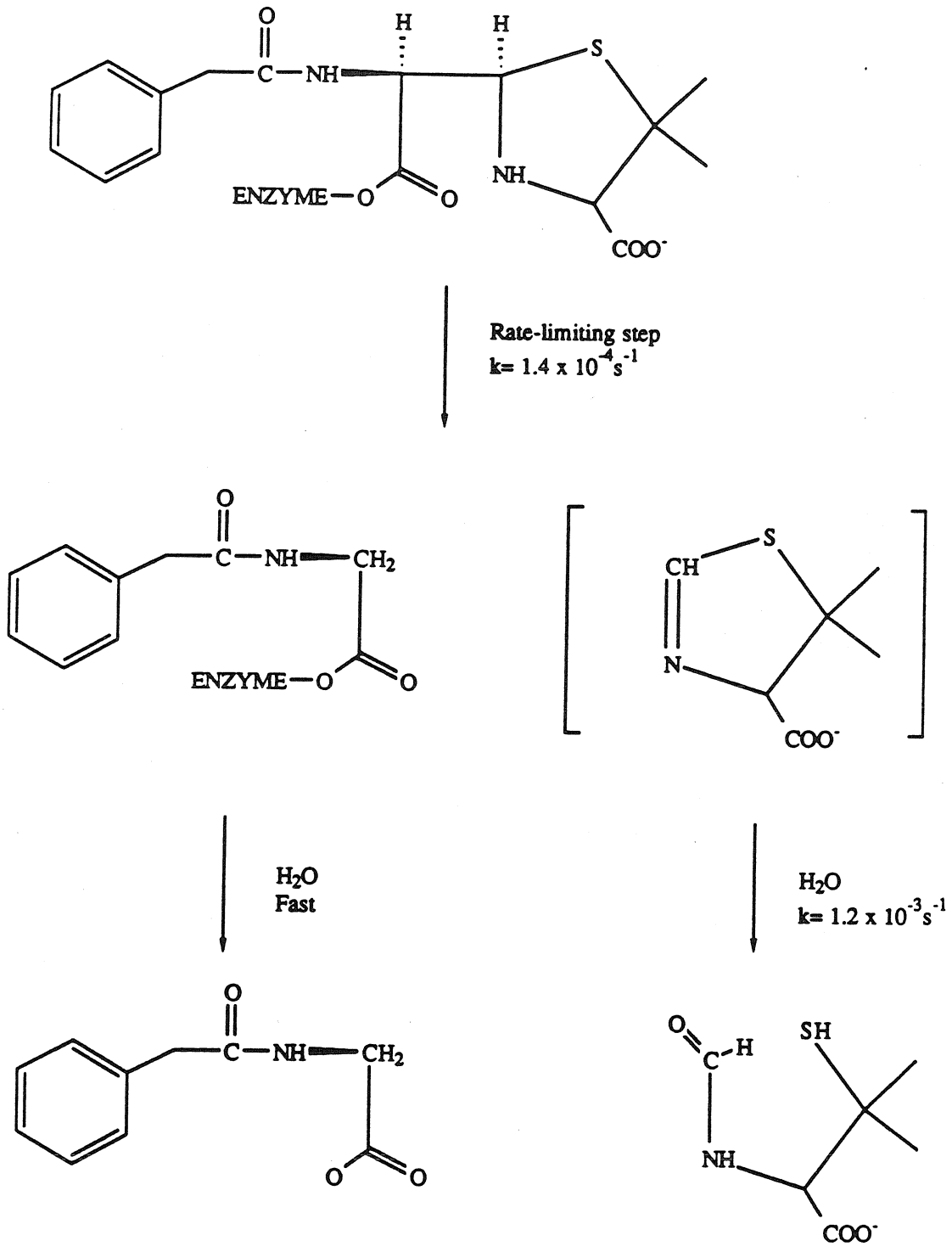



**Figure 1** - Degradation of benzyl penicillin by the R61 carboxypeptidase involves fragmentation of the penam's $C_5$-$C_6$ bond, followed by hydrolysis (or aminolysis) of the phenylacetylglycyl intermediate.

determined by sequence alignments (6) (Figure 2). Residues were chosen for mutagenesis on the basis of their: *i*) known (or suspected) role in catalysis, *ii*) location and orientation within the ß-lactamase cavity, and *iii*) homology to those residues present in the R61 carboxypeptidase/trans-peptidase.

To generate the chimeric binding cavity, four general sites within the RTEM-1 cavity were chosen for mutation (Figure 4). A synthetic scheme was designed in which a convergent stepwise synthesis would allow for the examination of the physical and catalytic effects of each mutation, both individually and in combination with each other. As with the PBP-5 chimera, the α-helix containing the active serine was targeted for cassette mutagenesis. However, unlike the PBP-5 chimera, the exchange of residues was designed to be limited primarily to those residues which would be exposed to solvent (Figure 5). This was intended to minimize disruption of the hydrophobic packing and maintain intrinsic protein stability. The three remaining regions are: *i*) Glu 166, *ii*) Lys 234 and Ser 235, and *iii*) Asp 131 and Asn 132 (Ambler convention of ß-lactamase numbering (17)).

Glutamate 166 was chosen because of this residue's known involvement in deacylation of the acyl-enzyme intermediate formed with ß-lactam antibiotics (18). The corresponding residue in R61 is an aspartic acid. While this is a conservative mutation, it has been shown that the E166D mutant has a greatly reduced deacylation rate--presumably due to the decrease in chain length (~1Å). Four out of five class A ß-lactamases contain a glutamate at this position, as compared to six out of seven PBPs which contain an aspartate (Figure 2, Box V).

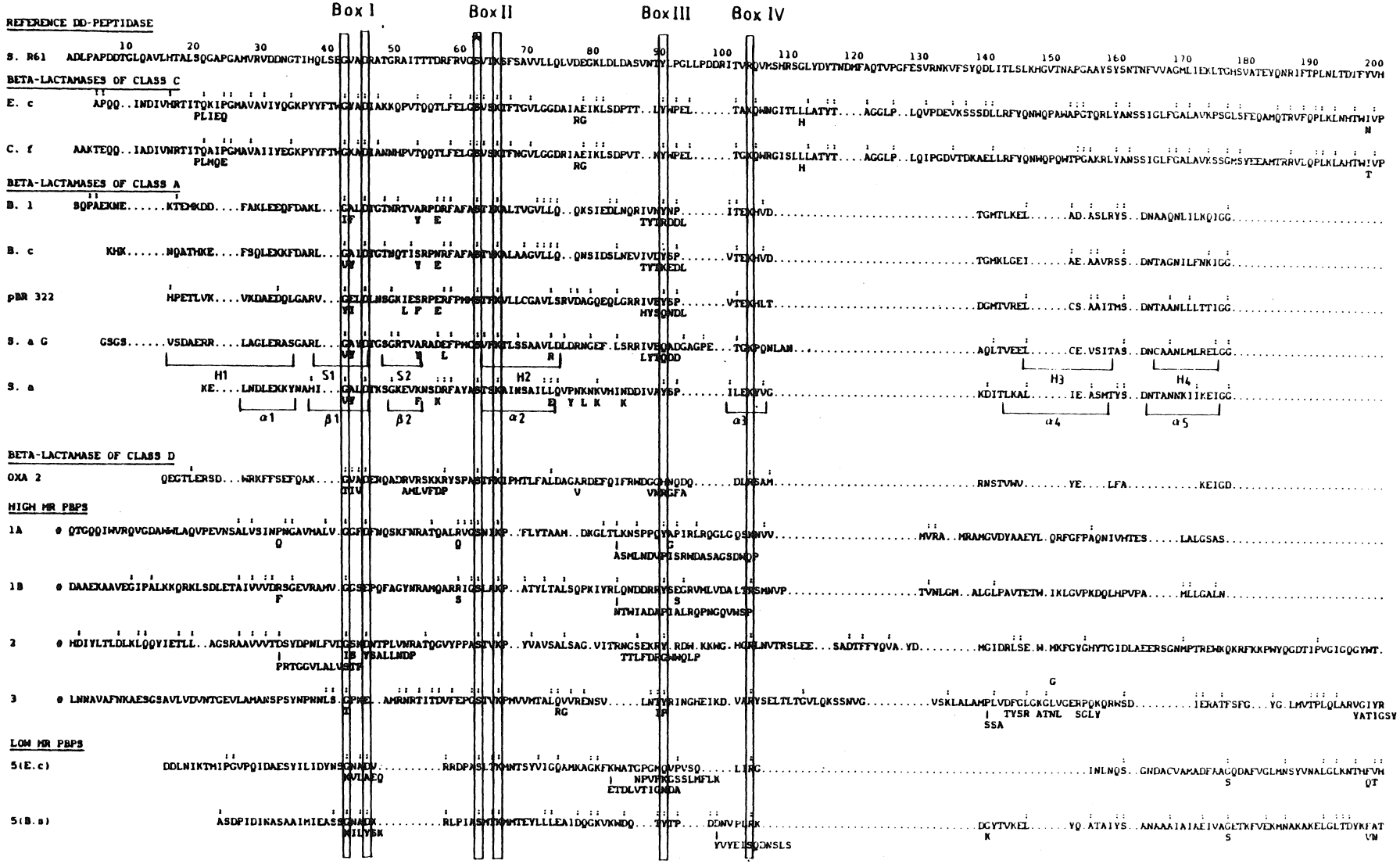



**Figure 2** - Proposed alignments of the amino acid sequences of the ß-lactamases and the penicillin-binding proteins. Streptomyces R61 D,D-carboxypeptidase is the reference sequence (6).

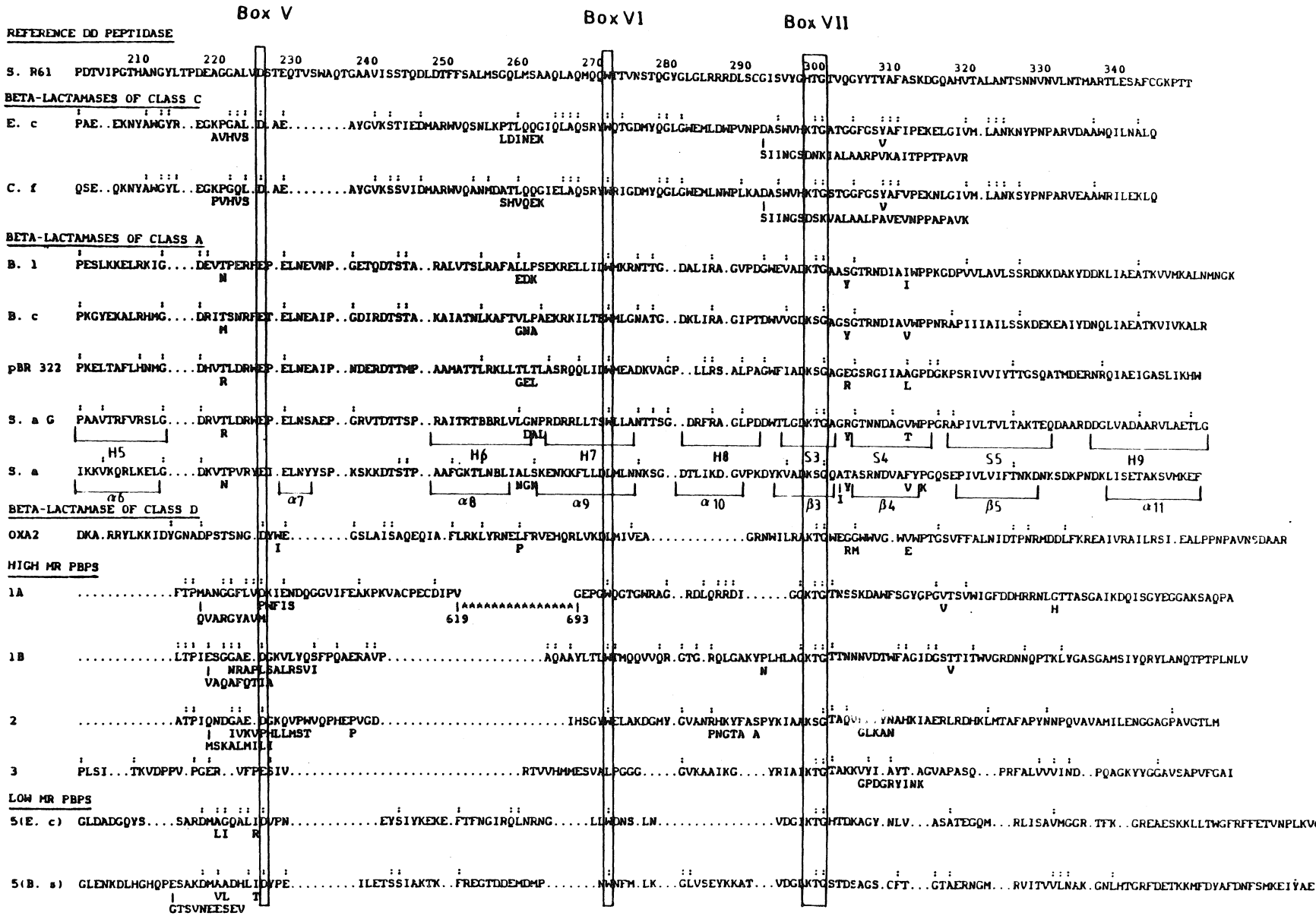



**Figure 2** - continued from page 74.

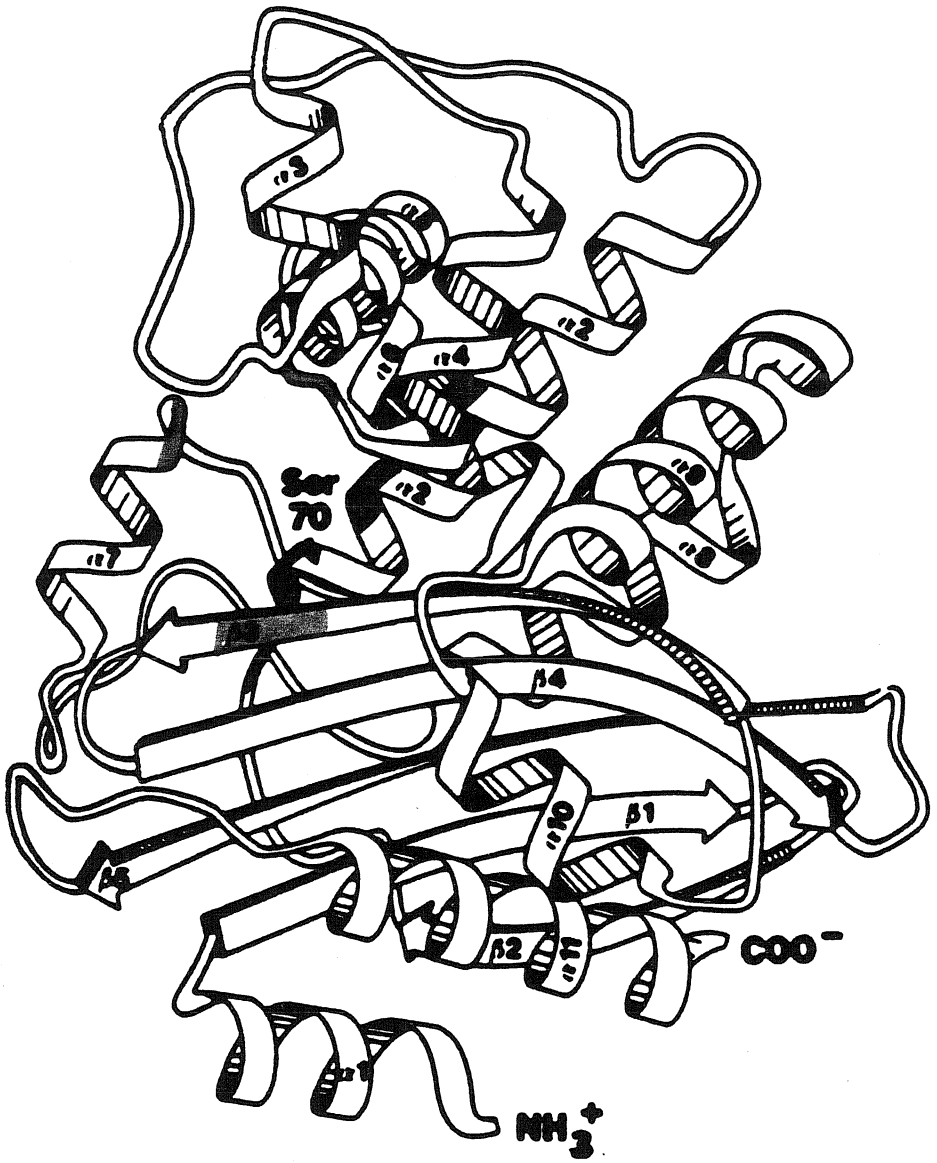



**Figure 3** - Four regions of the ß-lactamase binding cavity were selected to be mutated: i) the α-helix containing the active serine (P67R, M68V, M69G, F72I, V74S, L76S), ii) Asp 131 & Asn 132, iii) Glu 166, and iv) Lys 234 & Ser 235.

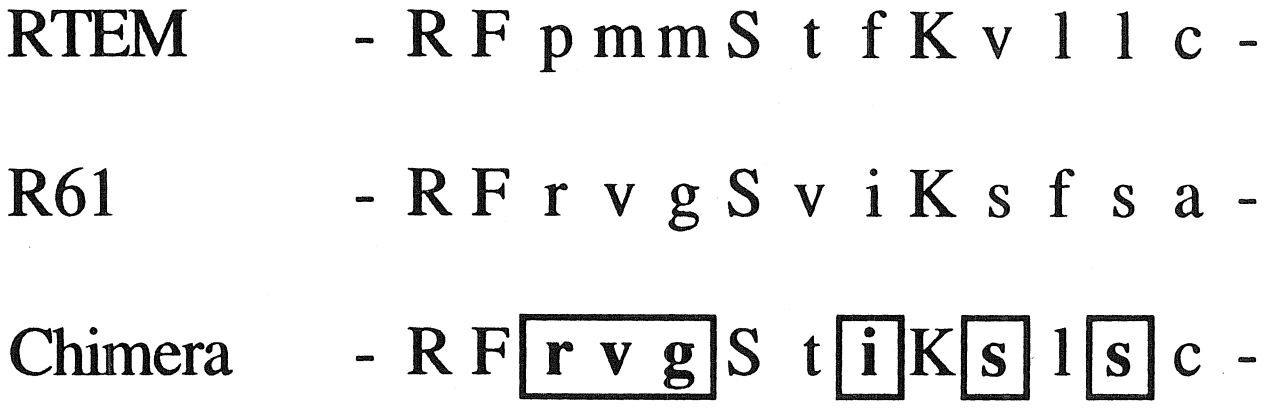





**Figure 4** - The sequences of RTEM ß-lactamase, R61 carboxypeptidase and the resulting R61.1 chimera. Residues on the α-helix containing the active serine were chosen for mutagenesis because of their access to solvent or catalytic potential.

Lysine 234 and serine 235 were chosen because of their involvement in a semi-conserved triad (Figure 2, Box VII) which is present in all class A and class C ß-lactamases (Lys-Ser/Thr-Gly) in addition to all PBP's (His/Lys-Thr/Ser-Gly) (6). Site saturation at residue 234 (19) has revealed that this residue is involved with substrate binding ($K_M$) while having little or no effect on catalysis ($k_{cat}$). The role of residue 235 has never been investigated. Directly following this triad in linear sequence, residue 237 has already been shown to be involved in altering ß-lactamase substrate specificity (20). It is believed that the amide nitrogen of residue 237 participates in the stabilization of the oxyanion intermediate, in a motif similar to that observed in the serine proteases (21, 22).

The roles, either structurally or catalytically, of residues 131 and 132 have never been investigated. These sites follow a serine (Ser 130 in ß-lactamase, Ser 160 in R61) conserved in all class A ß-lactamases and low molecular weight PBPs (6). Two factors were dominant in the decision to mutate the 131-132 dyad. First, the side chain from residue 132 (Asn in RTEM-1, Phe in R61) is juxtaposed in the folded structure to the side chain from Glu 166. The tremendous disparity in hydrophobic character of asparagine and phenylalanine side chains could have an enormous effect on the ability of Glu 166 to access the water necessary to hydrolyse the acyl-enzyme intermediate. Second, while the side-chain of residue 131 (Asp in RTEM-1, Asn in R61) is not exposed to the solvent accessible cavity but projected into the hydrophobic core, superimposition of the α-carbon traces of R61 (9) and the PC1 ß-lactamase (14) from *Staphylococcus aureus* (a closely related enzyme) shows nearly identical placement of residues 130, 131, and 132 (160, 163, and 164 in R61) despite the insertion of two additional residues in R61 between the serine and aspartate residues. For

completeness, the single mutation at residue 132 (N132F) would also be made should it be determined that residue 131 was solely involved in protein stability.

In total, ten chimeras were constructed. The synthesis and partial characterization of the chimeric enzymes are described. One set of mutations, Asp 131 Asn and Asn 132 Phe, result in chimeras with altered catalytic properties.

## MATERIALS AND METHODS

### Enzymes and Chemicals

Restriction enzyme Mlu I was purchased from New England Biolabs (NEBL). All other restriction enzymes, T4 DNA ligase, and T4 polynucleotide kinase were purchased from Boehringer Mannheim Biochemicals (BMB). Kanamycin sulfate was purchased from BMB; all other antibiotics were supplied by Sigma Chemical Company. [$\alpha$-$^{35}$S]-dATP, [$^{14}$C]-benzyl penicillin, and [$^{3}$H]-glycine were supplied by Amersham. [$^{35}$S]-Benzyl penicillin was obtained from Dupont/New England Nuclear. Isopropyl-β-D-thiogalactoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal), 10x ligase buffer, and molecular biology grade agarose were purchased from International Biotechnologies, Inc. (IBI). Phenol and chloroform were purchased from Fisher Scientific, Inc. Q-Sepharose and Sephacryl-HR100 chromatography media were purchased from Pharmacia; Sephadex G-25 was purchased from Sigma Chemical Company. Low melting-point agarose was purchased from FMC Bioproducts.

### Bacterial Strains

The pJN plasmids (23), which were utilized as expression vectors, were harbored in *E. coli* strain D1210. The D1210 strain contains the kanamycin resistance factor and a *lac* repressor (lac i$^{Q}$) necessary for the control of the *tac* promotor (24) present in pJN. Bacteriophage were propagated in *E. coli* strain TG1 obtained from Amersham. *E. coli* strain XL1-Blue (25) were purchased from Stratagene. This strain has a tetracycline resistance maintained on the F-plasmid and a *rec A*$^{-}$ genotype (26) which greatly reduces the level of homologous recombination; this

deters gene elimination from the M13 vectors. Culture medium was L broth (10 g tryptone, 5 g yeast extract, 5 g NaCl in 1 liter; add 15 g bacto-agar to make L plates) for plasmid preparations and 2 x YT broth (16 g tryptone, 10 g yeast extract, 5 g NaCl in 1 liter; add 15 g bacto-agar to make 2xYT plates) for bacteriophage preparations.

Transformation and Plating

Cells were made competent for transformation of plasmid DNA using a process adapted from Hanahan (27). A 250 ml Erlenmyer flask containing 25 ml of SOB media (20 g tryptone, 5 g yeast extract, 0.6 g NaCl, 0.2 g KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ in 1 liter) was inoculated with *E. coli* and incubated with shaking at 37°C to $OD_{550}$~0.3. The cells were then centrifuged at 3,000 rpm for 5 minutes at 4°C; the 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 $CaCl_2 \cdot H_2O$, 150g glycerol, 30 ml of 1M KOAc, pH 7.5 in 1 liter. Adjust to pH 5.8 with 0.2 M acetic acid.). The cells were then incubated on ice for 15 minutes followed by centrifugation (3,000 rpm for 5 minutes). The resulting pellet was resuspended in 4 ml transformation buffer 2 (1.2 g RbCl, 11 g $CaCl_2 \cdot H_2O$, 150 g glycerol, 20 ml of 0.5 M MOPS, pH 6.8 in 1 liter). Cells were transformed within two hours of preparation or frozen in liquid nitrogen and stored at -70°C. The frozen cells could then be used by thawing on ice for 15 minutes. Comparable efficiencies were observed with frozen cells used within 2 months.

DNA was added to 300 µl of competent cells in a 1.5 ml eppendorf tube with gentle mixing. These were left on ice for 40 minutes, followed by heat-shock at 42°C for 90 seconds. For plasmid DNA, 800 µl SOC media (20 g tryptone, 5 g yeast extract, 0.6 g NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 10

mM glucose in 1 liter) was added and the tubes were incubated for 1 hour at 37 °C and then plated onto L-agar containing the appropriate antibiotic. For single-stranded DNA, the heat-shocked sample was added to 200 µl log phase *E. coli* TG1 cells 3 ml of melted (45°C) top agar (10g bactotryptone, 8g NaCl, 8g bacto agar in 1 liter), mixed by inverting gently 3-4 times, and poured onto preheated (37°C) H-plates (10g bactotryptone, 8g NaCl, 10g bacto agar in 1 liter). Plates were left at room temperature for 10-15 minutes before inverting.

DNA

All DNA concentrations were estimated from absorbance at 260 nm and purity estimated by $A_{260}/A_{280}$. Ethanol precipitation of DNA was accomplished by adding 3 M sodium acetate (0.1 volume), absolute ethanol (2.5 volumes) and precipitating on dry ice for 15 minutes. The sample was centrifuged (14,000 rpm for 15 minutes) at room temperature. The resulting pellet was then washed with 100 µl of 70% ethanol, and dried *in vacuo*. The DNA is then resuspended in TE.

Wild-type plasmid pBR322 and bacteriophage M13 mp19 replicative form (RF) DNA were purchased from Bethesda Research Laboratories (BRL). Mutant plasmids and RF phage were purified from *E. coli* by the alkaline lysis method (28). Mini preps (2 ml) were performed by growing the cultures to saturation. The cells were pelleted and resuspended in Solution I (100 µl ; 50 mM glucose, 25 mM Tris (pH 8.0), 10 mM EDTA, 1 mg/ml lysozyme) and allowed to react for five minutes at room temperature. Solution II was added (200 µl ; 0.2 N NaOH, 1 % SDS) followed by incubation on ice for five minutes. The pH was neutralized with cold (4°C) Solution III (150 µl ; 60 ml 5M KOAc, 11.5 ml glacial AcOH in 100 ml), and the solution

was centrifuged to remove the precipitated cellular debris. A single phenol/chloroform extraction (100 µl each) was performed followed by ethanol precipitation. This DNA was then suitable for sequencing or reaction with restriction endonucleases. Large scale preparations were further purified using ultracentrifugation in cesium chloride (0.95g/ml) / ethidium bromide (75µl/ml) gradients (single spin: 20 hours, 45,000 rpm) (29) to remove the large amounts of contaminating RNA and protein. Single-stranded phage DNA was prepared from phage supernatant by precipitation with 20% polyethylene glycol-6000 / 2.5 M NaCl (30) followed by phenol/chloroform extraction and ethanol precipitation.

Synthetic oligonucleotides (0.2 µmol) were synthesized by the Caltech Microchemical Facility using phosphoramidite chemistry (31) on an Applied Biosystems automated DNA synthesizer, Model 380A. Oligonucleotides (1/3 of synthesis) were resuspended in 25 µl TE and 25 µl Maxam-Gilbert (32) loading buffer (80% (w/v) deionized formamide, 50 mM Tris-borate (pH 8.3), 1 mM EDTA (pH 8.0), 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue.), and purified by preparative polyacrylamide gel electrophoresis (15-20%, 0.1 x 20 x 40 cm gel; 500 volts; 12 hours) in TBE buffer (10.8g Tris base, 5.5g boric acid, 4 ml 0.5M EDTA, pH 8.0, in 1 liter.). DNA was visualized with UV light reflected from a fluorescent indicator coated silica TLC plate. The appropriate bands were excised, the gel crushed, and then suspended in 1 ml of 2 M NaCl overnight at 37°C. The samples were desalted using two sequential G-25 Sephadex spin columns (10 minutes @ 2,500 rpm).

Restriction Digests

Restriction digests typically were performed on 5 μg plasmid DNA with 5-20 units of restriction enzyme, and 3 μl 10X digest buffer (as suggested by the supplier) in 30 μl at 37°C for 1-2 hours. DNA restriction fragments were separated on 1.2% agarose gels in TAE buffer (4.8g Tris base, 1.14 ml glacial acetic acid, 2.0 ml 0.5 M EDTA, pH 8.0, in 1 liter). The agarose gels contained 0.005% ethidium bromide (v/v), which allowed for fragment visualization by UV irradiation. DNA was isolated from low melting-point 1.2% agarose gels (Seakem). The excised gel fragment was melted by incubation at 65°C for 15 minutes in a 1.5 ml eppendorf tube. An equal volume of cold (4°C) TE-equilibrated phenol was added and the mixture was incubated on ice for 30 minutes. The reaction tube was centrifuged (5 minutes @ 14,000 rpm) followed by chloroform extraction of the supernatant. The DNA was ethanol precipitated as a final purification.

Kinasing and Annealing Synthetic Oligonucleotides

The purified oligonucleotides (100 pmol each) were added to 3 μl 1M Tris (8.0), 1 μl 100mM DTT, 3 μl 10mM ATP/100mM $MgCl_2$, and 2-5 units of T4 polynucleotide kinase in a total volume of 30 μl. The reaction was incubated for 30 minutes at 37°C, followed by 10 minutes at 65°C to inactivate the enzyme.

Complementary oligonucleotides (100 pmol each) were annealed with 10 μl 10x medium salt buffer (10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT) in a total volume of 100 μl, by heating to 95°C and allowing the temperature to cool to room temperature slowly. It is best to use a larger vessel (2-4 liters) for the annealing so that the temperature drop is gradual. No further purification was necessary for ligation.

Oligonucleotide-Directed Mutagenesis - **pJN-XMS**

Oligonucleotide-directed mutagenesis was performed on wild-type pJN to generate the necessary restriction sites for construction of the R61/RTEM-1 chimeras. The Eco RI/Sal I 3712 base-pair fragment from wild-type pJN plasmid was subcloned into Eco RI/Sal I digested M13 mp18 RF DNA. The fragments (10 pmol each) were ligated with using 2 units T4 DNA ligase, 5 µl 10x IBI ligase buffer, in a total volume of 50 µl. The reaction was incubated at 25°C for 2.5 hours. Competent *E. coli* TG1 cells were transformed with aliquots of 1 and 5 µl of the ligation mixture. The transformed cells were plated on top agar H plates with X-gal and IPTG. Clear plaques on a lawn of TG1 represented successful clones containing the pJN Eco RI/Sal I insert; blue plaques indicated background wild-type M13 mp18 phage.

Three restriction sites (Xho I @ 5032, Mlu I @ 4955, & Sac I @ 4898 in pJN) were designed to be produced by silent mutations (Figure 5) using the Amersham *in vitro* mutagenesis kit (30), which employs the Eckstein selection (33) method. All concentrations and volumes were those specified by the manufacturer unless otherwise noted. Single-stranded phage (5 pmol) was isolated and annealed to an equimolar mixture of the three kinased mutagenic primers (8.25 pmol). Chain extension from this oligonucleotide was accomplished with the Klenow fragment of DNA polymerase I (6 units), T4 DNA ligase (6 units), and a mixture of dATP, dGTP, dTTP, and αS-dCTP as supplied. The reaction was incubated overnight at 15°C. The reaction mixture was filtered through a double nitrocellulose disk filter (Schleicher & Schuell) to remove the remaining single-stranded DNA. Double-stranded DNA was recovered from the filtrate by ethanol precipitation. The heteroduplex DNA was subsequently

Xho I @ 5032 5' - AAAACT**CTCGAG**GATCTTAC - 3'

Mlu I @ 4955 5' - GGCGTCAAC**GCGTGA**TAATACC - 3'

Sac I @ 4898 5' - TGAGTACTCAAC**GAGCTC**ATTCTGAGAATA - 3'

**Figure 5** - Synthetic oligonucleotides used to produce the Xho I, Mlu I, and Sac I restriction sites by silent mutation. The recognition site is boxed and the mismatched bases shown in italics.

digested with the restriction endonuclease Nci I (16 units) for 90 minutes at 37°C. Due to the the incorporation of αS-dCTP in the synthetic strand, only the native strand is cleaved resulting in nicked duplex DNA. Exonuclease III (50 units; 25 minutes at 37°C) was then employed to excise the native DNA in the region of the mismatch. It is important to limit the time of reaction with Exo III to that stated in order to maintain some native-strand DNA necessary for synthesis priming. Repolymerization was accomplished by incubating the partially digested heteroduplex with all dNTP's, DNA polymerase I (6 units), and T4 DNA ligase (6 units), yielding double-stranded DNA in which both strands carried the correct mutated sequence. Competent *E. coli* TG1 cells were transformed with the final DNA solution (5 & 20 μl). RF DNA was prepared from the growth of 14 plaques and screened by restriction analysis. Out of 14, 4 were positive for the generation of all 3 desired mutations. One of these was then subcloned into pJN using Eco RI and Sal I, producing pJN-XMS (Figure 6). The entire ß-lactamase gene was then sequenced to insure the absence of secondary mutations.

Cassette Mutagenesis to Produce the R61/RTEM-1 Active-Site Helix Chimera (P67R, M68V, M69G, F72I, V74S, L76S) - **R61.1**

A synthetic cassette (34, 35) was designed to replace selected amino acids in RTEM-1 ß-lactamase with those residues present in the R61 carboxypeptidase of *Streptomyces*. The complementary synthetic oligonucleotides (Figure 7) for the cassette (100 pmol each) were kinased and annealed as above. A two-fragment ligation was then carried out, as outlined in Figure 8. Annealed oligonucleotides (0.4 pmol) were combined with pJN-XMS vector that had been over-digested with Xho I and Mlu I

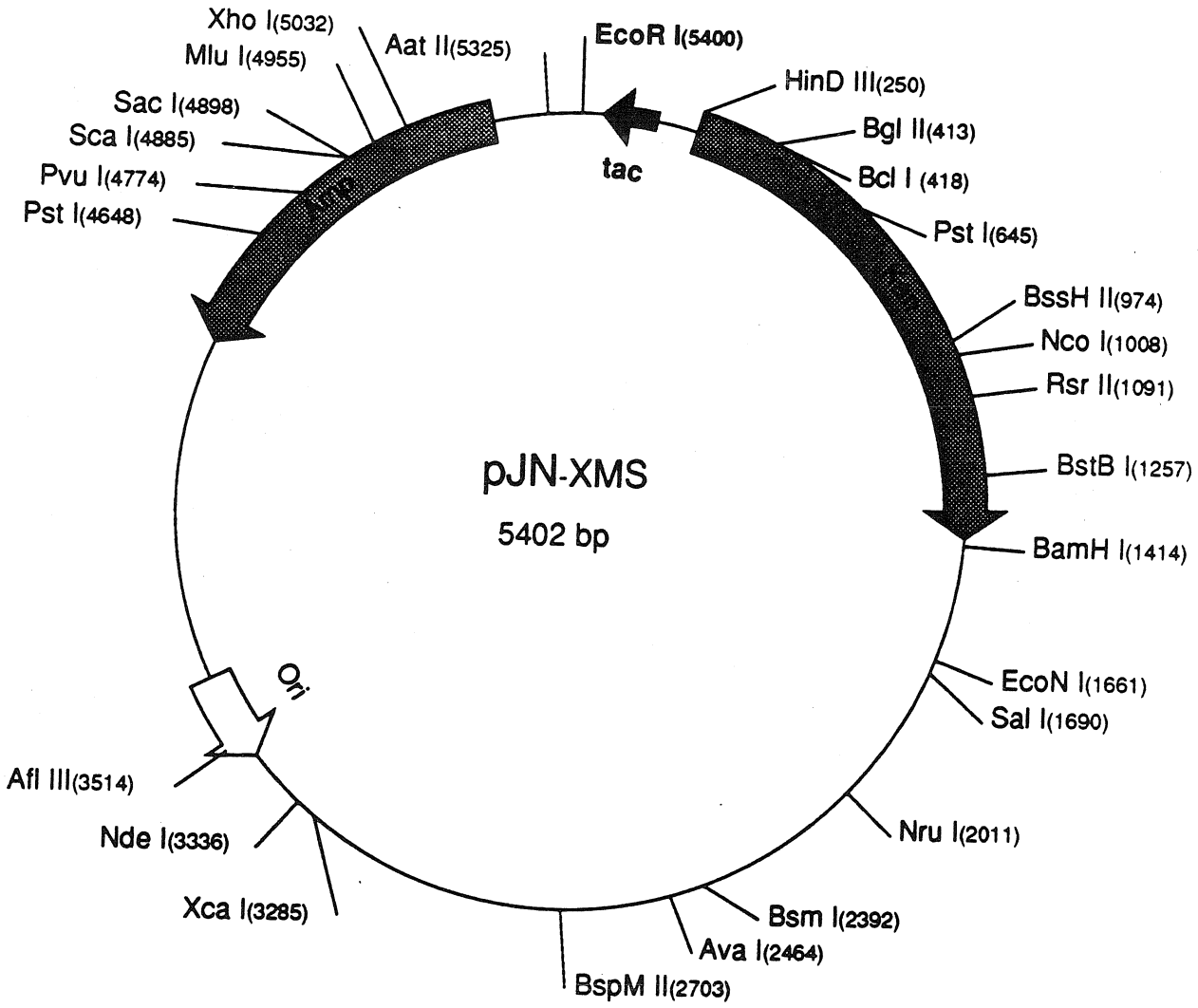



**Figure 6** - Partial restriction map of pJN-XMS.

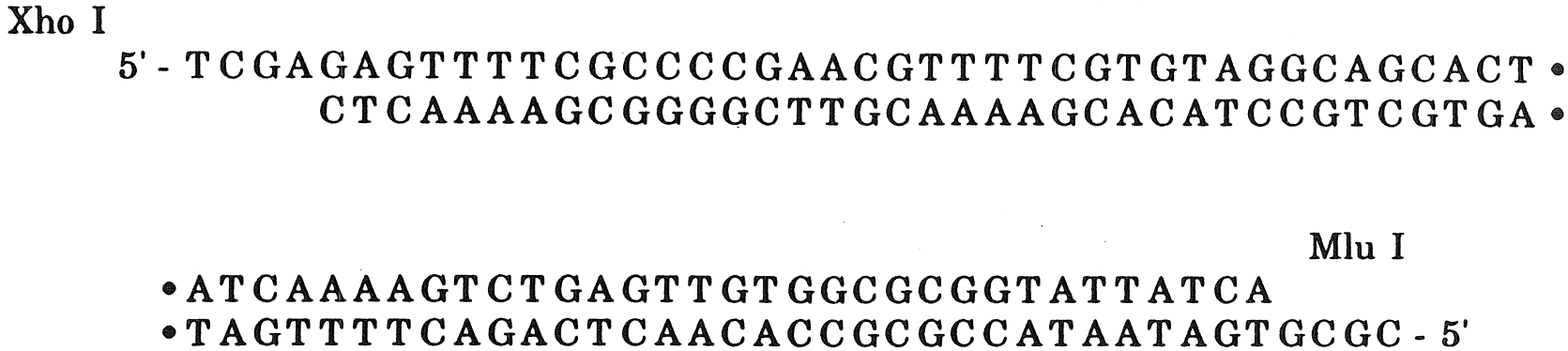



**Figure 7** - Synthetic oligonucleotide cassette used to produce the R61.1 chimera (P67R, M68V, M69G, F72I, V74S, L76S).

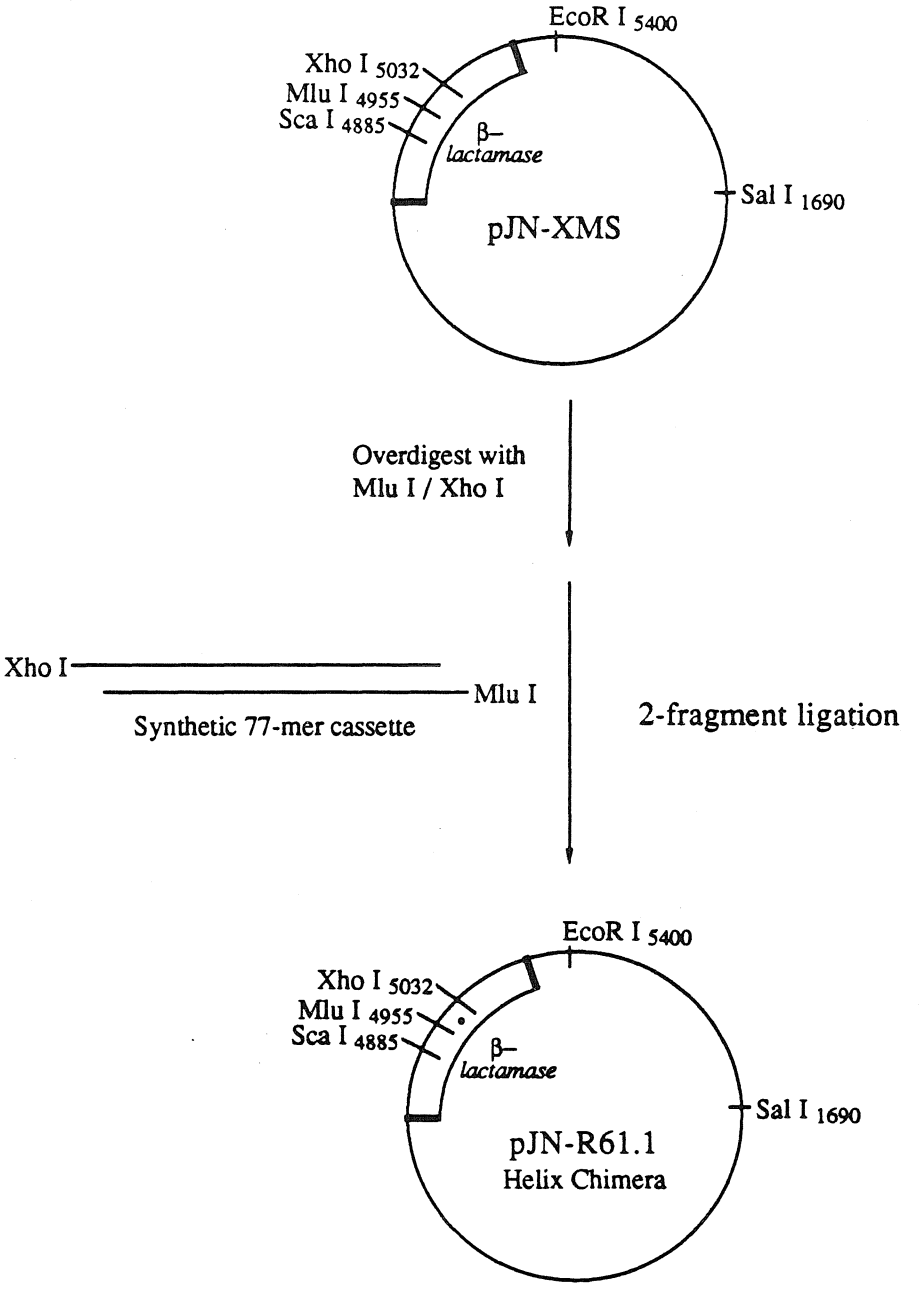



**Figure 8** - Three-fragment ligation scheme for the synthesis of the R61.1 chimera (P67R, M68V, M69G, F72I, V74S, L76S).

restriction endonucleases. The DNA was incubated with 1 unit T4 DNA ligase and 2.5 µl IBI 10x ligation buffer in a total volume of 25 µl for 12-14 hours at 15°C followed by transformation of competent *E. coli* D1210 with aliquots of 1-5 µl of the ligation mixtures. The cells were plated onto kanamycin sulfate (50 mg/l) L-agar plates and incubated overnight at 37°C. The resulting colonies were picked onto kanamycin sulfate (50 mg/l) and ampicillin (100 mg/l) master plates. Sixty percent of the colonies were unable to grow in the presence of ampicillin; six out of six sequences from these colonies were verified to be the desired ligation product.

Glu 166 Asp - **R61.A**

Oligonucleotide-directed mutagenesis was performed on the pJN-XMS /M13 mp18 single stranded DNA. A single base change was designed to convert the glutamate at residue 166 to an aspartate. Mutagenesis was attempted using the modified Amersham protocol as detailed above. *E. coli* strain XL1B were used instead of strain TG1. Single-stranded phage (5 pmol) was annealed with the kinased mutagenic 21-mer 5'-CAGCTCCGGATCCCAACGATC-3' (8.25 pmol, mismatch underlined). Eight plaques were chosen for sequence analysis. None of the colonies yielded a readable sequence possibly due to the loss of the primer site by spontaneous deletion. Due to persistent deletion problems, the gene for the E166D mutation was obtained from frozen samples prepared by W.J. Healey (18).

Lys 234 His, Ser 235 Thr - **R61.B**

Oligonucleotide-directed mutagenesis was performed on the pJN-XMS /M13 mp18 single stranded DNA. A single synthetic oligonucleotide

incorporating three base changes was designed to convert the lysine at residue 234 to a histidine and the serine at residue 235 to a threonine. Mutagenesis was accomplished using the modified Amersham protocol as detailed above. *E. coli* strain XL1B were used instead of strain TG1. Single-stranded phage (5 pmol) was annealed with the kinased mutagenic 30-mer 5'-CTCACCGGCGCCAGTGTGATCAGCAATAAA-3' (8.25 pmol, mismatches underlined). Eight plaques were chosen for sequence analysis; seven out of eight were positive for the desired mutation. The Eco RI / Sal I fragment containing the mutation was subsequently subcloned back into pJN, producing pJN-R61.B.

Asp 131 Asn, Asn 132 Phe - **R61.C**

Oligonucleotide-directed mutagenesis was performed on the pJN-XMS /M13 mp18 single stranded DNA. A single synthetic oligonucleotide incorporating four base changes was designed to convert the aspartate at residue 131 to an asparagine and the asparagine at residue 132 to a phenylalanine. Mutagenesis was accomplished using the modified Amersham protocol as detailed above. *E. coli* strain XL1B were used instead of strain TG1. Several attempts to perform this mutagenesis were unsuccessful. Due to a presumably unfavorable DNA conformation, it was necessary to alter the oligonucleotide annealing conditions; single-stranded phage (5 pmol) was annealed with the kinased mutagenic 39-mer 5'-AAGT AAGTTGGCCGCAGTGAAGTTACTCATGGTTATGGC-3' (8.25 pmol, mismatches underlined) by incubating the mixture at 90°C for 15 minutes followed by 37°C for 30 minutes. Six plaques were chosen for sequence analysis; five out of six were positive for the desired mutation. The Eco RI /

Sal I fragment containing the mutation was subsequently subcloned back into pJN, producing pJN-R61.C.

Asn 132 Phe - **R61.D**

Cassette mutagenesis was employed to generate the single residue mutation at residue 132. A new pJN vector (pJN-Sax) was developed by T. Richmond of this laboratory (36), which contains Sac I (@ 4787) and Sac II (@ 4751) restriction sites in the RTEM-1 ß-lactamase gene. The complimentary synthetic oligonucleotides (100 pmol each) were kinased and annealed as above. A two-fragment ligation was then carried out as outlined in Figure 9. Annealed oligonucleotides (0.4 pmol) were combined with pJN-XMS (5 µg) vector that had been overdigested with a 20-fold excess of Sac I and Sac II restriction endonucleases. The ligation conditions were as described above. Competent *E. coli* D1210 cells were transformed with the ligation mixture and plated onto kanamycin sulfate (50 mg/l) L agar plates. The resulting colonies were picked onto kanamycin sulfate (50 mg/l) and ampicillin (100 mg/l) master plates. Thirty percent of the colonies were unable to grow in the presence of ampicillin; One out of six sequences from these colonies was verified to be the desired ligation product.

Chimeric Helix, Asp 131 Asn, Asn 132 Phe - **R61.2**

The R61.2 chimera was produced by the condensation ligation scheme shown in Figure 10. pJN-R61.1 (5 µg) was digested with restriction endonucleases Sca I and Sal I to yield a 2207 base pair fragment encompassing the R61.1 mutation. Subsequently, pJN-R61.C was subjected to restriction digestion with the same endonucleases to yield a 3195 base pair fragment containing the R61.C mutation. The isolated fragments (0.5

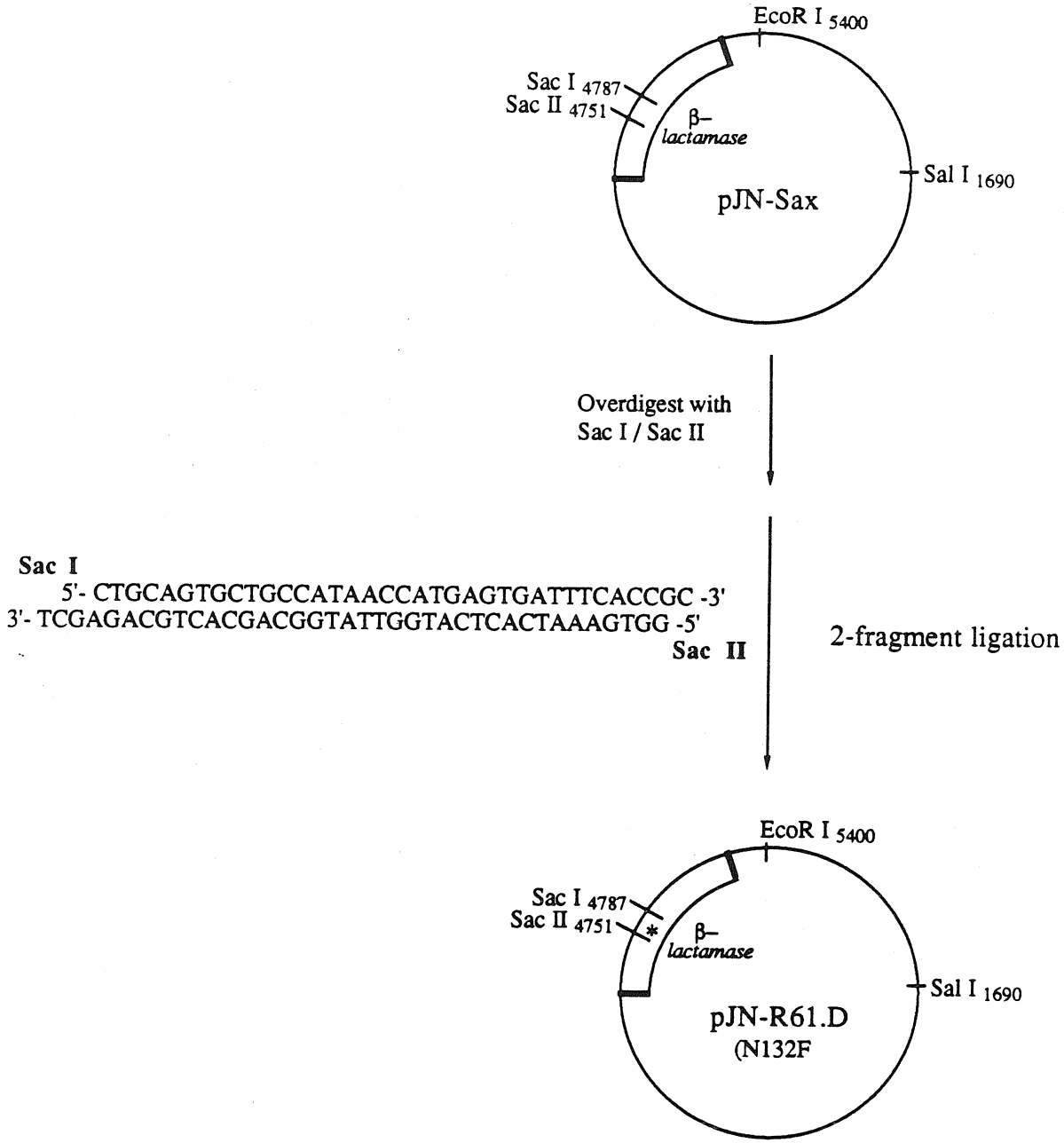



**Figure 9** - Two fragment ligation scheme employed to generate the R61.D (N132F) chimera. pJN-Sax was used because of its convenient Sac I and Sac II site.

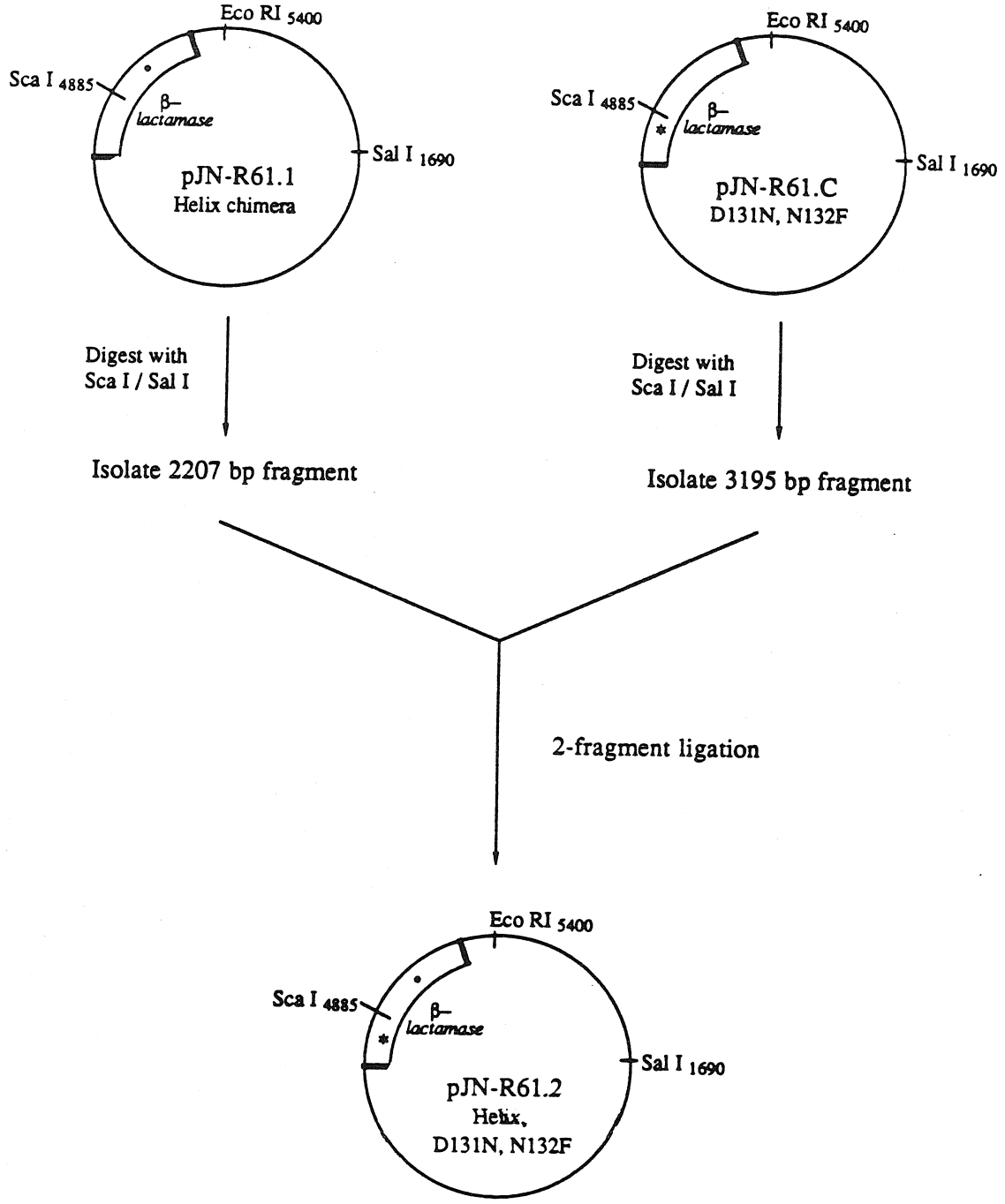



**Figure 10** - The cloning scheme used to combine the R61.1 (P67R, M68V, M69G, F72I, V74S, L76S) and R61.C (D131N, N132F) mutations and thereby generate the R61.2 chimera.

pmol each) were ligated as described above. Competent *E. coli* D1210 cells were transformed with the ligation mixture and plated onto kanamycin sulfate (50 mg/l) L-agar plates. Four colonies were chosen for sequence analysis; four out of four sequences were correct for both the Ser 70 and Asn 132 regions.

Glu 166 Asp, Lys 234 His, Ser 235 Thr - **R61.3**

The R61.3 chimera was produced by the three-fragment ligation (37) scheme depicted in Figure 11. Due to the presence of two Pst I restriction sites in the pJN vector, it was necessary to first digest pJN-R61.A (5 μg) with restriction endonuclease Hind III. This reaction was subdivided into two tubes, one digested with Sal I to yield a 1440 base pair fragment and the other digested with Pst I to yield a 1004 base pair fragment. pJN-R61.B (5 μg) was digested with restriction endonucleases Pst I and Sal I to yield a 2958 base pair fragment. The isolated fragments (0.5 pmol each) were ligated as described above. Competent *E. coli* D1210 cells were transformed with the ligation mixture and plated onto kanamycin sulfate (50 mg/l) L-agar plates. Eight colonies were chosen for plasmid preparation; four out of six sequences were correct for both the Glu 166 and Lys 234 regions.

Glu 166 Asp, Lys 234 His, Ser 235 Thr, Asp 131 Asn, Asn 132 Phe - **R61.4**

The R61.4 chimera was produced by the condensation ligation scheme shown in Figure 12. pJN-R61.C (5 μg) was digested with restriction endonucleases Pvu I and Sal I to yield a 2318 base pair fragment encompassing the R61.C mutation. Subsequently, pJN-R61.3 was subjected to restriction digestion with the same endonucleases to yield a

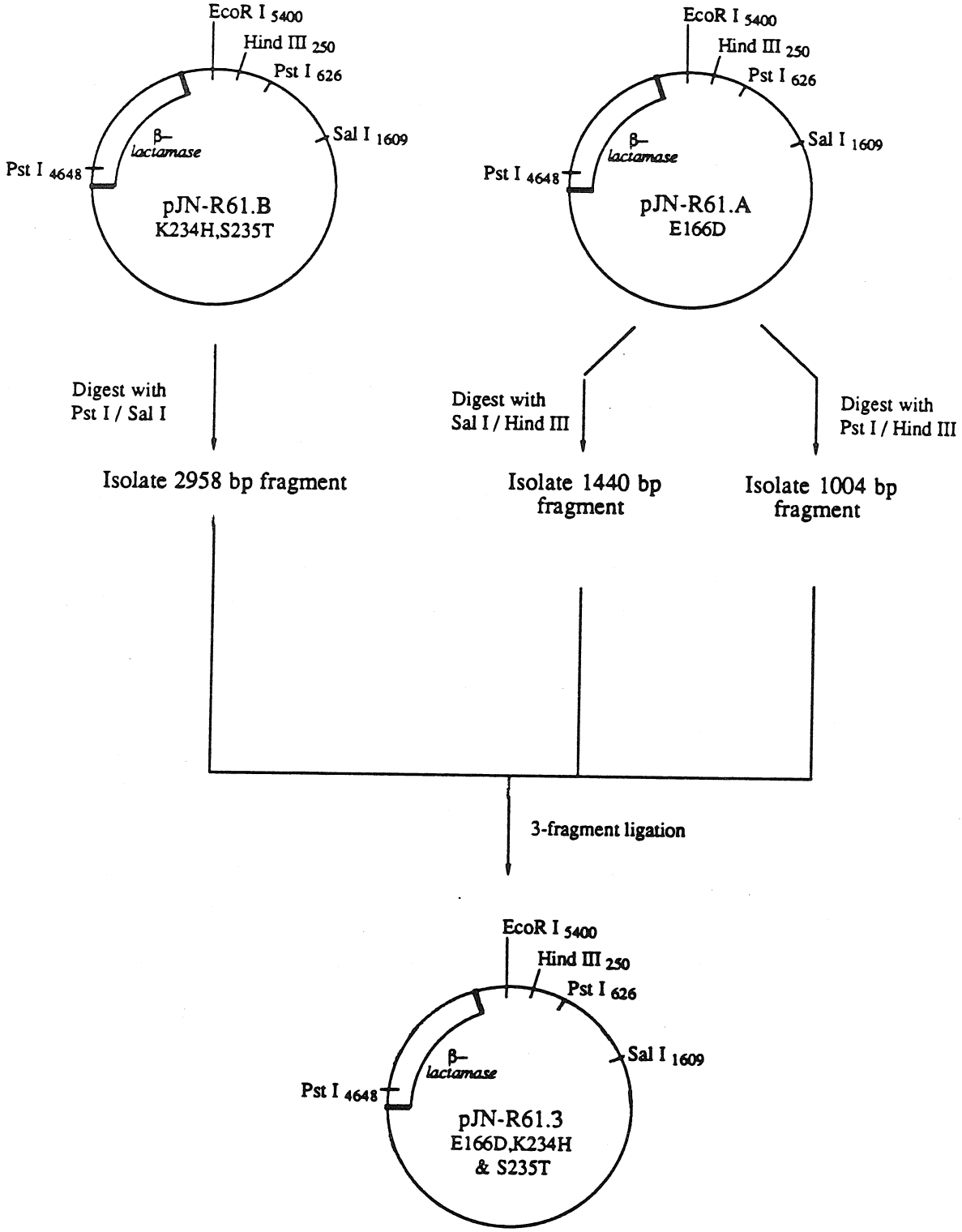



**Figure 11** - The cloning scheme used to combine the R61.A (E166D) and R61.B (K234H, S235T) mutations and thereby generate the R61.3 chimera. Due to the presence of two Pst I sites in pJN, it was necessary to perform the three-fragment ligation shown.

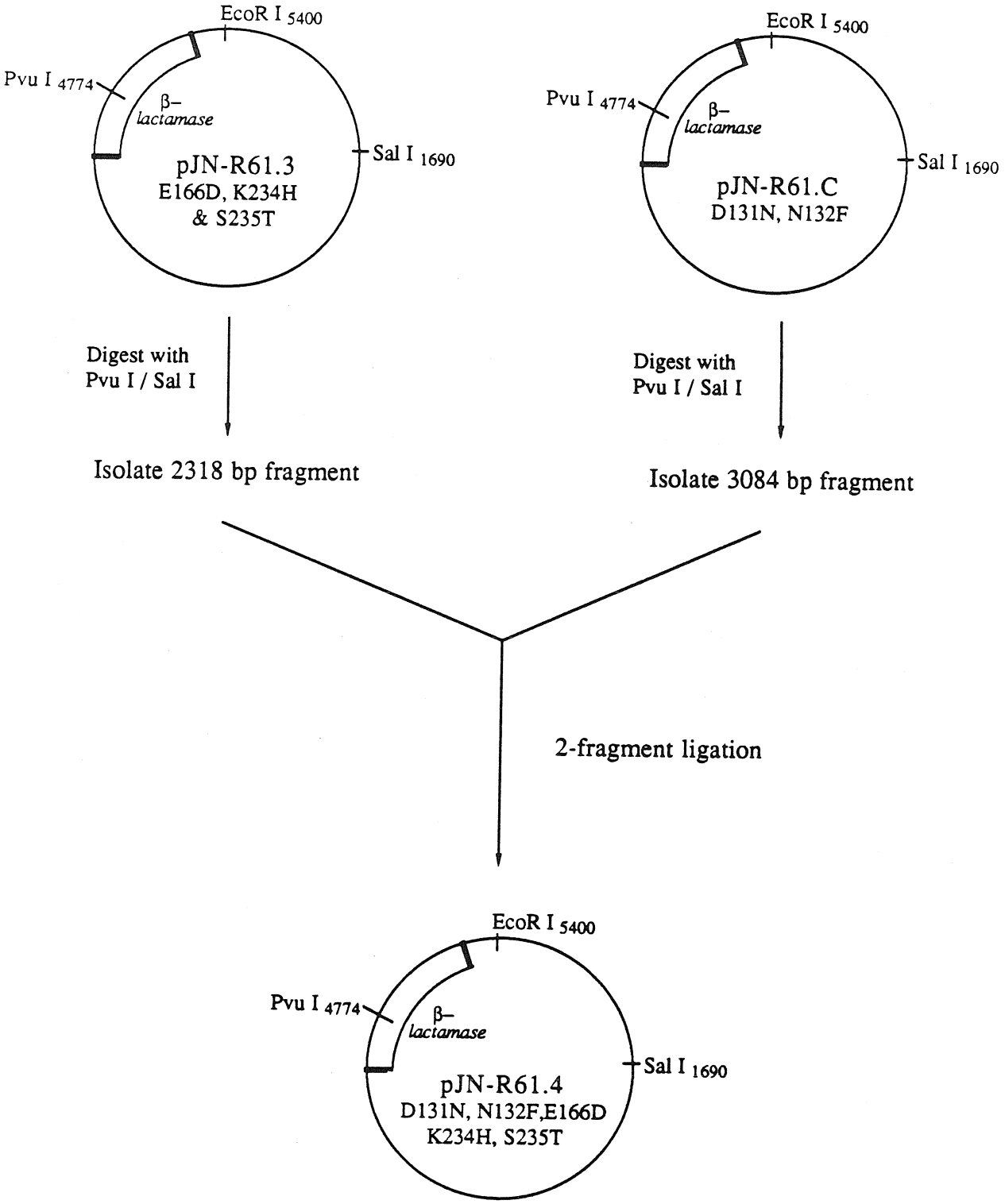



**Figure 12** - The cloning scheme used to combine the R61.C (D131N, N132F) and R61.3 (E166D, K234H, S235T) mutations and thereby generate the R61.4 chimera.

3084 base pair fragment containing the R61.3 mutation. The isolated fragments (0.5 pmol each) were ligated as described above. Competent *E. coli* D1210 cells were transformed with the ligation mixture and plated onto kanamycin sulfate (50 mg/l) L-agar plates. Six colonies were chosen for plasmid preparation; three out of three sequences were correct for all three mutated regions.

Chimeric Helix, Glu 166 Asp, Lys 234 His, Ser 235 Thr, Asp 131 Asn, Asn 132 Phe - **R61.5**

The R61.5 chimera was produced by the condensation ligation scheme shown in Figure 13. pJN-R61.2 (5 μg) was digested with restriction endonucleases Pvu I and Sal I to yield a 2318 base pair fragment encompassing the R61.1 and R61.C mutations. Subsequently, pJN-R61.3 was subjected to restriction digestion with the same endonucleases to yield a 3084 base pair fragment containing the R61.3 mutations. The isolated fragments (0.5 pmol each) were ligated as described above. Competent *E. coli* D1210 cells were transformed with the ligation mixture and plated onto kanamycin sulfate (50 mg/l) L-agar plates. Six colonies were chosen for plasmid preparation; two out of three sequences were correct for all four mutated regions.

Double-Stranded Plasmid Sequencing

Plasmid DNA was sequenced by the dideoxy method (38) for denatured, double-stranded DNA (39). The sequencing primers were synthesized to be 17-18 bases in length and lie ~100 base pairs upstream of the point of mutation. Plasmid DNA was denatured and annealed with the sequencing primer in a single step; double-stranded template DNA (1-5 μg CsCl

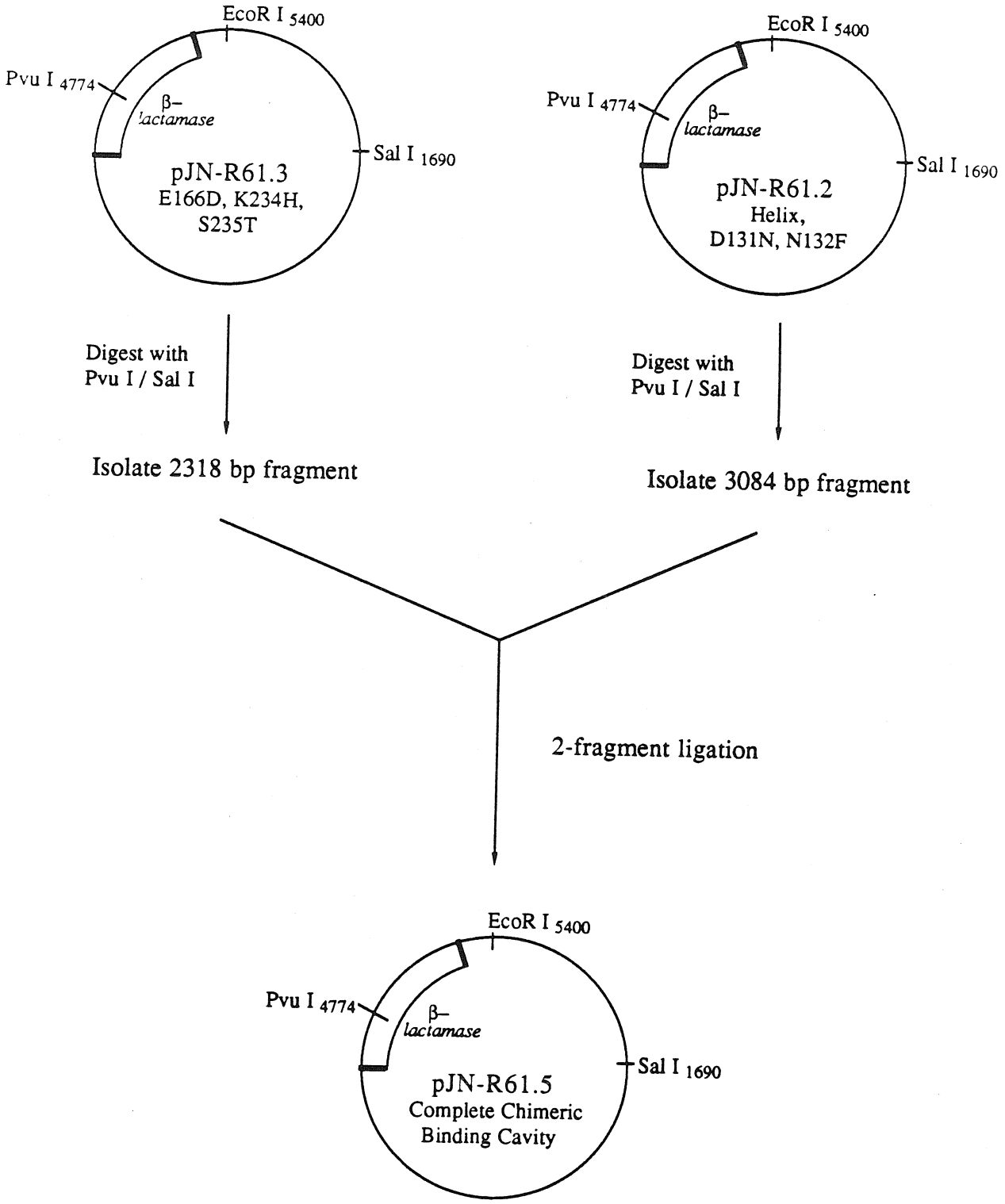



**Figure 11** - The R61.5 chimera containing the complete chimeric binding cavity was produced by a two-fragment ligation which combined the R61.2 and R61.3 mutations.

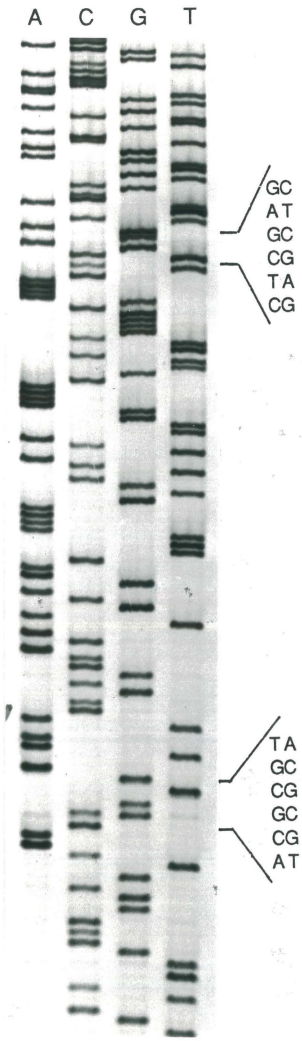
purified, 5 of 25 µl from a 2 ml prep) was combined with the synthetic oligonucleotide sequencing primer (100 ng) and the resulting mixture was boiled for 4 minutes. The tube was then snap-frozen in a dry ice/ethanol bath. Chain extension reactions were performed with small variations on the protocol described in the SEQUENASE™ kit from United States Biochemical (40). To the frozen sample containing the annealed primer-DNA complex was added 0.5 µl labeling mix, 2 µl 100 mM DTT, 2 µl sequenase reaction buffer, 1 µl [$\alpha$–$^{35}$S]-dATP (1000 Ci/mmol), and 2 µl diluted sequenase (1 in 8 dilution) followed by reaction for 3 minutes at 25°C. The reaction mixtures were then added to the termination mixes containing the dideoxynucleotides (2.5 µl) and incubated for 10 minutes at 37°C. Reactions were stopped by adding Maxam-Gilbert (32) loading buffer (4 µl). Samples were loaded onto 5-8% polyacrylamide / TBE gels, run at 50 mA for 3 hours, and autoradiographed 12-24 hours using Kodak XAR film (Figure 14).

Western Blots

Western blots were performed to examine *in vivo* stability of the mutant enzyme. Colonies harboring the chimeric plasmid were grown to late log phase ($OD_{600}$ = 1.0); a 1.0 ml sample pelleted by centrifugation and resuspended in 100 µl protein sample buffer (10% v/v glycerol, 5% v/v 2-mercaptoethanol, 3% w/v SDS, 62.5 mM Tris-HCl, pH 6.8, 1.0 mM EDTA, 0.05% w/v bromophenol blue) per absorbance unit (i.e., $OD_{600}$ = 0.95 ; 95 µl buffer) and boiled for 5-10 minutes to lyse the cells. Aliquots of 15 µl each were loaded onto a 15 cm, 12% polyacrylamide stacking gel (4% stack) and run at a constant current of 10 mA for 10-12 hours in Tris-glycine buffer (12 g Tris base, 57.6 g glycine, 40 ml 10% SDS (w/v) in 4 liters). The protein was

**Figure 14** - Autoradiographs of sequencing gels verifying the production of the desired mutations in the RTEM-1/R61 chimeras. Labeling was accomplished with [$\alpha$-$^{35}$S]-dATP.

pJN-XMS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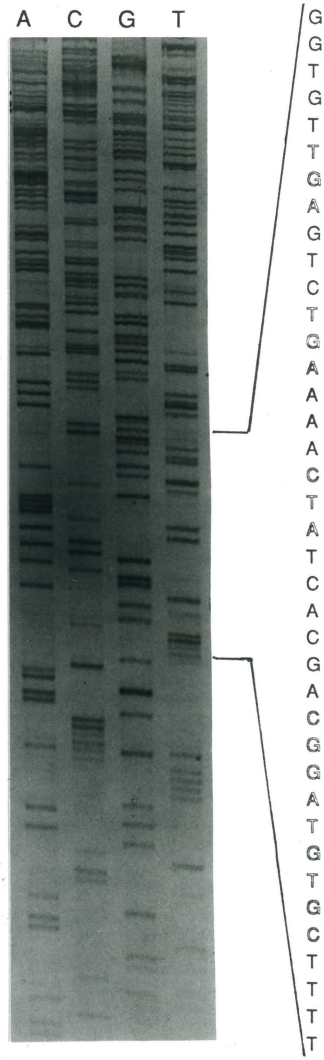
R61.1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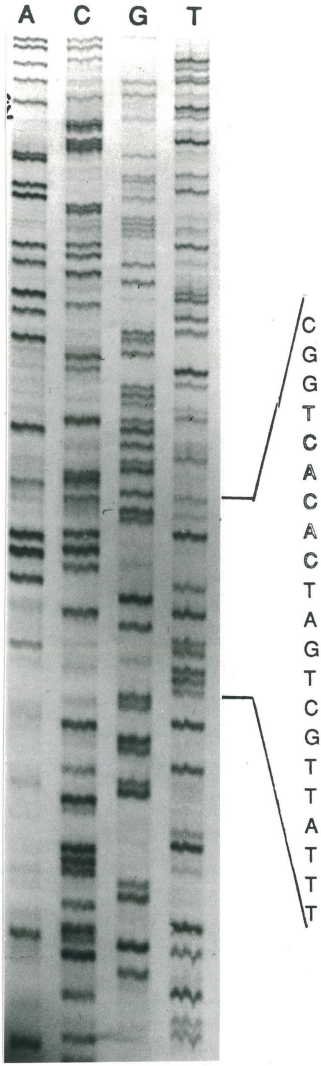
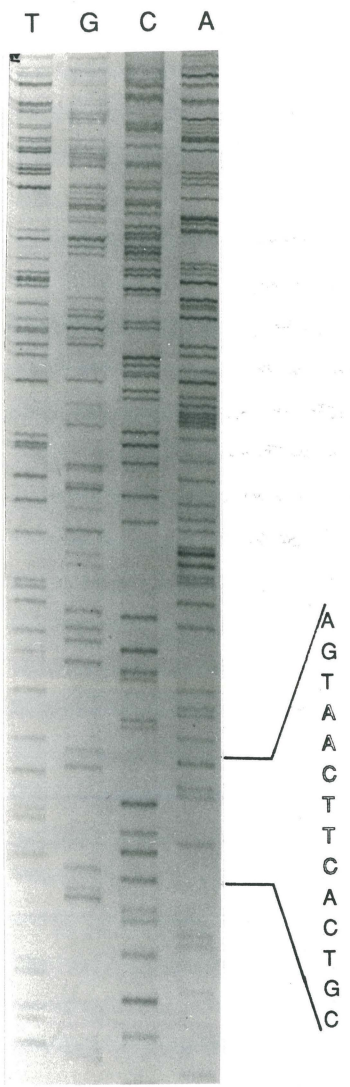
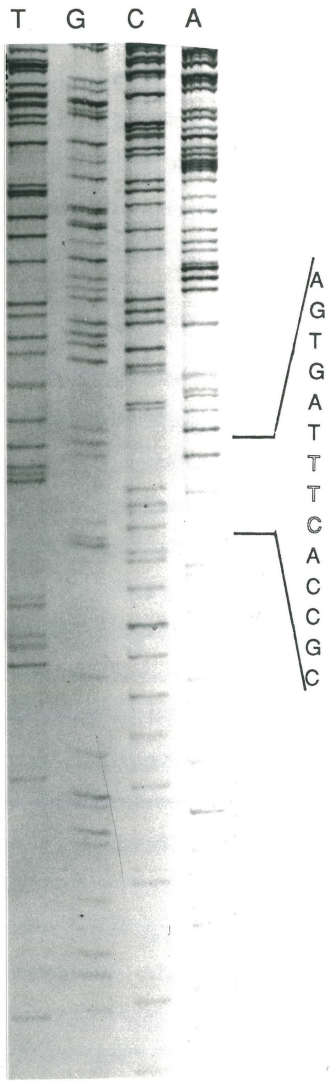
**104**
R61.B



R61.C




R61.D



then transferred from the acrylamide gel onto nitrocellulose (Schleicher & Schuell) using a Bio-Rad Transblot™ cell equipped with plate electrodes for 1.5 hours at 100 mA. ß-Lactamase was visualized following binding of rabbit anti-ß-lactamase (41) with the Vectastain™ ABC immuno-peroxidase system (42). Western blots to examine the *in vivo* stabilization effects of ß-lactam antibiotics were also performed; the procedure was the same as above with the addition of 6 mg/l ampicillin in the growth media.

Protein Expression and Purification

Mutant genes were all contained on pJN derived plasmids (23). pJN is an expression vector utilizing the IPTG inducible *tac* promoter (24) for the overproduction of ß-lactamase. *E. coli* D1210 cells containing the chimeric pJN were grown in XB media (25 g Bactotryptone, 7.5 g yeast extract, 50 ml 1 M Tris (pH 7.5) per liter) at 37°C until saturated. Typical preparation volumes were 1 - 10 liters. IPTG (0.1 M) was added followed by cooling to 0°C. At no point after this was the protein exposed to temperatures exceeding 4°C throughout the remaining purifications. After 30 minutes the cells were harvested by centrifugation in 250 ml bottles in a GSA rotor for 10 minutes at 10,000 rpm. Perplasmic proteins, including the mutant ß-lactamase, were released by osmotic extrusion (43); the pellets (1 liter) were resuspended in 40 ml sucrose solution (25 ml 1.0 M Tris-HCl, pH 7.0, 450 g sucrose, 0.5 g $Na_2EDTA$ in 1 liter) and shaken at 4°C for 30 minutes. Samples were centrifuged at 10,000 rpm for 20 minutes. The supernatant was decanted and discarded, the pellets were resuspended in 40 ml of chilled $H_2O$ (4°C), and shaken at 4°C for 30 minutes. Samples were then centrifuged at 13,000 rpm for 30 minutes. The crude ß-lactamase, contained in the supernatant, was collected and filtered

through a Nalgene 0.22 μm filter unit to remove insoluable cellular debris. The supernatant was loaded directly onto an anion exchange (Q-sepharose) gravity column (5 x 10 cm) and washed through with distilled water (~150 ml). The mutant ß-lactamases were eluted with aqueous 0.4 M NaCl. The volume of the solution was reduced and dialysed against 25 mM triethanolamine-HCl (TEA), pH 7.25, using an Amicon ultrafiltration apparatus. The semi purified protein was subjected to FPLC purification using a Mono-Q™ 10/10 anion exchange column. The protein was eluted in a linear NaCl gradient. Protein (10 ml) was loaded onto the column in 25 mM 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 as follows: t=0 minutes, 100% A; t=5, 100% A; t=62, 19% B; t=75, 100% B. The flow rate was 2.5 ml/min. The ß-lactamase containing fractions were pooled and concentrated by ultrafiltration. The concentrated protein was then applied to a 2.5 x 60 cm gel filtration (Sephacryl HR-100) column, and eluted with 0.1 M potassium phosphate, pH 7.0. Purity was checked by 12% SDS-PAGE stained with Coomassie blue (R250). The protein concentration was estimated by $OD_{281}$ using the extinction coefficient 29,400 M-1cm-1 (3).

Kinetics

Michaelis-Menten (44) kinetic parameters ($k_{cat}$, $K_M$, and $k_{cat}/K_M$) for the chimeric β-lactamase activity were determined by Eadie-Hofstee (45) replots of initial velocity data. Measurements were obtained using a Beckman DU7 spectrophotometer with 1 cm pathlength optical quartz cells. Kinetic analyses of ß-lactamase activities were performed at 30°C, pH 7.0, 0.1 M potassium phosphate. The hydrolysis of the lactam bond was monitored at 232 nm to obtain initial velocities ($\Delta\varepsilon$ = 500 for benzyl

penicillin) (46). Buffers were maintained at 30°C prior to beginning the assay to avoid error from temperature flux.

Transpeptidase activities for the transfer of the benzylpenicilloyl acyl-enzyme hydrolysis product to glycine (or similar amino acceptor), was measured using potassium [$^{14}$C]-benzyl penicillin (58.5 mCi/mmol). The radioactive substrate was diluted with nonradioactive benzyl penicillin (1:100). Reactions were initiated with 24 μg enzyme, 50 mM benzyl penicillin, 0 to 200 mM glycine, in 0.05 M potassium phosphate, pH 7.5. The reactions were incubated overnight at 37°C; after which time 2 μg RTEM-1 ß-lactamase was added to remove any remaining unhydrolysed substrate. The percent transpeptidation was determined by separation of the two products, phenylacetylglycine and phenylacetylglycylglycine, by thin-layer chromatography (buffer: 100 ml AcOH, 825 ml BuOH, 75 ml $H_2O$). Bands were excised from the TLC plates and analyzed for radioactive content using a Beckman LS9000 scintillation counter. The products were verified by proton NMR.

A fluorescence assay (47, 48) was employed to assay for the presence of carboxypeptidase activity , as observed by the liberation of D-alanine from the hydrolysis of the synthetic diacetyl-L-Lys-D-Ala-D-Ala substrate (49). Reactions (0.25 ml) were initiated at a single substrate concentration (10 mM). After reaction overnight (37°C, 0.1 M Tris-HCl, pH 7.5) the sample was added to 2 mls of buffered reagent [1.5 ml *o*-phthaldialdehyde (5 mg/ml ethanol), 1.5 ml 2-mercaptoethanol (5 μl/ml ethanol), 90 ml aqueous 0.05 M sodium tetraborate, pH 9.5]. The samples were analyzed within 15 minutes of mixing on a SLM-4800 fluorescence spectrophotometer. Excitation and emission monochromators were set at 340 nm and 455 nm, respectively, with a 2 nm slit width. The fluorescence value was corrected against the

time-zero value and the concentration of D-alanine determined from a calibration curve.

To assay the hydrolysis of the depsipeptide diacetyl-L-Lys-D-Ala-D-Lac (50), parallel reactions were initiated at a single substrate concentration (10mM) and allowed to react overnight (37°C, 0.1 M Tris-HCl, pH 7.5). Reactions were quenched at timed intervals by snap-freezing in dry ice/ethanol. The remaining quantity of depsipeptide starting material in the samples was compared to controls using an ABI/Kratos HPLC with a C-18 reversed phase column and linear gradient of acetonitrile/water (0.1% trifluoroacetic acid). The gradient profile was as follows: Buffer A=0.1% TFA in water, Buffer B= 0.1% TFA, 80% acetonitrile in water; t = 0, 100% A; t = 3, 100% A; t=33, 20% B; t=41, 100% B. To assay the chimeras for transpeptidase activity utilizing the acyclic peptide substrates, depsipeptide digests (0.1 M Tris-HCl, pH 7.5, 37°C) were initiated with [$^3$H]-glycine present in the reaction mixture. The reaction was allowed to react overnight and the mixture analyzed by HPLC for the formation of the tripeptide, diacetyl-Lys-D-Ala-Gly and the incorporation of the radiolabel.

Thermal Stability of R61 / RTEM-1 Chimeras

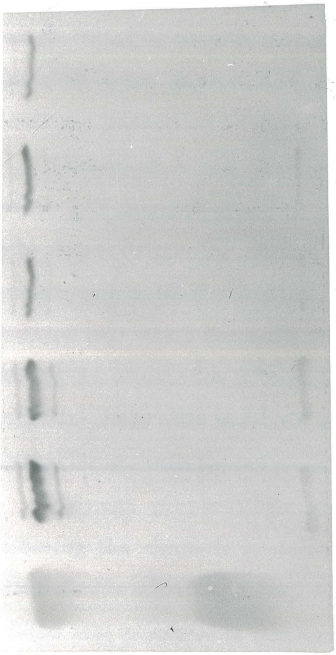
The thermal stability of the R61/RTEM-1 chimeric proteins were assessed by circular dichroism using a Jasco J600 spectropolarimeter. Samples were incubated in 100 mM potassium phosphate buffer in a 1 mm water jacketed quartz cell. The change in molar elipticity was monitored at 222 nm. Samples were subjected to a linear temperature ramp (10-85 °C). The temperature of the water circulator was adjusted to the desired temperature and allowed to equilibrate for 3-5 minutes, after which time data points were collected.

## RESULTS AND DISCUSSION

The results obtained from the PBP-5 chimera project lead to the design of a series of second generation chimeric enzymes to further investigate the proposed evolutionary relationship between the class A ß-lactamases and the bacterial cell wall carboxypeptidases/transpeptidases (4-8). Construction of these chimeras involved replacement of selected residues in RTEM-1 ß-lactamase, with the corresponding residues present in the R61 carboxpeptidase of *Streptomyces* as determined by sequence alignment (6).

Oligonucleotide-directed mutagenesis was first performed on the Eco RI/Sal I 3712 base pair fragment of pJN in M13 mp18, to produce three unique restriction sites (Xho I at 5032, Mlu I at 4955, and Sac I at 4898 in pJN). The resulting plasmid, pJN-XMS, was subsequently employed for cassette mutagenesis to produce an R61/RTEM-1 chimera consisting of six exchanged residues in the α-helix containing the active-site serine (P67R, M68V, M69G, F72I, V74S, L76S, Figure 5). Unlike the PBP-5/RTEM-1 chimera, primarily only those residues which are exposed to solvent were exchanged, thereby reducing the disruption of the hydrophobic core of the enzyme while maintaining intrinsic protein stability. While residues 67 and 68 (Ambler convention (17) for ß-lactamase numbering) are not presumed to be directly involved in catalysis, I proposed to replace them to aid in orienting the helix to the proper geometry for carboxypeptidase catalysis. Not all of the mutations are conservative, yet this approach yielded an enzyme with reduced yet reasonable stability compared to the wild-type RTEM ß-lactamase and seemingly improved over the PBP-5/RTEM-1 chimera (Figure 15).

**Figure 15** - Western blots of the R61.1 chimera as compared to wild-type ß-lactamase (pJN) and the PBP-5 chimera. The R61.1 chimera displayed significantly reduced *in vivo* stability compared with the wild-type ß-lactamase, yet seems to be improved relative to the PBP-5 chimera.




**PBP-5 / RTEM Hybrid (30°C)**

**R61.1 / RTEM Hybrid (30°C)**

**R61.1 / RTEM Hybrid (37°C)**

**pJN - XMS (37°C)**

**wild-type pJN (37°C)**

**MW Marker**

In addition to the mutagenesis performed on the α-helix to create R61.1, mutations were made to other regions of the ß-lactamase substrate binding cavity. The synthetic scheme was designed to allow for the creation of each mutation singularly to examine the individual and combinatorial effects of each mutation. The chimeras R61.B (K234H, S235T) and R61.C (D131N, N132F) were produced by oligonucleotide directed mutagenesis on the Eco RI/Sal I 3712 base pair fragment of pJN-XMS in M13 mp18. The single-site mutant R61.A (E166D) had been previously constructed by cassette mutagenesis (18). The final single-site mutant, R61.D (N132F), was generated by cassette mutagenesis using pJN-Sax (36). The remaining chimeras were constructed from condensation of these individual mutations, generating a total of ten chimeric enzymes (Table II).

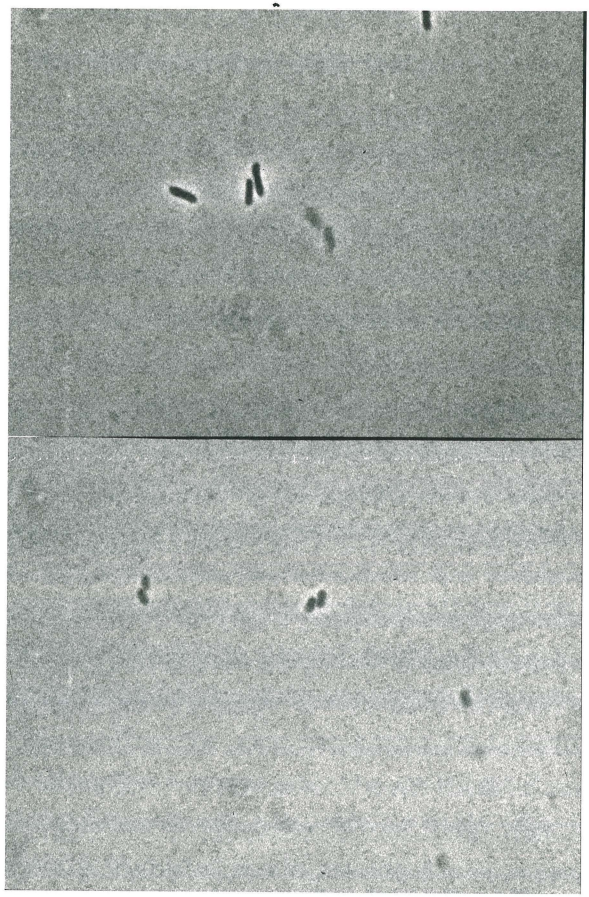
Competent *E. coli* XL1-Blue (30) cells were transformed with each of the ten pJN plasmids encoding the chimeric proteins. The strain XL1-Blue cells were originally obtained for use in oligonucleotide-directed mutagenesis with the bacteriophage M13. These cells have a *rec A*$^-$ genotype (26) which decreases the rate of homologous recombination, thus limiting the elimination of large inserts from M13. In addition to the *rec A*$^-$ mutation, these cells maintain a *lac* repressor (lac $I^Q$) and are therefore suitable for use with the *tac* promotor (24) present in pJN.

Plasmid preparations were performed on the XL1-Blue cells harboring the chimeric proteins; however, cells containing the D131N, N132F double mutation (R61.C, R61.2, and R61.5) multiplied at a markedly reduced rate compared to non-transformed cells. The doubling time of the chimeric cells was ~80 minutes versus ~25 minutes for the normal cells. Examination of these cells by light microscopy revealed an altered ovoid cell morphology (Figure 16). None of the other remaining chimeric genes

**Table I** - The complete set of ten RTEM-1/ R61 chimeras. Individual mutations were performed to produce the basic chimeras, then condensation ligation schemes were employed to build the complete chimeric binding cavity in a stepwise synthesis.

| Name | Description |
|---|---|
| pJN-XMS | Xho I @ 5032, Mlu I @ 4955, Sac I @ 4898 |
| R61.A | pJN-XMS mutagenesis with:<br>E166D |
| R61.B | pJN-XMS mutagenesis with:<br>K234H, S235T |
| R61.C | pJN-XMS mutagenesis with:<br>D131N, N132F |
| R61.D | pJN-XMS mutagenesis with:<br>N132F |
| R61.1 | **Base Hybrid.**<br>Xho I --> Mlu I insert into active site.<br>P67R, M68V, M69G, F72I, V74S, L76S |
| R61.2 | R61.1 + R61.C:<br>P67R, M68V, M69G, F72I, V74S, L76S, D131N, N132F |
| R61.3 | R61.A + R61.B:<br>E166D, K234H, S235T |
| R61.4 | R61.A + R61.B + R61.C:<br>D131N, N132F, E166D, K234H, S235T |
| R61.5 | R61.1 + R61.4:<br>P67R, M68V, M69G, F72I, V74S, L76S, D131N,<br>N132F, E166D , K234H, S235T |

**Figure 16** - Expression of the R61.C (D131N, N132F) chimera in E. coli XL1-Blue cells resulted in an altered, ovoid, cell morphology. Normal rod-shaped E. coli at the same magnification are shown for comparison.



induced this altered morphology, but rather were present in normal rod shaped cells. Work has been performed by Spratt and coworkers which overexpressed PBP-5 of *E. coli* as a soluble enzyme (51). A spherical cell morphology was observed in *E. coli* harboring the PBP-5 containing plasmid. The viability of these cells was greatly reduced by the overproduction of the PBP-5 gene product, even when cloned into the low copy number plasmid pSC105 (52). It is presumed that the presence of the external source of transpeptidase activity results in a hyper-crosslinked cell wall. The resulting cell wall structure impedes cell division, thereby resulting in an increased doubling time. Thus the increased doubling time and altered cell morphology would appear to be an indication of hyper-crosslinking as catalyzed by the chimeric (D131N, N132F) proteins.

Interestingly, competent D1210 cells were transformed with the R61.C (N132F) plasmid with only slight ill effects on growth or morphology. A primary difference between the two strains is that the XL1-Blue cells contain the gene responsible for tetracycline resistance, maintained on the F-plasmid. The tetracycline resistance factor is a membrane bound enzyme that functions by pumping the antibiotic out of the cells (52). To investigate the possibility that the presence of these proteins in the cell wall may initially destabilize the cell wall, thereby magnifying the subtle effect of the R61.C gene product, the gene was subcloned into pBR322 which contains the tetracycline resistance gene. The pBR322-R61.C plasmid was then propagated in both *E. coli* strains TG1 and D1210. Cells from both strains revealed a diminished growth rate and altered phenotype when transformed with the R61.C plasmid. It is important to note that the experiments involving the over-expression of PBP-5 were also performed in the presence of tetracycline.

*E. coli* cells containing the plasmid encoding the chimera exhibit a greatly reduced or non-resistant phenotype to penam antibiotics (Table II). Western blots (Figure 17) performed to examine the *in vivo* stability of the chimeras revealed high levels of proteolytic degradation of several chimeras, especially the R61.5 chimera, which cannot be detected. No substrate induced stabilization was observed for cells grown in the presence of sub-lethal levels of ampicillin. The chimeric proteins (with the exceptions of R61.2 and R61.5) were isolated from the periplasm of the *E. coli* strain D1210 cells (grown in the presence of 50 μg/ml of kanamycin) by osmotic extrusion (2). The crude protein was first purified using an anion exchange gravity column with Q-Sepharose resin. This pre-purification is performed to protect the Mono-Q FPLC column from damage due to large concentrations of contaminating cellular proteins and lipids. The chimeric protein was further purified by FPLC purification using a Mono-Q anion exchange column, followed by removal of small amounts (> 5% by weight) of contaminating proteins via gel filtration chromatography (Sephacryl HR-100). Purity was confirmed by analysis using 12% SDS-PAGE stained with Coomassie blue (R250). Protein concentrations for kinetic analyses were estimated at 281 nm using the ß-lactamase extinction coefficient (3) of 29,400 $M^{-1}cm^{-1}$.

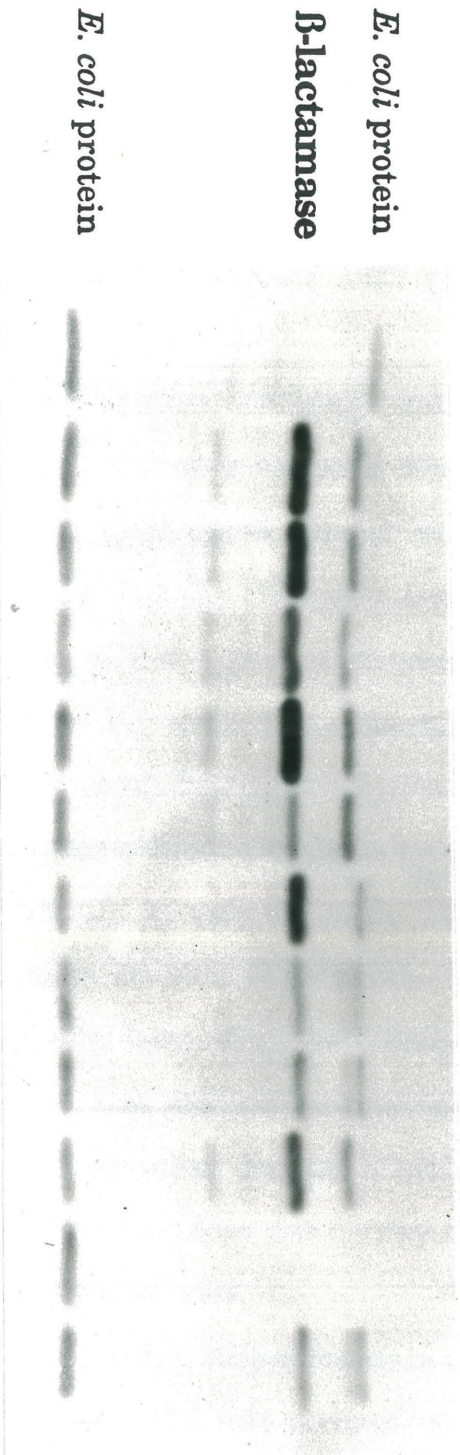
The purified chimeric proteins were partially characterized to facilitate identification of mutants which exhibit interesting catalytic or structural properties. The general structural characteristics of each chimera were examined by circular dichroism (CD). CD scans (180-300 nm) were performed to compare the gross structural content of the chimeras relative to the wild-type ß-lactamase. All enzymes, with the exclusion of the R61.C and R61.1 chimeras, showed CD spectra nearly

**TABLE II**

Phenotypic activities of the R61/RTEM-1 chimeras. Numbers indicate the highest concentration on which colonies grew (mg/l).

| Chimera | Ampicillin | Cephalothin |
|---|---|---|
| R61.1 (α-helix) | 20 | 0 |
| R61.2 (α-helix, D131N,N132F) | 5 | 0 |
| R61.3 (E166D, K234H, S235T) | 5 | 0 |
| R61.4 (E166D, K234H, S235T, D131N, N132F) | 5 | 0 |
| R61.5 (α-helix, E166D, K234H, S235T, D131N, N132F) | 5 | 0 |
| R61.A (E166D) | 100 | 0 |
| R61.B (K234H, S235T) | 20 | 0 |
| R61.C (D131N, N132F) | 5 | 0 |
| R61.D (N132F) | 5 | 0 |
| ß-lactamase | 2000 | 100 |
| *E. coli* D1210 | 5 | 0 |

**Figure 17** - Western blots comparing the *in vivo* stability of the R61/RTEM-1 chimeras at 37°C to wild-type ß-lactamase. The R61.5 chimera is not present in any measurable amount. The band corresponding to the R61.C (D131N, N132F) chimera is diminished compared to the R61.D (N132F) chimera, yet they both are thermally stable at 37°C as determined by melting experiments.




*E. coli* D1210

wild-type pJN

pJN-XMS

R61. A (E166D)

R61. B (K234H, S235T)

R61. C (D131N, N132F)

R61. D (N132F)

R61. 1 (α-helix chimera)

R61. 2 (α-helix, D131N, N132F)

R61. 3 (E166D, K234H, S235)

R61. 5 (Complete chimera)

R61. 4 (D131N, N132F, E166D, K234H, S235)

superimposable with those of the wild-type ß-lactamase at 25°C. Due to a strong temperature instability, assessment of the R61.1 enzyme was performed at 15°C to insure accurate measurements. At this reduced temperature, while there was a mild decrease in the molar elipticity in the region of α-helix content, the resulting spectrum is remarkably similar to the wild type. This demonstrates that the mutations performed were accommodated, albeit with reduced stability, within the native protein conformation.

The most striking results were obtained with the R61.C (D131N, N132F) chimera (Figure 18). While an exact determination of the structural changes cannot be determined without x-ray crystal data, it appears that the chimera's overall structure is markedly altered compared to the RTEM-1 parent enzyme. Temperature dependent melting curves (Figure 19) were also determined by CD spectropolarimetry. With the exception of the chimeras with the mutations at the active-site serine, the chimeras exhibited temperature melting points either equal to the wild-type or reduced by only 5 - 15°C. The R61.1 chimera melted substantially lower at about 30-35°C (Table III). The R61.C chimera melts at a temperature only ~5°C lower than the wild-type RTEM-1 parent, but periplasmic levels of the chimera are low as determined by western blots (Figure 17). It would therefore appear that the D131N, N132F mutations induce a conformational change which does not decrease protein stability at 37°C, yet increases the rate of proteolysis.

Cursory measurements of ß-lactamase activities were determined (Table IV). The R61 carboxypeptidase does not hydrolyze benzyl penicillin to the corresponding penicilloic acid, the ß-lactamase hydrolysis product, but rather generates phenylacetylglycine (1, 12, 13). This "altered" catalytic

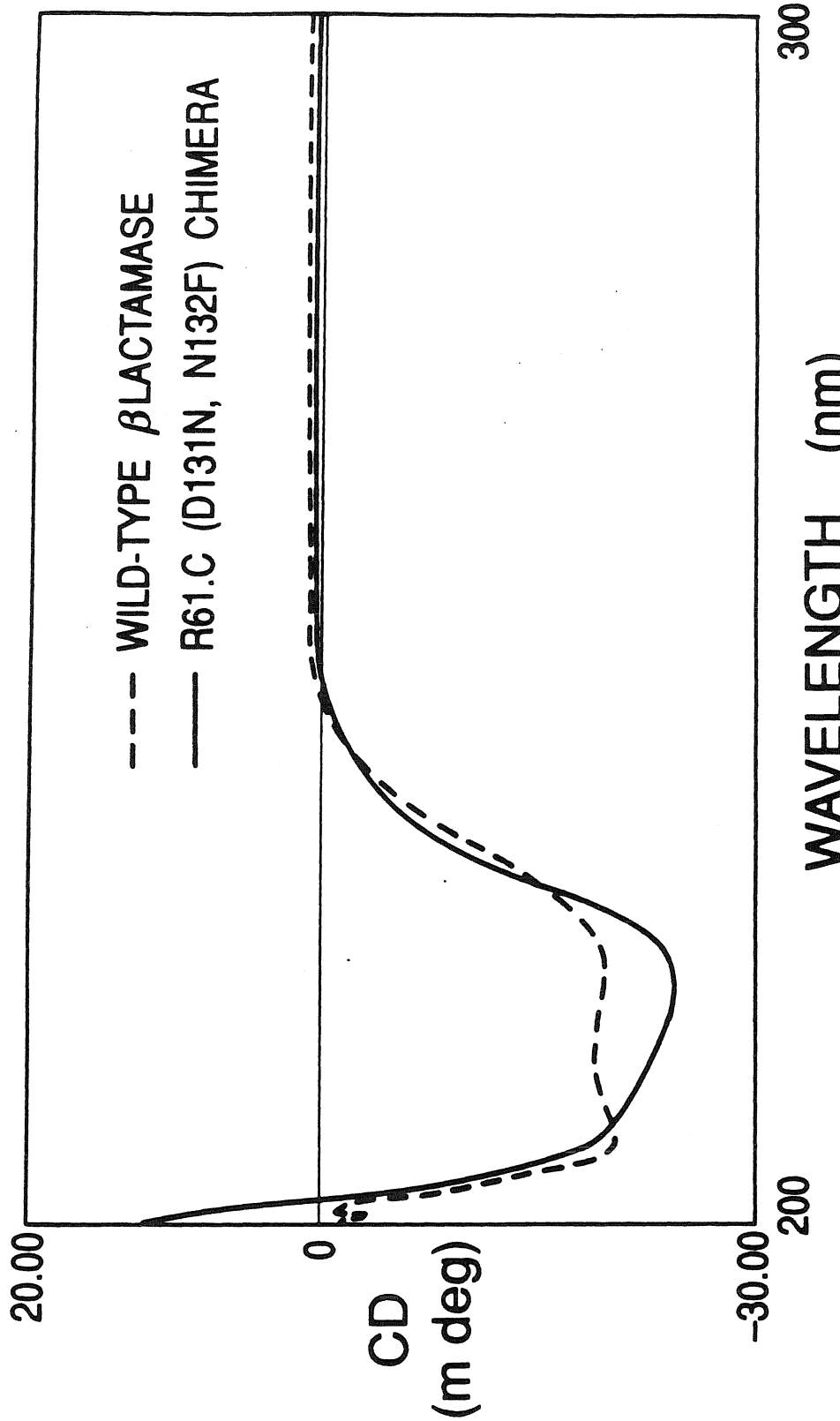



**Figure 18 -** CD scans comparing the overall structural similarity between the wild-type ß-lactamase and the R61.C chimera. Only the R61.C chimera exhibited a CD spectra markedly different from the wild-type.

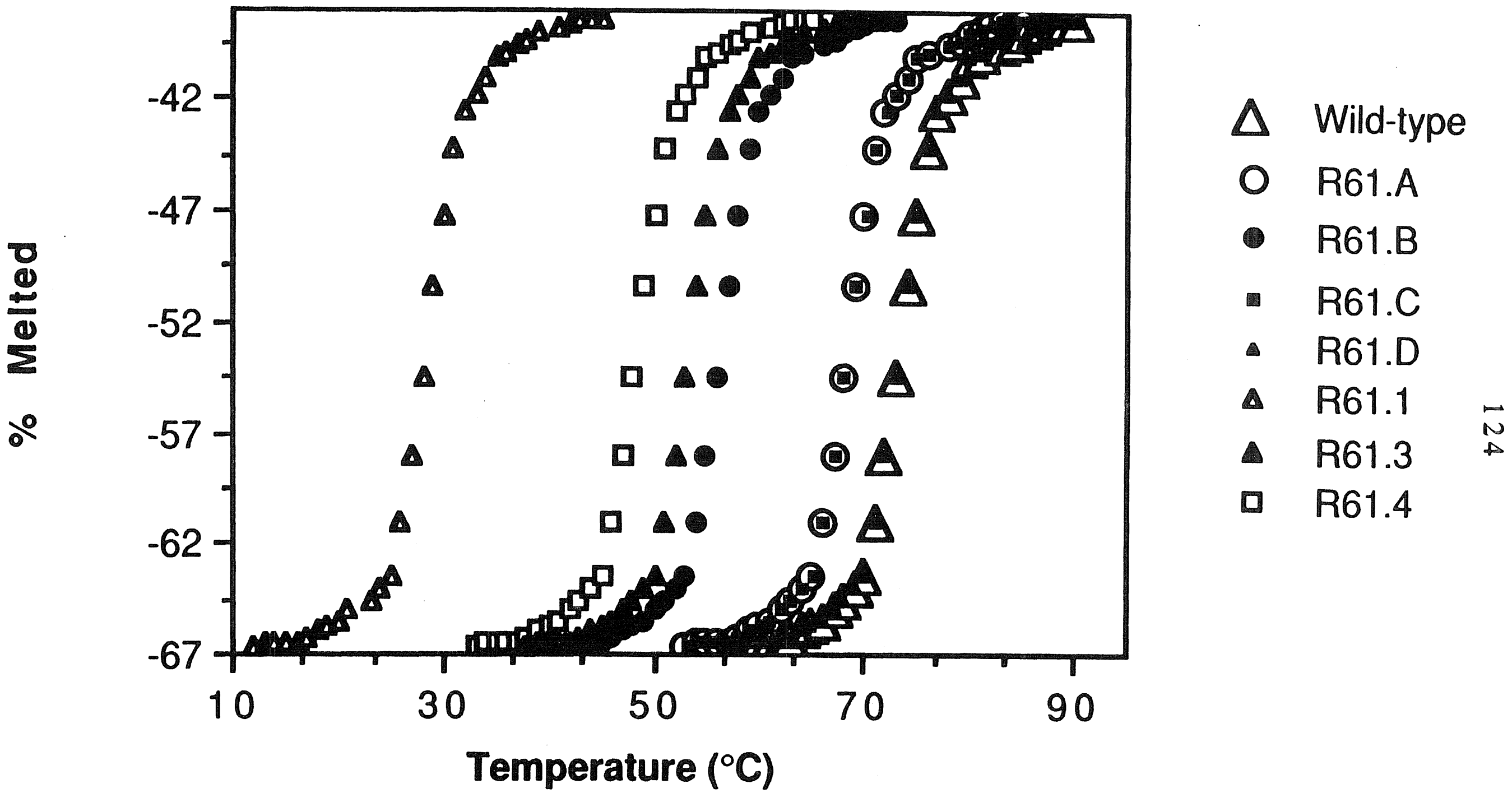





**Figure 19** - Thermal melting curves comparing the chimeras to the wild-type ß-lactamase. With the exception of the chimeras containing mutations in the active site α-helix (R61.1 shown, R61.2 and R61.5 were not determined), all the chimeras were thermally stable at 37°C, with melting points lowered only 5-15°C.

**TABLE III**

Melting temperatures of the R61/RTEM-1 chimeras as determined by decrease in molar elipticity at 222 nm. ND - not determined.

| Chimera | Temperature (°C) |
|---|---|
| R61.1 | 30 |
| R61.2 | ND |
| R61.3 | 55 |
| R61.4 | 50 |
| R61.5 | ND |
| R61.A | 70 |
| R61.B | 58 |
| R61.C | 70 |
| R61.D | 75 |
| ß-lactamase | 75 |

## TABLE IV

Preliminary kinetic parameters of the R61/RTEM-1 chimeras utilizing benzyl penicillin as a substrate. The products of the substrate hydrolysis and the corresponding product with the reaction in the presence of glycine are given. Parameters were obtained by UV spectrophotometry. Due to extremely low levels of activities for some mutants, detailed parameters could not be obtained by this method. Activities for the R61.C and R61.D chimeras are given in Chapter 4. ***ND*** - not determined, *pen* - penicilloic acid, *pag* - phenylacetylglycine, *pagg* - phenylacetylglycylglycine.

| Chimera | $K_M$ (mM) | $k_{cat}$ ($sec^{-1}$) | $k_{cat}/K_M$ | Product | w/glycine |
|---|---|---|---|---|---|
| R61.1 | ND | $>10^{-3}$ | ND | pen | pen |
| R61.2 | ND | ND | ND | ----- | ----- |
| R61.3 | $65.5 \pm 2.2$ | $6.8 \times 10^{-3} \pm 8.2 \times 10^{-4}$ | $1.04 \times 10^{2}$ | pen | pen |
| R61.4 | ND | $>10^{-3}$ | ND | pen | pen |
| R61.5 | ND | ND | ND | ----- | ----- |
| R61.A | $21 \pm 9$ | $1.1 \pm 0.02$ | $5.1 \times 10^{4}$ | pen | pen |
| R61.B | $342 \pm 22$ | $0.53 \pm 0.1$ | $1.56 \times 10^{3}$ | pen | pen |
| R61.C | ND | $>10^{-3}$ | ND | pag | pagg |
| R61.D | ND | $>10^{-3}$ | ND | pen | pen |
| ß-lactamase | 20 | 2000 | $1 \times 10^{8}$ | pen | pen |
| R61 Cpase | | $10^{-4}$ | | pag | pagg |

activity was a strong factor in the choice of the R61 carboxypeptidase. In the original PBP-5 chimera it was indeterminable whether the decreased activity of the chimera utilizing penam antibiotics was a result of enhanced carboxypeptidase character or whether the mutations simply destroyed ß-lactamase catalysis. Reactions with excess benzyl penicillin were initiated with the purified chimeric enzymes at 37°C and incubated for 12 hours. Similar reactions were performed in the presence of 100 mM glycine. Once again, all enzymes yielded varying amounts of penicilloic acid with the exception of the R61.C enzyme. This chimera degraded benzyl penicillin to phenyl-acetylglycine and yielded the corresponding transpeptidase product, phenylacetylglycylglycine (54), when incubated in the presence of glycine. No penicilloic acid could be detected above background hydrolysis. The degradation products were isolated by thin-layer chromatography and verified by proton NMR. The phenylacetylglycine produced by the chimera was identical to synthetic phenylacetylglycine under all conditions examined.

The chimeras were tested for carboxypeptidase and transpeptidase activities, utilizing the synthetic peptide diacetyl-L-Lys-D-Ala-D-Ala (49) and the depsipeptide diacetyl-L-Lys-D-Ala-D-lactate (50), in reactions similar to those with benzyl penicillin (Table V). Once again only the R61.C chimera exhibited a level of substrate hydrolysis over that of background hydrolysis. This activity, however, was exceedingly low even when compared to the PBP-5/ RTEM-1 chimera.

**TABLE V**

Preliminary screening for the presence of carboxypeptidase and transpeptidase activities utilizing the peptide diacetyl-L-Lys-D-Ala-D-Ala (KAA) and the depsipeptide diacetyl-L-Lys-D-Ala-D-lactate (KAL) as substrates. Purified enzymes were incubated with the substrates (37°C, 0.1M Tris-HCl, pH 7.5) and analyzed by HPLC. Transpeptidation reactions used glycine (100mM) as an amino acceptor. (-) - no observed reaction, (+) - reaction observed, (±) - low levels of activity observed, (ND) - not determined.

| | Hydrolysis | | Transpeptidation |
|---|---|---|---|
| Chimera | KAA | KAL | (w/glycine) |
| R61.1 | - | - | - |
| R61.2 | ND | ND | ND |
| R61.3 | - | - | - |
| R61.4 | - | - | - |
| R61.5 | ND | ND | ND |
| R61.A | - | - | - |
| R61.B | - | - | - |
| R61.C | ± | ± | ± |
| R61.D | - | - | - |
| ß-lactamase | - | - | - |
| R61 Cpase | + | + | + |

## CONCLUSION

Site-directed mutagenesis was successfully performed to create a series of ten chimeric enzymes by exchanging selected residues in RTEM-1 ß-lactamase with those present in the R61 carboxypeptidase from *Streptomyces*. The goal of this project was to use the information gained from the PBP-5 chimeras in designing and isolating a chimeric enzyme capable of carboxypetidase/transpeptidase activity. In contrast to the original PBP-5 chimera, the R61 project targeted residues for mutagenesis in several regions of the ß-lactamse substrate-binding cavity in addition to the α-helix containing the active-site serine. The mutations on the α-helix were chosen to exchange primarily those residues which are exposed to the solvent-accessible hydrophilic cavity. The goal of this design was to exchange the residues capable of substrate interaction while maintaining intrinsic protein stability. A similar technique was employed in the substitution of five amino acids an the exposed surface of the 434 repressor DNA recognition helix with those present in the P22 repressor (55). The resulting chimera produced P22 recognition in the 434 repressor framework with no loss of stability. While the resulting R61.1 chimera does not exhibit any measurable carboxypeptidase/transpeptidase activity, the attempts to stabilize the R61.1 chimera were modestly successful, compared to the PBP-5 chimera.

The most prominent results were obtained from the chimeras containing the D131N, N132F double mutation. The first indication of a possible carboxypeptidase/transpeptidase activity was observed by changes in cell morphology and reduced rate of cellular division involved with the chimeras. It has been shown that overproduction of PBP-5 of *E. coli* results in a spherical cell morphology and reduced growth rate due to hyper-

crosslinking of the cell wall (51). Preliminary assessment of ß-lactamase, carboxypeptidase, and transpeptidase activities indicate the presence of activities distinct from those observed in the RTEM-1 ß-lactamase parent enzyme (3) and noticeably similar to the activity of the R61 carboxypeptidase (1) from *Streptomyces*. Detailed kinetic and structural analysis comparing the properties of the R61.C (D131N, N132F) and the R61.D (N132F) chimeras are discussed in Chapter 4. The complete implications of these finding on the catalytic role of residue 132 in class A ß-lactamases and a hypothesis regarding the evolutionary relationship between the class A ß-lactamases and the D-Ala-D-Ala-carboxypeptidases will be discussed in detail.

In conclusion, I have extrapolated from the results obtained with the original PBP-5/RTEM-1 chimera with a goal of producing a chimera with novel catalytic activities. I have identified a mutant with only two amino acid changes from RTEM-1 ß-lactamase which exhibits greatly altered catalysis. These results verify the importance and power of the techniques of site-directed mutagenesis for the understanding of enzyme structure and function.

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# Chapter 4

## Understanding the Mechanism of ß-Lactamase Activity and the Evolutionary Relationship With the Penicillin Binding Proteins: Kinetic Analysis of Two RTEM-1/R61 Chimeras

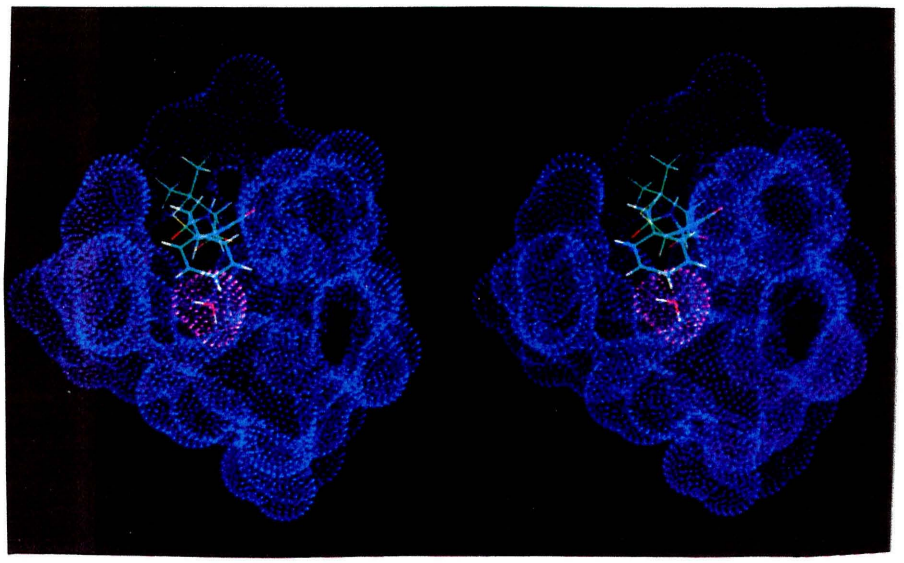
## INTRODUCTION

The design of the R61/RTEM-1 chimera project was based on an extrapolation from the original PBP-5/RTEM-1 chimera (1,2). By exchanging selected residues throughout the substrate binding cavity of RTEM-1 ß-lactamase with the corresponding residues present in the R61 carboxypeptidase/transpeptidase of *Streptomyces*, a chimeric binding cavity was generated. One selected double mutation, Asp 131 Asn and Asn 132 Phe (Ambler convention (3)) yielded a chimeric enzyme (R61.C) with altered catalytic activity remarkably similar to that present in the R61 enzyme (4).

These two residues were initially chosen for mutation because of their proximity and possible influence on the catalytically important glutamate 166 residue. Site-saturation of residue 166 was previously performed (5) to demonstrate the mechanistic importance of this site. Of all 20 amino acids, only aspartate, histidine and tyrosine conveyed any resistance to ß-lactam antibiotics, with the Glu 166 Asp mutant proving the most active. Yet even this conservative mutation decreased the catalytic reactivity ($k_{cat}/K_M$) by two orders of magnitude (6). The side-chain of the 166 residue has been proposed to have two functions in ß-lactamase. It is believed to be involved in a salt-bridge with lysine 73 (7) and directly involved in the hydrolysis of the acyl enzyme intermediate formed between the active-site serine 70 and the ß-lactam substrates, utilizing a water molecule present in the cavity beneath the substrate (Figure 1). Geometries and distances observed in modeling studies predict that this water is hydrogen bonded to either asparagine 132 or asparagine 170.

Detailed examination of the orientation of the asparagine side-chain of residue 132 was performed using the crystal structures of the class A ß-lactamases from *Staphylococcus aureus* (7) and *Bacillus licheniformis* (8)

**Figure 1** - Stereo plot of the active site of a class A ß-lactamase showing the bound water molecule beneath the bound substrate.

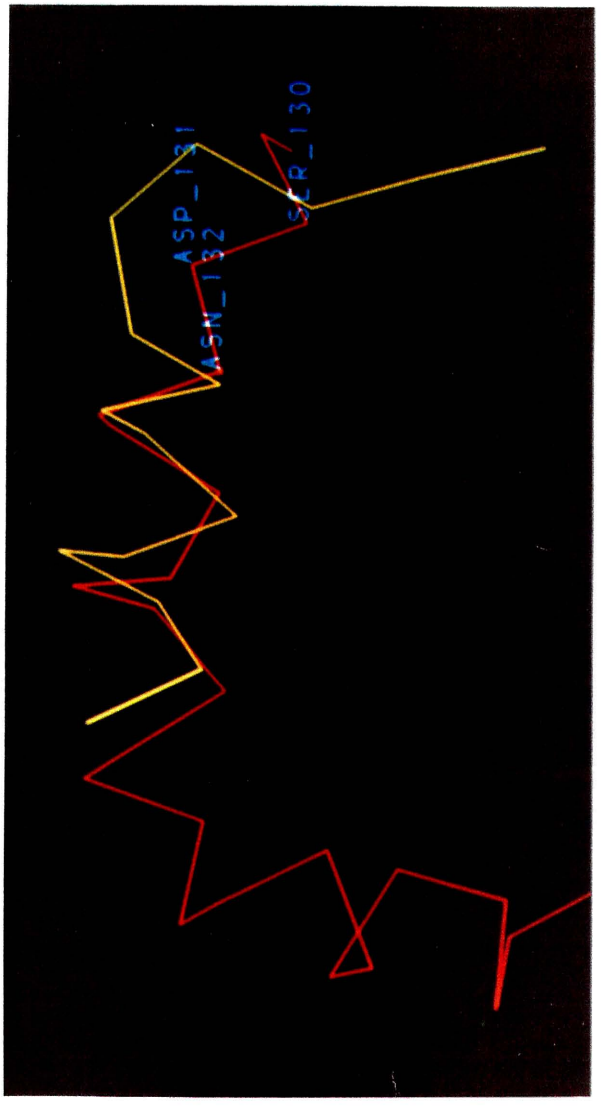


and the corresponding phenylalanine side chain of residue 164 in the R61 carboxypeptidase (9). In all three enzymes these side-chains are proximal to that of Glu 166 (Asp 225 in R61). However, the placement of the large hydrophobic phenyl ring prohibits the hydrogen bonding of a water molecule within an accessible distance to the residue involved in substrate hydrolysis.

The rationale to perform the secondary mutation of RTEM-1 residue 131 (163 in R61) is not immediately apparent. The side chain of this residue in both enzymes is buried within the hydrophobic core of the protein. The 131/132 amino acid pair is proceeded in linear sequence by a conserved serine residue (Ser 130 in RTEM-1, Ser 160 in R61). Superimposition of the α-carbon traces of the two enzymes (Figure 2) reveals a similar orientation and geometry of this serine in relationship to the active-site serine (70 in RTEM-1, 62 in R61). Despite the insertion of two amino acids following serine 160 in R61, residues 131 and 132 superimpose with their R61 counterparts; this is accomplished even at the expense of adopting an unfavorable peptide chain conformation (10).

The resulting R61.C (D131N, N132F) chimera exhibited tremendously different activities and effects on cellular metabolism compared to the other R61/RTEM-1 chimeras and the parent RTEM-1 enzyme (11,12). *E. coli* cells harboring the plasmid encoding the chimeric gene exhibit an altered, ovoid, cell morphology in addition to a reduced growth rate (see chapter 3). Furthermore, preliminary kinetic analysis revealed catalytic activity remarkable similar to that of the R61 enzyme (4), particularly in the ability to degrade benzyl penicillin to phenylacetylglycine, and the corresponding transpeptidation products when reacted in the presence of an amino acceptor.

**Figure 2** - Computer generated representation of the superimposed α-carbon traces of the class A ß-lactamase for *S. aureus* and the R61 carboxypeptidase from *Streptomyces*. Note that while two residues are inserted between the serine and asparagine in R61, the asparagine and phenylalanine residues overlap with the corresponding aspartate 131 and asparagine 132 residues in ß-lactamase.


SER_130
ASP_131
ASN_132

I describe here the detailed kinetic analysis of the R61.C (D131N, N132F) and R61.D (N132F) chimeras, and the implications of these results on both the role of residue 132 in ß-lactamse activity and the evolutionary relationship between the class A ß-lactamases and the D-Ala-D-Ala-carboxypeptidases.

## MATERIALS AND METHODS

### Enzymes and Chemicals

Kanamycin sulfate was purchased from BMB; all other antibiotics were supplied by Sigma Chemical Company. [α-$^{35}$S]-dATP, [$^{14}$C]-benzyl penicillin, and [$^{3}$H]-glycine were supplied by Amersham. [$^{35}$S]-Benzyl penicillin was obtained from Dupont/New England Nuclear. Q-Sepharose and Sephacryl-HR100 chromatography media were purchased from Pharmacia. Low melting-point agarose was purchased from FMC Bioproducts.

### Bacterial Strains

The pJN plasmids (13), which were utilized as expression vectors, were harbored in *E. coli* strain D1210. The D1210 strain contains the kanamycin resistance factor and a *lac* repressor (lac i$^{Q}$) necessary for the control of the *tac* promotor (14) present in pJN. *E. coli* strain XL1-Blue (15) were purchased from Stratagene. This strain has a tetracycline resistance maintained on the F-plasmid and a *rec A*$^{-}$ genotype (16) which greatly reduces the level of homologous recombination; this deters gene elimination from the M13 vectors.

### Double-Stranded Plasmid Sequencing

Plasmid DNA was sequenced by the dideoxy method (17) for denatured, double-stranded DNA (18). The sequencing primers were synthesized to be 17-18 bases in length and lie ~100 bases upstream of the point of mutation. Plasmid DNA was denatured and annealed with the sequencing primer in a single step. Double stranded template DNA (1-5 μg CsCl, 5 of 25 μl from a 2 ml prep) was combined with the synthetic oligonucleotide sequencing

primer (100 ng) and the resulting mixture was boiled for 4 minutes. The tube was then snap-frozen in a dry ice/ethanol bath. Chain extension reactions were performed with small variations on the protocol described in the SEQUENASE™ kit from United States Biochemical (19). To the frozen sample containing the annealed primer-DNA complex was added 0.5 µl labeling mix, 2 µl 100 mM DTT, 2 µl sequenase reaction buffer, 1 µl [α–$^{35}$S]-dATP (1000 Ci/mmol), and 2 µl diluted sequenase (1 in 8 dilution) and reacted for 3 minutes at 25 °C. The reaction mixtures were then added to the termination mixes containing the dideoxynucleotides (2.5 µl) and incubated for 10 minutes at 37°C. Reactions were stopped by adding Maxam-Gilbert (20) loading buffer (4 µl). Samples were loaded onto 5-8% polyacrylamide / TBE gels, run at 50 mA for 3 hours, and autoradiographed 12-24 hours using Kodak XAR film.

Protein Expression and Purification

Mutant genes were all contained on pJN derived plasmids (13). pJN is an expression vector utilizing the IPTG inducible *tac* promoter (14) for the overproduction of ß-lactamase. *E. coli* D1210 cells containing the chimeric pJN were grown in XB media (25 g Bactotryptone, 7.5 g yeast extract, 50 ml 1 M Tris (pH 7.5) per liter) at 37°C until saturated. Typical preparation volumes were 5 - 10 liters. IPTG (0.1 M) was added followed by cooling to 0°C. At no point after this was the protein exposed to temperatures exceeding 4°C throughout the remaining purifications. After 30 minutes the cells were harvested by centrifugation in 250 ml bottles in a GSA rotor for 10 minutes at 10,000 rpm. Perplasmic proteins, including the mutant ß-lactamase, were released by osmotic extrusion (21); the pellets (1 liter) were resuspended in 40 ml sucrose solution (25 ml 1.0 M

Tris-HCl, pH 7.0, 450 g sucrose, 0.5 g $Na_2EDTA$ in 1 liter) and shaken at 4°C for 30 minutes. Samples were centrifuged at 10,000 rpm for 20 minutes. The supernatant was decanted and discarded, the pellets resuspended in 40 ml of chilled $H_2O$ (4°C) and shaken at 4°C for 30 minutes. Samples were then centrifuged at 13,000 rpm for 30 minutes. The crude ß-lactamase contained in the supernatant was collected and filtered through a Nalgene 0.22 μm filter unit to remove insoluable cellular debris. The supernatant was loaded directly onto an anion exchange (Q-sepharose) gravity column (5 x 10 cm) and washed through with distilled water (~150 ml). The mutant ß-lactamases were eluted with aqueous 0.4 M NaCl. The volume of the solution was reduced and dialysed against 25 mM triethanolamine-HCl (TEA), pH 7.25, using an Amicon ultrafiltration apparatus. The semi-purified protein was subjected to FPLC purification using a Mono-Q™ 10/10 anion exchange column. The protein was eluted in a linear NaCl gradient. Protein (10 ml) was loaded onto the column in 25 mM 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 as follows: t=0 minutes, 100% A; t=5, 100% A; t=62, 19% B; t=75, 100% B. The flow rate was 2.5 ml/min. The ß-lactamase containing fractions were pooled and concentrated by ultrafiltration. The concentrated protein was then applied to a 2.5 x 60 cm gel filtration (Sephacryl HR-100) column, and eluted with 0.1 M potassium phosphate, pH 7.0. Purity was checked by 12% SDS-PAGE stained with Coomassie blue (R250). The protein concentration was estimated by $OD_{281}$ using the extinction coefficient 29,400 $M^{-1}cm^{-1}$ (11).

Kinetics

Michaelis-Menten (22) kinetic parameters ($k_{cat}$, $K_M$, and $k_{cat}/K_M$) for the chimeric β-lactamase activity were determined by initial velocity methods using an Eadie-Hofstee replot (23). Measurements were obtained using a Jasco J600 spectropolarimeter with 1 mm pathlength optical quartz cells. Kinetic analyses of ß-lactamase activities were performed at 30°C, pH 7.0, 0.1 M potassium phosphate. The hydrolysis of the lactam bond was monitored at 231.8 nm to obtain initial velocities ($\Delta\theta$ = 391.4 mM $^{-1}$ cm$^{-1}$ for benzyl penicillin) (24). Buffers were maintained at 30°C prior to beginning the assay to avoid error from temperature flux.

Transpeptidase activities for the transfer of the benzylpenicilloyl acyl-enzyme hydrolysis product to glycine or similar amino acceptor were measured using potassium [$^{14}$C]-benzyl penicillin (58.5 mCi/mmol). The radioactive substrate was diluted with nonradioactive benzyl penicillin (1:100). Reactions were initiated with 24 μg enzyme, 50 mM benzyl penicillin, 0 to 200 mM glycine, in 0.05 M potassium phosphate, pH 7.5. The reactions were incubated for 6 hours at 37°C, after which time 2 μg RTEM-1 ß-lactamase was added to remove any remaining unhydrolysed substrate (25). The percent transpeptidation was determined by separation of the two products, phenylacetylglycine and phenylacetylglycylglycine, by thin-layer chromatography (buffer: 100 ml AcOH, 825 ml BuOH, 75 ml $H_2O$). Bands were excised from the TLC plates and analyzed for radioactive content using a Beckman LS9000 scintillation counter. To obtain the pH vs. activity plot, reactions were performed at 50 mM benzyl penicillin, varying pH and buffer systems: 0.1 M KOAc, pH 5.0 and 6.0; 0.1 M Tris-HCl, pH 7.0, 7.5, 8.0, 8.5 and 9.0; 0.1 M $KH_2PO_4$, pH 7.0 and 8.0; 0.1 M $K_2CO_3$, pH 8.5 and 9.0.

D,D-carboxypeptidase activities were determined at 37°C, pH 7.5, 0.1 M Tris-HCl. A fluorescence assay (26, 27) was employed to measure the carboxypeptidase activity utilizing diacetyl-L-Lys-D-Ala-D-Ala (28) as a substrate. Reactions (0.25 ml) were initiated at several substrate concentrations. Aliquots of 50 µl were collected at intervals of 30 minutes and added to 2 mls of buffered reagent [1.5 ml *o*-phthaldialdehyde (5 mg/ml ethanol), 1.5 ml 2-mercaptoethanol (5 µl/ml ethanol), 90 ml aqueous 0.05 M sodium tetraborate, pH 9.5]. The samples were analyzed within 15 minutes of mixing, on a SLM-4800 fluorescence spectrophotometer. Excitation and emission monochromators were set at 340 nm and 455 nm, respectively, with a 2 nm slit width. The fluorescence value was corrected against the time-zero value and the concentration of D-alanine determined from a calibration curve. Kinetic parameters were determined from Eadie-Hofstee plots (23) of initial velocity data. To assay the hydrolysis of the depsipeptide diacetyl-L-Lys-D-Ala-D-Lac (29), parallel reactions were initiated at a single substrate concentration. Reactions were quenched at timed intervals by snap-freezing in dry ice/ethanol. The remaining quantity of depsipeptide starting material in the samples was determined by isolation on ABI/Kratos HPLC using a C-18 reversed phase column and linear gradient of acetonitrile/water (0.1% trifluoroacetic acid). The gradient was as follows: Buffer A = 0.1% TFA in water, Buffer B = 0.1% TFA, 80% acetonitrile in water; t = 0, 100% A; t = 3, 100% A; t = 33, 20% B; t = 41, 100% B. The protein content of the samples was quantified by $OD_{214}$. A blank run (starting material only) was performed for calibration. To assay the chimeras for transpeptidase activity utilizing the acyclic peptide substrates, depsipeptide digests (0.1 M Tris-HCl, pH 7.5, 37°C) were initiated with [$^3$H]-glycine present in the reaction mixture. The reaction was quenched at 3

hours and the mixture analyzed by HPLC for the formation of the tripeptide, diacetyl-Lys-D-Ala-Gly and the incorporation of the radiolabel. Control reactions using S70G-RTEM-1 ß-lactamase (30) and bovine serum albumin (BSA) were performed to insure the absence of activity from external sources.

Trapping the Acyl-Enzyme Intermediate

Attempts to isolate a stable acyl-enzyme intermediate, involving the chimeras with benzyl penicillin, were performed with [$^{14}$C]- and [$^{35}$S]-labeled substrates (Figure 7). Enzyme (50 µg) was incubated at 37°C with a 1 in 100 dilution of radiolabled substrate in 50mM benzyl penicillin (100 µl; 0.1 M $KH_2PO_4$, pH 7.0). The reactions were quenched (31) at 1, 3, 5, and 10 minutes with 50% (v/v) triflouroacetic acid (10 µl) and dialyzed against distilled water. The radioactive content was quantified using a Beckman LS9000 scintillation counter.

## RESULTS AND DISCUSSION

The R61/RTEM-1 chimeric proteins were expressed in *E. coli* strain D1210 cells and recovered from the periplasm by osmotic extrusion (21). The chimeras were purified from the crude extract by anion exchange chromatography followed by gel filtration. The purity of the enzymes was checked by 12% SDS-PAGE stained with Coomassie blue (R250).

Due to the extremely low rate of ß-lactam hydrolysis by the chimeras, the hydrolysis of these substrates was measured by the loss of molar elipticity at 231.8 nm using a Jasco J600 spectropolarimeter (24). Use of this CD technique was advantageous because this method allows extremely high enzyme concentrations to be used (>1 mg/ml) without the prohibitively high protein background absorbance encountered with UV spectrophotometry. Controls were performed for all kinetic determinations with equal concentrations of S70G ß-lactamase (30) and BSA substituted for the chimeras to insure the activity observed was catalysis by the chimeras.

Table I collects the rates of ß-lactam hydrolysis catalyzed by the R61.C (D131N, N132F) and R61.D (N132F) chimeras compared to the RTEM-1 and R61 parent enzymes. The rate of substrate turnover was reduced by four and three orders of magnitude, respectively. While these rates differ from each other by only one order of magnitude, the mechanism of the ß-lactam hydrolysis by the two chimeras is different. R61.D (N132F) yields a single hydrolysis product from benzyl penicillin, benzyl penicilloic acid--the same product produced by the class A ß-lactamases. In contrast, R61.C (D131N, N132F) yields phenylacetylglycine--the corresponding hydrolysis product formed by the PBPs (4). No penicilloic acid was observed even in low concentrations, as determined by the use of radiolabeled substrates. This finding has direct mechanistic implications which will be discussed below.

## TABLE I

Kinetic parameters of the R61.C & R61.D chimeras utilizing benzyl penicillin as a substrate. The products of the substrate hydrolysis and the corresponding product with the reaction in the presence of glycine are given. Parameters were obtained by CD spectropolarimetry. *Pen* - penicilloic acid, *pag* - phenylacetylglycine, *pagg* - phenylacetylglycyl-glycine.

| Chimera | $K_M$ (mM) | $k_{cat}$ ($sec^{-1}$) | $k_{cat}/K_M$ | Product | w/glycine |
|---|---|---|---|---|---|
| R61.C | $1.2 \pm 0.1$ | $7.5 \times 10^{-4} \pm 2.5 \times 10^{-4}$ | $6.25 \times 10^{-1}$ | pag | pagg |
| R61.D | $1.7 \pm 0.14$ | $2.1 \times 10^{-3} \pm 1.3 \times 10^{-4}$ | 1.24 | pen | pen |
| ß-lactamase | 0.02 | 2000 | $1 \times 10^{8}$ | pen | pen |
| R61 Cpase | ------ | $1.4 \times 10^{-4}$ | ------- | pag | pagg |

The presence of glycine, or a similar amino acceptor, has no effect on the rate of ß-lactam hydrolysis, nor does it alter the reaction products in either RTEM-1 ß-lactamase or R61.D. However, like the PBPs, the double mutant R61.C yields a mixture of phenylacetylglycine and phenylacetyl-glycylglycine (Figure 3) when reacted under the same conditions (4,25). Thus the double mutation recruits a transpeptidase activity into the chimera. The effects of pH and glycine concentration on this transpeptidase activity were measured. Not surprisingly, the pH profile for this reaction is similar to that observed in the R61 parent enzyme (25), with activity decreases below pH 5.0 and increases as pH is increased (Figure 4), thereby increasing the nucleophilicity of the free amino group. What is surprising is that the transpeptidase activity increases with increasing glycine concentrations up to ~50 mM glycine, after which it levels out, with ~60% of the products being the result of transpeptidation (Figure 5). This result implies that there is a binding site for glycine that is distinct from the binding site for water. Therefore, above 50 mM glycine the relative rate of hydrolysis to aminolysis is controlled by the individual rates of the two reactions and not by substrate concentration. The R61 carboxypeptidase exhibits a similar reaction profile (25) with a saturation concentration of 25 mM glycine. In the native enzyme, however, the transpeptidation reaction is dominant, as seen by a ratio of hydrolysis to transpeptidase products of 1:10.

The synthetic substrates, diacetyl-L-Lys-D-Ala-D-alanine (28) and diacetyl-L-Lys-D-Ala-D-lactate (29), were employed to measure D-Ala-D-Ala-carboxypeptidase/transpeptidase activities. Despite the use of large concentrations of either substrate, it was not possible to detect hydrolysis above background. Two commercially available cell wall precursors were

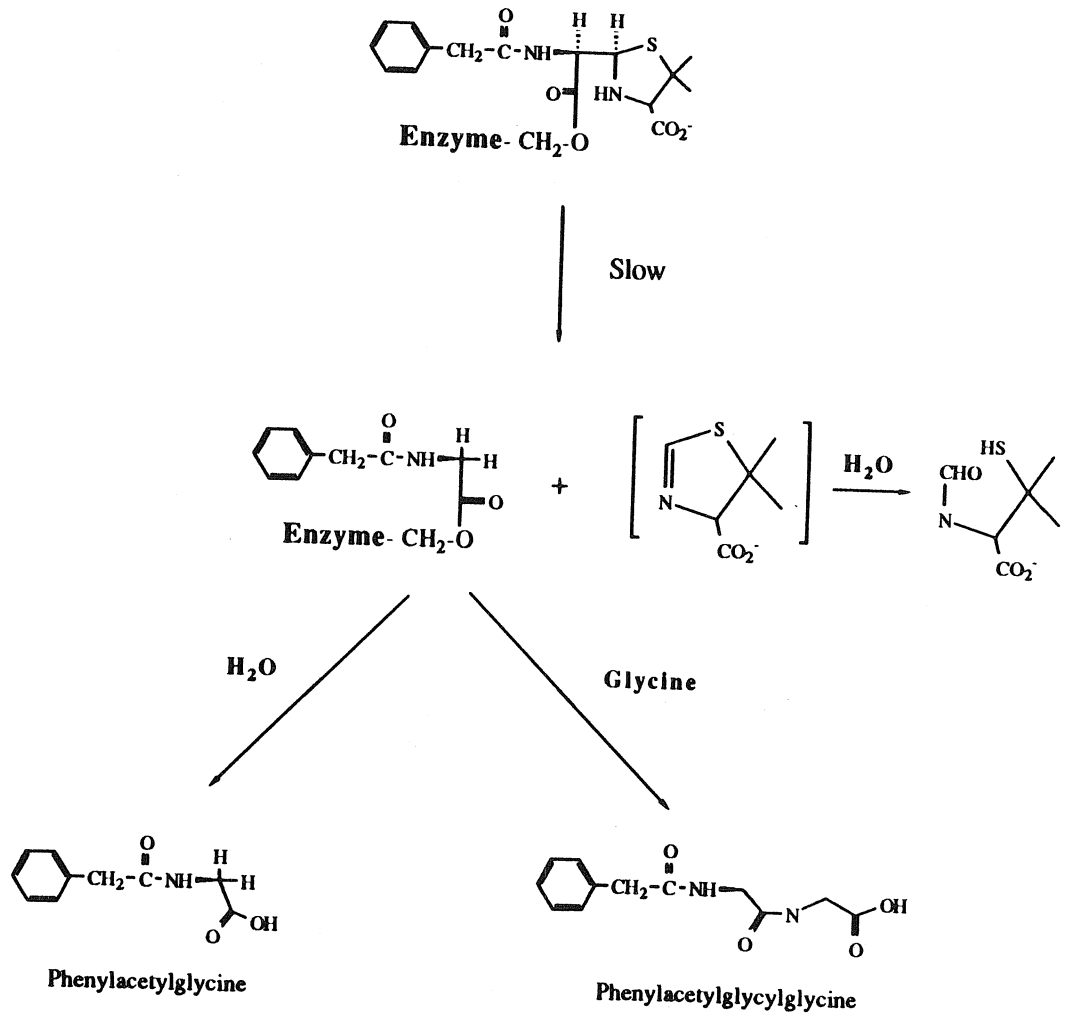



**Figure 3** - Reaction of the R61.C chimera with benzyl penicillin yields phenylacetylglycine; in the presence of glycine the transpeptidation product, phenylacetylglycine, is also produced.

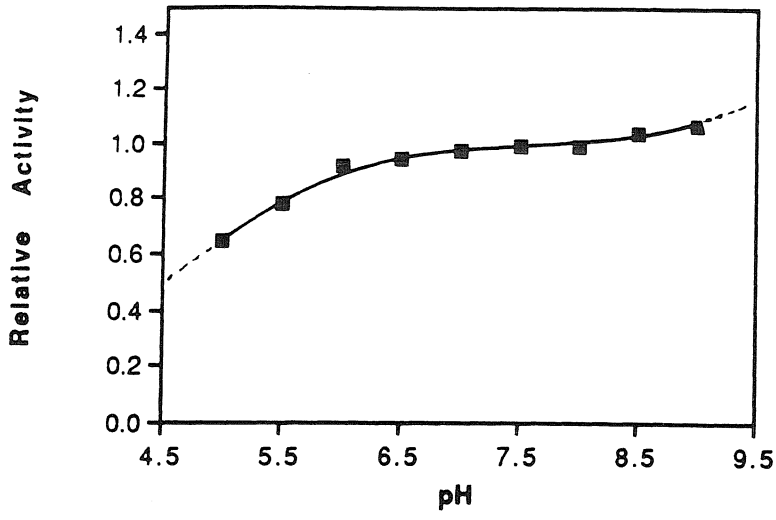



**Figure 4** - pH dependency of transpeptidation observed with the R61.C chimera utilizing benzyl penicillin as a substrate and glycine as an amino acceptor.

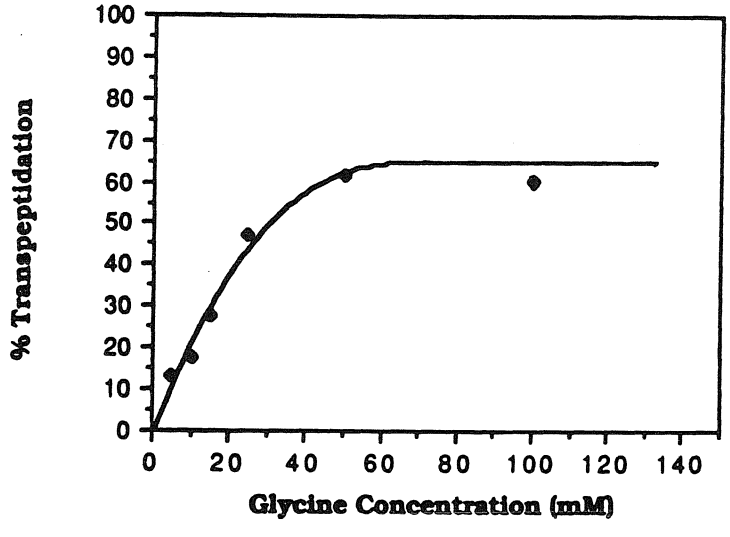



**Figure 5** - Transpeptidation activity (% of total products) observed with the R61.C chimera utilizing benzyl penicillin as a substrate and glycine as an amino acceptor.

also tried; neither Ala-D-isoglutaminyl-Lys-D-Ala-D-Ala (32) or Ala-D-γ-Glu-Lys-D-Ala-D-Ala (33) yielded any more definitive results. However, reaction of the R61.C (D131N, N132F) chimera with the depsipeptide diacetyl-L-Lys-D-Ala-D-lactate in the presence of 100 mM glycine yielded a tripeptide peak (Figure 6) not observed with R61.D (D131N), wild-type ß-lactamase or the PBP-5 RTEM-1 chimera. Use of [$^{3}$H]-glycine revealed an incorporation of the radioactive label in the tripeptide peak. While this carboxypeptidase/transpeptidase activity was low, the lack of similar activity in any of the other enzymes tested suggests that the observed activity is intrinsic to the R61.C chimera. Why the activity is low is not obvious; it is possibly a result of improper or inefficient substrate binding or geometry, or possibly the inability to deacylate the acyl-enzyme intermediate which is generated.

The chimeric enzymes were incubated with [$^{14}$C]- and [$^{35}$S]-benzyl penicillins (Figure 7) in an attempt to isolate a stable acyl-enzyme intermediate implied by the low turnover rate ($t_{1/2}$ = 15 minutes). Unless the intermediate could be isolated, it is not safe to assume that it is deacylation and not acylation that is hindered. The use of the two labeled substrates was designed to reveal whether the acyl-enzyme formed was the serine ester-linked benzyl penicilloic acid species (both labels would be present) or the fragmented serine ester-linked phenylacetylglycine species (in which case only the [$^{14}$C]-label would be present). Incubation of the enzymes with a mixture of radioactive and non-radioactive substrates for several timed intervals was followed by quenching, yielding reproducible results demonstrating the presence of the [$^{14}$C]-substrate for both the R61.C (D131N, N132F) and the R61.D (N132F) chimeras. Therefore, deacylation is blocked in both chimeras by the presence of the phenylalanine side-chain.

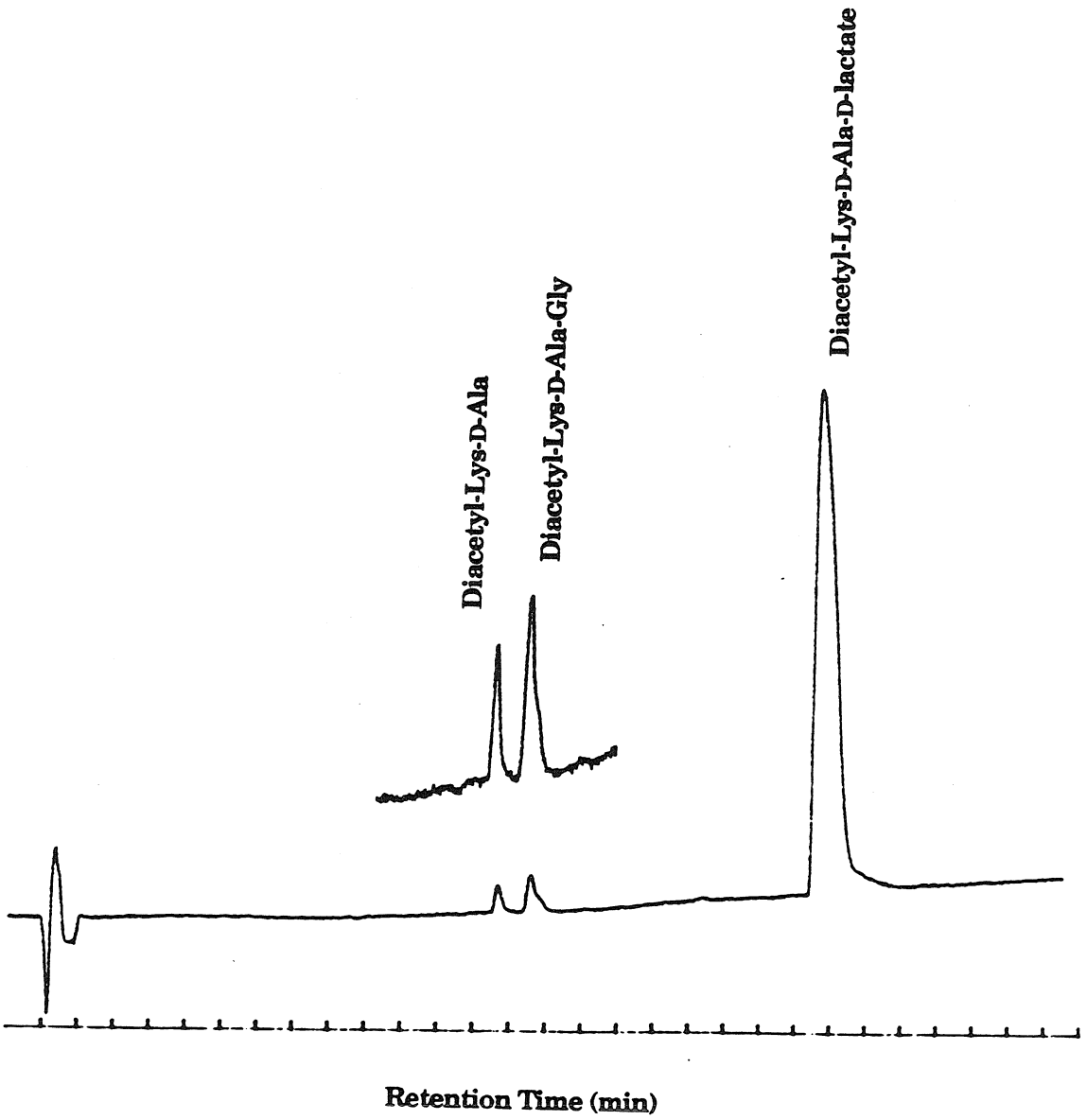



**Figure 6** - HPLC trace of the reaction of the R61.C chimera with diacetyl-L-Lys-D-Ala-D-lactate in the presence of [$^3$H]-glycine. The tripeptide peak was found to contain the radioactive label, thus showing a low level of D-Ala-D-Ala transpeptidase activity by the chimera.

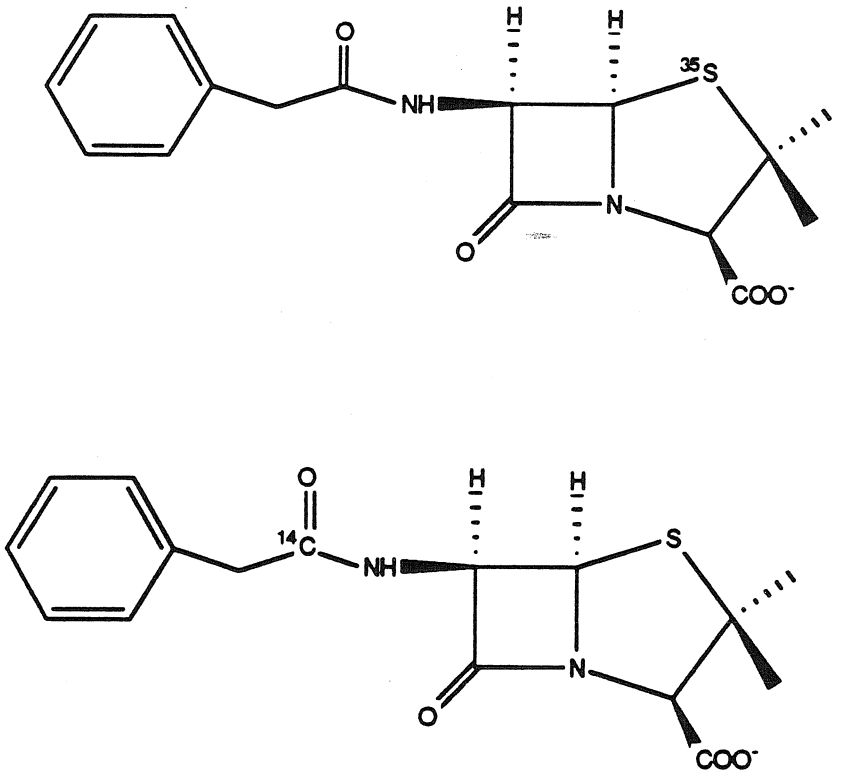



**Figure 7** - Isotopically labeled substrates used to trap the acyl-enzyme intermediate formed between the chimera and the ß-lactams.

The wild-type RTEM-1 ß-lactamase control showed no incorporation of the radioactive label. The same experiments with the [$^{35}$S]-substrate were inconclusive due to extremely high background radioactivity with the chimeric and wild-type enzymes. Attempts to isolate the fragmented thiazolidine ring hydrolysis product, N-formyl-D-penicillamine, using the [$^{35}$S]-substrate were also unsuccessful due to this high background level of radioactivity.

It is imperative, especially with low levels of activity, to insure that the observed activities are due to the chimera and not a contamination. Based on the following criteria, I am confident that the activities observed with the R61.C (D131N, N132F) chimera are properties of the enzyme: i) cells transformed with the plasmid encoding the R61.C chimera demonstrate an altered cell morphology and reduced growth rate unobserved with other plasmids, ii) the R61.C chimera coelutes during the protein purification process with the R61.D chimera and wild-type ß-lactamase, yet neither R61.D nor wild-type ß-lactamase exhibit any of the noted carboxypeptidase or transpeptidase activities, iii) the lack of activity in control reactions with S70G ß-lactamase and BSA show the reactivity is catalysed by the chimera.

## CONCLUSION

The results obtained from the comparison of the R61.C and R61.D chimeras are not only important to the investigation of the proposed evolutionary relationship (34) between the class A ß-lactamases and the bacterial cell wall D-Ala-D-Ala carboxypeptidases, but also have elucidated an important feature in the mechanism of ß-lactam hydrolysis by the ß-lactamases.

The wild-type RTEM-1 ß-lactamase reacts with the ß-lactam antibiotics via nucleophilic attack on the lactam carbonyl group by the active-site serine hydroxyl group. This results in an acyl-enzyme intermediate which rapidly deacylates to yield penicilloic acid. This reaction course is not altered by external nucleophiles such as methanol, ethylene glycol, or hydroxylamine (35). In contrast, the R61 carboxypeptidase forms an acyl-enzyme intermediate by a similar nucleophilic attack (36, 37), yet is unable to deacylate at the rates observed in the ß-lactamase. Before the deacylation can be accomplished, the $C_5$-$C_6$ bond is broken and the thiazolidine ring is separated from the penam substrate (36, 38). After this fragmentation of substrate occurs the enzyme is able to deacylate. It is partially due to these mechanistically different reaction profiles that the evolutionary relationship between the two enzymes has been challenged (4).

An R61/RTEM-1 chimera, containing the D131N, N132F double mutation, has shed light on the relationship of the two groups of enzymes. The chimera is capable of forming the serine ester-linked acyl-enzyme intermediate with the ß-lactam antibiotics (11). (It sould be noted that one cannot state with absolute confidence that the residue involved in the nucleophilic attack of the substrate is still serine 70, however the sequence homologies between the PBPs and the ß-lactamases imply that serine 70 is

still the active-site residue.) However, after this point the chimera no longer functions as a ß-lactamase. Similar to its R61 parent, the chimera is unable to deacylate prior to dissociation of the thiazolidine ring. After fragmentation occurs, the product is then hydrolysed or transferred to an amino acceptor such as glycine, just as with the R61 acyl-enzyme (25).

The finding that no penicilloic acid is released from the chimera, in conjunction with the fact that the wild-type ß-lactamase rapidly deacylates yet cannot transfer the acyl-enzyme intermediate to any species (i.e., hydroxlamine, methanol, etc.) except water (35), suggests that the water necessary for ß-lactam hydrolysis must be present in the binding cavity prior to substrate binding, and further suggests that this water is absent in the chimera because its binding site has been destroyed by the replacement of the carboxamide side chain of asparagine with the hydrophobic phenyl ring.

Unlike the ß-lactamase, the R61 carboxypeptidase is unable to hydrolyse the benzyl penicilloyl acyl-enzyme intermediate prior to substrate fragmentation (25). Joris *et al.* (4) have theorized that the R61 acyl-enzyme intermediate is stabilized by "geometric protection" of the ester bond. Only after fragmentation of the penam's $C_5$-$C_6$ bond occurs, resulting in a half vacant binding cavity by the loss of the thiazolidine ring and undoubtedly a change in conformation based on newly acquired degrees of freedom, will the ester be accessible to external nucleophiles. The observation that transpeptidation is increased in the R61 enzyme by a decrease in water content of the reaction mixture (39) supports the theory of the absence of a prebound water in the R61 enzyme. In the chimeras, therefore, insertion of the hydrophobic phenyl ring into the ß-lactamase binding cavity denies the residue which is involved in deacylation access to the water molecule

necessary for deacylation to occur. Consequently, both the chimera and wild-type R61 are able to catalyze the transpeptidation of the phenylacetylglycyl intermediate.

The presence of saturation kinetics demonstrates that the process of transpeptidation is unequivocally enzyme dependent and not simply a function of diffusion. This saturation also implies a separate and distinct binding site for the hydrolysing water and amino acceptor molecules (25). It could be possible that the thiazolidine ring and the amino acceptor share a common binding site; the glycyl carboxyl group could bind, with a necessarily lower affinity than the substrate, to the amino group of residue 234. However, this is not clear since the effects of high concentrations of glycine vary among the PBP's.

While the extremely low levels of carboxypeptidase activities with the D-Ala-D-Ala substrates could be interpreted as a shortfall of the R61.C chimera, some transpeptidation was observed using the depsipeptide in the presence of [$^3$H]-glycine. This is a dramatic result considering only two amino acids were exchanged in the ß-lactamase. These results, in combination with the transpeptidation observed with the ß-lactams, thereby show that the additional helix G (40) observed in the low resolution R61 crystal structure need not be present for transpeptidation.

However concise these findings may be, there is the question of why the R61.C (D131N, N132F) chimera has such radically altered catalytic activities, whereas the R61.D (N132F) chimera merely exhibits ß-lactamase kinetics at a sharply reduced rate. It is reasonable to conclude that the N132F mutation blocks deacylation in both the R61.C (D131N, N132F) and R61.D (N132F) chimeras (acyl-enzyme trapping experiments), presumably by creating a hydrophobic environment proximal to Glu 166, but this alone,

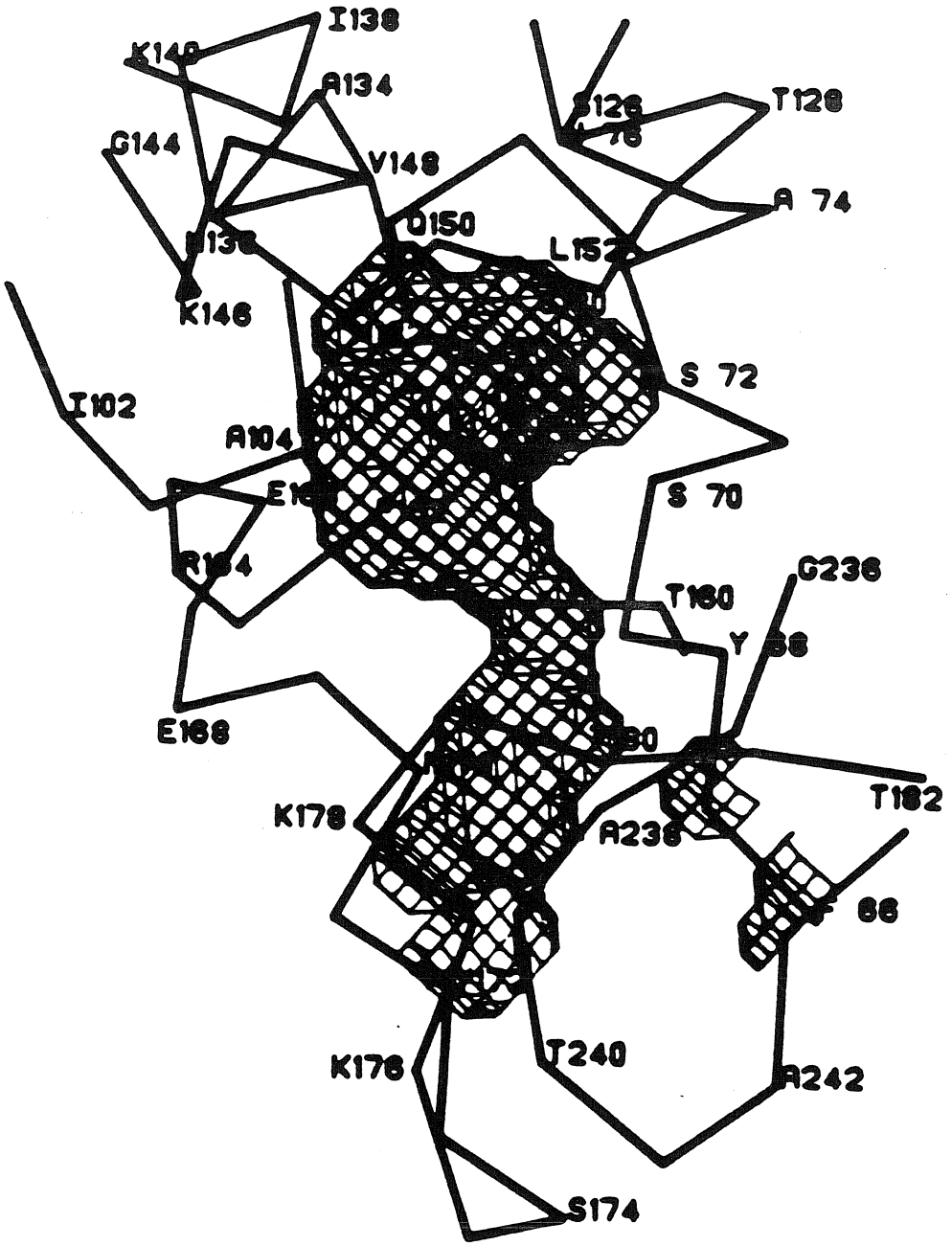



**Figure 8** - The solvent accessible space of the class A ß-lactamases is remarkably large as seen by the shaded areas of the figure. It is believed that the water molecule necessary for deacylation of the acyl-enzyme complex is present in the binding site prior to substrate binding.

as demonstrated, is not enough to alter the mechanism of hydrolysis. The solvent accessible space (Figure 8) in the class A ß-lactamases is remarkably large (7). Evidently, water must be able to remain present in the binding site of the R61.D chimera, possibly hydrogen bonded to Asn 170. If water were entering the cavity, other small nucleophiles (i.e., hydroxylamine, glycine, etc.) might also be able to do so; this is not observed with the R61.D chimera. The mutation of Asp 131 must result in a conformational change in the protein that excludes this water from the active site.

The side chain of residue 131 is buried within the hydrophobic core of the protein (8). Research which has investigated the role of charged residues within the hydrophobic cores of proteins (41), has shown that 80% of the charged residues were involved in salt-bridges and the remaining 20% of charges are involved in hydrogen bonding to 2 or 3 other residues. This second type of hydrogen bonding motif is observed in the crystal structure of the ß-lactamase from *B. licheniformis* (8) for the aspartic acid residue in question. CD spectra demonstrate that the structure of the R61.C chimera is different from both the R61.D and wild-type enzymes. Also, although the melting temperatures differ by only five degrees, the periplasmic levels of the chimeras are vastly different (Chapter 3, page 121). It would therefore appear that the increased proteolytic cleavage of the otherwise thermally stable protein is a result of this conformational change. These combined results are evidence that the isosteric replacement of the aspartic acid with the asparagine results in a change in hydrogen bonding which is propagated through the protein. I believe it is the combination of these two factors, hydrophobicity and conformation, that ultimately results in the altered catalysis.

The further investigation of other residues in the ß-lactamases may yield a chimera capable of utilizing the D-Ala-D-Ala substrates more efficiently and thereby lend greater credence to the theory of a close evolutionary development. Site saturation experiments to fully investigate the roles of residues 131 and 132 are presently ongoing (42) in our laboratory. Why the ß-lactamases exhibit no activity toward the D-Ala-D-Ala dipeptide is still an open question. If the ß-lactamases did evolve from a common PBP ancestor, this lack of activity is surely not without purpose. At the point of evolutionary divergence from the PBPs, the early inefficient ß-lactamases would need to be present in reasonably high cellular concentrations for full effectiveness. It has already been demonstrated that the overexpression of an enzyme capable of transpeptidation is detrimental to bacterial cell function. Thus the selective pressures would favor ß-lactamase activity while selecting against carboxypeptidase and transpeptidase activities. I feel the results from the R61 chimeras have added the most concrete proof to date that the class A ß-lactamases have evolved from the D,D-carboxypeptidases by demonstrating that the necessary catalytic machinery for carboxypeptidase and transpeptidase activities is present in the ß-lactamase framework.

In conclusion, this work has once again shown the tremendous power of site-directed mutagenesis as applied to understanding the structure/function relationships in proteins and in the development of stable enzymes capable of functioning as novel biological catalysts. Work in these areas will afford new catalysts capable of performing simple, and complex, organic transformations and lead to a better understanding of protein design.

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# Appendix I

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# Substrate Specificities in Class A β-Lactamases: Preference for Penams vs. Cephems. The Role of Residue 237

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*ABSTRACT* **Site saturation mutagenesis has been carried out at Ala-237 in RTEM-1 β-lactamase to assess the role of this site in modulating differences in specificity of β-lactamases for penams vs. cephems as substrates. (An Ala-237 Thr mutation had previously been shown to increase activity on cephems by about 30–80%.[1,2]) Screening of all 19 possible mutants on penams and cephems revealed the even more active Ala-237 Asn mutant. Detailed kinetic analysis shows that this mutant has about four times the activity toward cephalothin and cephalosporin C as the wild-type enzyme. Both mutations reduce the activity toward penams to about 10% that of RTEM-1 β-lactamase and lower by about 5°C the temperature at which the enzyme denatures. Functional properties of the other mutants have also been surveyed. The most interesting aspect of these results is that two quite disparate amino acids, threonine and asparagine, when introduced for Ala-237, cause such similar changes in enzyme specificity while more similar residues do not alter the catalytic properties of the enzyme to such a significant degree.**

**Key words: mutagenesis, structure-function relationships, enzymatic catalysis**

## INTRODUCTION

Newly developing techniques of in vitro mutagenesis provide powerful approaches to assess the possible roles of various amino acid residues in determining the three-dimensional structure and function of a protein.[3] Broadly these approaches take two general forms. In one, a specific variant of the protein of interest is generated and its structure and function then examined.

To generate such specific mutants requires some relatively refined insights to design the changes to be introduced. In another family of approaches, many mutants can be generated at one time and, particularly in cases where active mutants confer a readily determined phenotype, their properties can be rapidly assessed. This second approach requires less sophisticated preliminary predictive evaluations and, because many more mutations can be examined than in the case of the generation of specific mutants, allows a much broader search of the effect of change in amino acid sequence on properties. The approach also leads to the examination of mutants that might never have been created in a more highly rationalized study and holds, thereby, considerable potential for surprises.

One form of the second approach entails "saturation" of a site (or sites) in a protein with all 20 natural amino acids followed by an assessment of the behavior of the resulting proteins, most conveniently if they confer an easily determined phenotype, as, for example, resistance to a particular antibiotic.[4] β-Lactamases are a family of enzymes that meets these criteria; they catalyze the hydrolysis of the lactam ring of β-lactam antibiotics (penams, such as penicillin, and cephems, such as cephalosporin C) (Figure 1) thereby inactivating the bactericidal action of these antibiotics. The class A β-lactamases, of which the RTEM-1 enzyme[5] is a member, are characterized by a preference for penams rather than cephems as substrates.[6] In an interesting experiment to alter the specificity of class A β-lactamases, the RTEM-2 enzyme (a close relative of RTEM-1 differing only in residue 39, Glu in RTEM-1, Lys in RTEM-2) was subjected to random chemical mutagenesis induced by treating a plasmid containing the β-lactamase gene with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG).[1] A mutant Ala-237 Thr was recovered that had somewhat increased resistance to cephems such as cephalosporin C and decreased activity toward penams such as benzyl penicillin.[2]

The recently published crystal structure of the related β-lactamase PC1 from *Staphylococcus aureus*[7] shows that residue 237 (Gln in the *S. aureus* enzyme) lies at the edge of the substrate-binding cavity and immediately follows a triad of residues that is highly conserved both in β-lactamases and in

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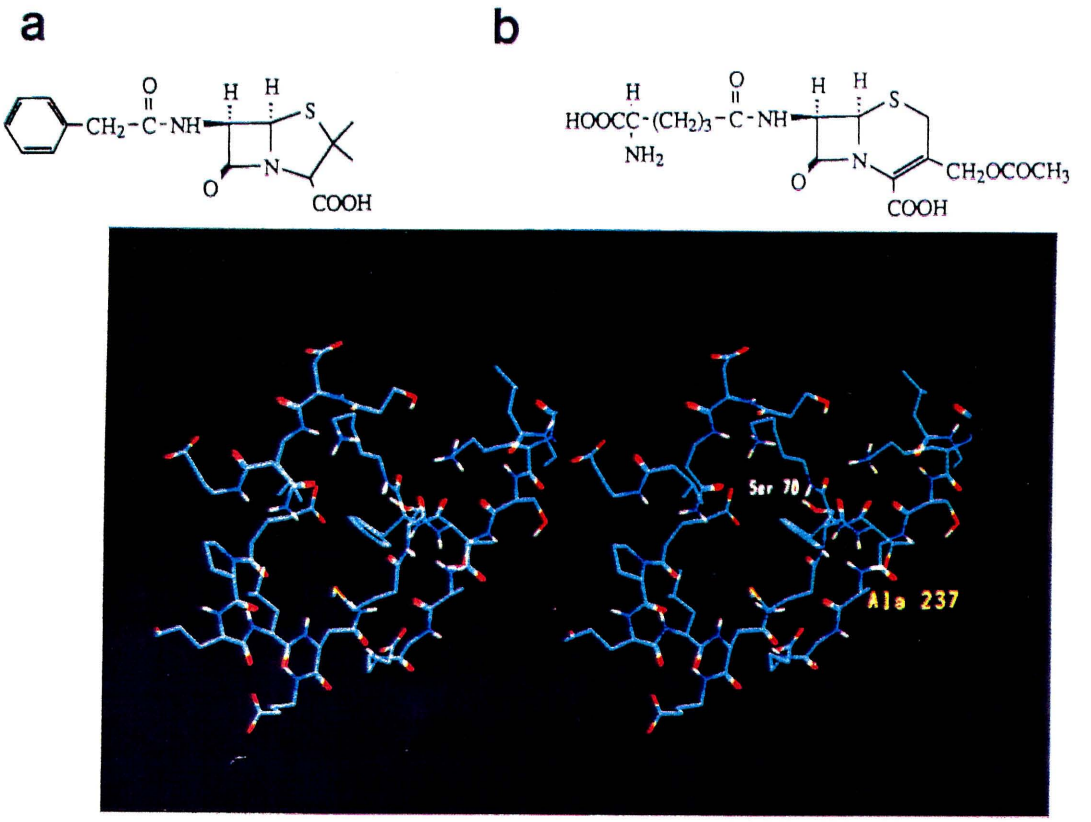


Fig. 1. Representative β-lactam antibiotics and the β-lactamase binding cavity: **(a)** benzyl penicillin (penam), **(b)** cephalosporin C (cephem). **(c)** Stereo representation of the active site of RTEM-1 β-lactamase. The representation was created on an Evans & Sutherland PS390 based on crystal structure data as provided by Herzberg and Moult.[7](Residue 237 in *S. aureus* is actually Gln.)

many penicillin-binding proteins with which the β-lactamases bear striking evolutionary relationships. In the structure of the *S. aureus* β-lactamase, the backbone amide NH of Gln-237, along with that of Ser-70, forms part of a putative oxyanion-binding site that stabilizes the likely tetrahedral species that occurs during the course of enzymatic hydrolysis and leads, upon opening of the β-lactam ring, to an acyl enzyme intermediate whose existence has been demonstrated. Such stabilization of an oxyanion mimics that encountered in catalysis by serine proteases.[8] Additionally, the main chain carbonyl oxygen of Gln-237 is thought to form hydrogen bonds both with the amide NH of the side chain of the β-lactam and with the water molecule that is then involved in the subsequent hydrolysis of the acyl enzyme.

The observation that changes at residue 237 may influence substrate specificity and the location of this residue in an apparently critical region of the protein draw attention to the study of the effect of changes to amino acids, other than those accessible by NTG treatment of the structural gene. Use of NTG as a mutagen results in G·C→A·T transitions,[9] thus allowing only for the conversion of Ala to Thr or Val. Accordingly, to explore the role of this residue in possible control of substrate specificity we have generated all 20 amino acids at residue 237. The approach we used involves cassette mutagenesis[10] to introduce codons encoding all 20 amino acids and an amber stop codon at position 237 of the β-lactamase gene followed by screening of resulting colonies of *E. coli* for their resistance to benzyl penicillin, ampicillin, cephalothin, and cephalosporin C. This approach identified Ala-237 Asn as a mutant whose $k_{cat}/K_m$ for cephalothin is increased by a factor of about 4 and for ampicillin is decreased by a factor of about 10 relative to wild type. This mutant is stable to about 50°C but denatures above this temperature, whereas wild-type RTEM-1 enzyme is stable at 55°C. The other mutant that showed significantly increased activity on cephalothin was Ala-237 Thr (previously described as a variant of the RTEM-2 enzyme).[1,2] Ala-237 Asp and Ala-237 Ser exhibited activities comparable to that of the wild-type enzyme.

## 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 grown in strain HB101[11]; the pJN expression vector was grown in strain D1210, which is lac iQ. Bacteriophage were propagated in strain JM101.[12] Culture media was L broth unless otherwise indicated. Cells were made competent for transformation of plasmid DNA using a process adapted from Hanahan.[13]

### DNA

Oligonucleotides were synthesized by the Caltech Microchemical Facility using phosphoramidite chemistry[14] 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.

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.[15,16] Large scale preparations were further purified using ultracentrifugation in cesium chloride/ethidium bromide gradients. Single-stranded phage DNA was prepared from phage supernatant by precipitation with 20% polyethylene glycol-6000/2.5 M NaCl[17] 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 hours. 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.

### Oligonucleotide-Directed Mutagenesis

To allow cassette mutagenesis at position 237, an *Xho*I site was first introduced at site 4035 in pBR322. This was done using the in vitro mutagenesis system™ by Amersham.[18] After cloning the 2958 bp fragment *Pst*I/*Sal*I from pBR322 into RF M13 mp18 using standard procedures, single-stranded phage was isolated and annealed to the septadecamer 5'-ATGATACCTCGAGACCC (mismatch underlined) by heating to 95°C and slowly cooling to room temperature. Mutagenesis was then accomplished using the Eckstein protocol.[17] Ten plaques were tested and sequenced using the Sanger dideoxy method.[19] Nine of the 10 were positive for the desired mutation. These were then subcloned back into pBR322 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 (NNG/C) for residue 237 were left unkinased and 50 pmol of each strand 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. A three-fragment ligation was then carried out as follows: 1 pmol of the annealed oligonucleotides was combined with approximately 0.12 pmol each of *Xho*I *Bam*HI 3061 base pair fragment and *Bgl*I *Bam*HI 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 hours at 16°C. Ten microliters of the ligation mixture was transformed directly into competent *E. coli* strain HB101 and cells were spread onto 15 mg liter tetracycline plates. In addition, control reactions were run in the absence of insert DNA to test for contamination by wild-type pBR322.

### Phenotypic screening

One hundred colonies from five 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 on the penams at the highest level tested (2000 mg/liter) and on cephalothin and cephalosporin C up to 100 mg/liter.

To avoid the error in estimating activity due to the inoculum effect (wherein colony survival can depend on the number of cells on the toothpick), 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 was spread onto plates containing concentrations of cephalothin ranging from 10 to 1,000 mg liter. 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 liter ceph-

alothin, but gave one or two colonies at up to 80 mg/liter. The most active mutant allowed full growth of *E. coli* at 225 mg liter cephalothin.

### Double-Stranded Plasmid Sequencing

Plasmid DNA was sequenced using a modification of the Sanger[19] dideoxy method for denatured, double-stranded DNA. Denaturation was carried out in 0.2 N NaOH for 5 minutes, then neutralized with $NH_4OH$, pH 4.5 and ethanol precipitated. The DNA pellet was suspended with 5 pmol 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.[20] This kit provides premixed deoxy- and dideoxy-nucleotides, as well as sequenase™—a modified version of T7 DNA polymerase. Labeling was accomplished with [α-$^{35}$S]dATP.

### 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 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 hours. Protein was then transferred from the gel onto DEAE nitrocellulose using a Bio-Rad Transblot® cell for 6 hours at 12 V. β-Lactamase was visualized following binding of rabbit anti-β-lactamase[21] using the highly sensitive Vectastain® ABC immunoperoxidase system.[22]

### Expression of β-Lactamase Mutants in pJN

To facilitate expression, the mutant β-lactamases were subcloned into pJN,[23] an expression vector which was developed in this lab. It contains the β-lactamase gene under the control of the *tac* promoter,[24] as well as a kanamycin gene for a selection marker. Synthesis of β-lactamase is induced by the addition of IPTG. The plasmids harboring the genes for the mutant proteins were digested with *Eco*RI and *Sal*I and the genes then ligated into pJN. Mutants were sequenced to verify the integrity of the cloned gene.

### Protein Purification

One-half liter cultures of *E. coli* D1210 containing mutant plasmid were grown in X broth containing 50 mg/liter kanamycin for 12–14 hours 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.[25] The sample containing the periplasmic proteins was reduced to 1 ml using a Diaflow cell (Amicon) under nitrogen. The resulting solution was then filtered through a Schleicher & Schuell 0.22 5m 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 $t = 0$ minute, 100% A; $t = 28$, 81% A, 19% B; $t = 33$, 100% B. The flow rate was 0.8 ml/minute. Elution was monitored by $A_{280}$; activity was assayed with the hydrolysis of benzylpenicillin. 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 $M^{-1}cm^{-1}$.[2] 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.[26] 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 280 nm for cephems. The enzyme concentration was adjusted so the reaction ran to completion in approximately 20 minutes. For highest accuracy, substrate concentrations 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, benzyl penicillin, cephalosporin C, 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 three fragment ligation scheme was used to insert a cassette of synthetic oligonucleotides containing degenerate bases at position 237 of β-lactamase. This ligation produced a total of 150 colonies on five

**TABLE I. Phenotypic Activity of Alanine 237 Mutants***

| Amino acid | Ampicillin | Benzylpenicillin | 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 |

*Number given is highest concentration of antibiotics (mg/liter) on which colonies grew.

tetracycline plates. Control ligations lacking the DNA insert 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.

Screening for phenotype was accomplished by picking colonies onto plates containing varying concentrations (50–2000 mg/liter) of ampicillin, benzylpenicillin, cephalosporin C, and cephalothin. Seventeen of the original 100 picked colonies were inactive and did not grow on any of the plates. Most of the other colonies had activities approaching that of wild-type. However, eight of the original 100 colonies grew on higher levels of cephalothin (500 mg/liter) than did wild-type (100 mg/liter). To gauge this increased activity more accurately, cells picked from these eight mutant colonies and wild-type were grown to saturation, diluted, and then grown on plates containing varying levels of cephalothin. In this way, the inoculum effect (wherein colony survival can depend on the number of cells on the toothpick) was avoided. In this assay, wild-type β-lactamase survived on 30 mg/liter cephalothin, although a few colonies were observed on plates of higher concentration. Of the eight colonies that had increased activity on cephalothin as determined by picking, four grew on plates with 140 mg/liter cephalothin and one produced colonies at levels of cephalothin as high as 225 mg/liter. This indicates a markedly increased resistance to cephem antibiotics. Of the other four mutants, two produced noticeably more colonies than wild-type up to 100 mg/liter, while the other two gave colonies that behaved essentially the same as colonies that have wild-type enzyme.

Sequencing of these mutants afforded all 20 residues at position 237, and the stop codon TAG. One sequence showed an absence of the insert and yielded a mutant that was inactive. Western blots (Fig. 2) confirmed that all mutants were thermally stable and present in amounts equal to wild-type, except the amber mutant—which showed no band for β-lactamase.

Table 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 Ala-237 Pro (the amber mutant also shows no activity). Of the rest, all grew on either 2000 mg/liter (the maximum level tested) or 1000 mg/liter 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 which show very considerable loss of activity toward cephems while exhibiting close to full activity toward penams. However, many mutants of RTEM-1 β-lactamase do confer a fully resistant phenotype to penicillin G since even though their activities are significantly reduced relative to wild-type. Cephalothin is such a poor substrate that it is a better indicator of minor activity loss. It is noteworthy that one of these mutants, Ala-237 Gln, has the wild-type residue 237 found in *S. aureus* β-lactamase, the only class A β-lactamase that has a residue at 237 other than alanine.

Four mutants which had wild-type activity on

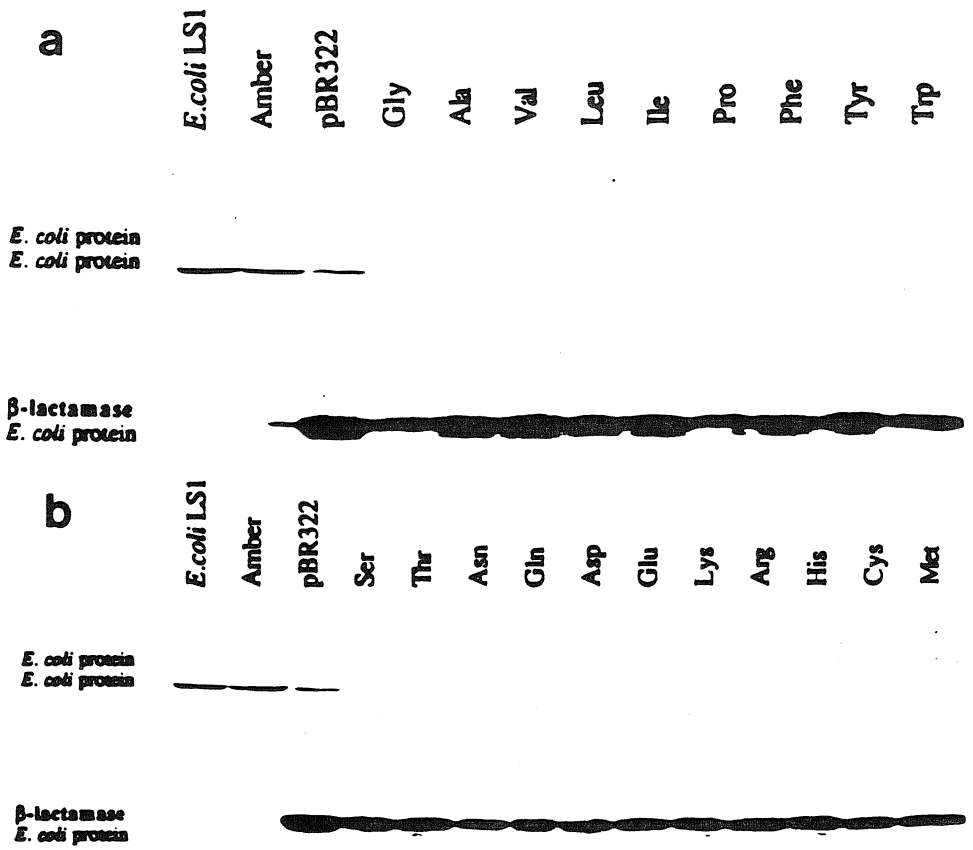


Fig. 2. Western blots of β-lactamase with 19 amino acid substitutions at residue 237. The blots were treated with rabbit anti-β-lactamase antibody and visualized using the Vectastain immunoperoxidase system.

benzylpenicillin and ampicillin showed a significant increase in activity on cephalothin (500 mg/liter): Ala-237 Asn, Ala-237 Asp, Ala-237 Ser, and Ala-237 Thr. These four were also the only mutants to show activity equal to that of wild-type on cephalosporin C (100 mg/liter). Since picking colonies onto antibiotic plates is at best only an approximate method for determining phenotypic activity, these mutants were also tested for activity by plating dilute cell growths onto varying concentrations of cephalothin. Table 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/liter cephalothin, as do the aspartate and serine mutants. However, colonies appear as high as 140 mg/liter cephalothin for threonine and 225 mg/liter for asparagine (intermediate concentrations were not tested).

The two mutants showing greatest resistance to cephem antibiotics, Ala-237 Thr and Ala-237 Asn, were chosen for further study. The genes encoding them were first subcloned into the expression vector pJN, which has the β-lactamase gene under control of the inducible *tac* promoter. Successful subcloning was verified by sequencing. Crude mutant enzymes were then isolated as described in Methods. Final purification involved FPLC, using a NaCl gradient. β-Lactamase eluted at about 20 minutes as seen by $OD_{280}$ and confirmed by assay on benzylpenicillin. Purity of the pooled fractions was assessed on a polyacrylamide gel and staining with Coomassie blue. The average protein yield was 1 mg of wild-type β-lactamase and 300 μg of mutant per 500 ml growth. The yields were lower for mutants since fewer fractions were collected from the FPLC due to a slight shift in peak retention resulting in a poorer 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.[26] The substrates used for the kinetic assays were benzylpenicillin, ampicillin, cephalothin, and cephalosporin C. The results for the penams are listed in Table III; results for cephems are in Table IV. Table V lists the comparative $k_{cat}/K_M$ on the four substrates, relative to wild-type β-

**TABLE II. Phenotypic Activity of Alanine 237 Mutants Plated on Cephalothin***

| | Cephalothin (mg/liter) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Amino acid | 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 |

*Number given is colonies counted after plating diluted cells onto varying amounts of cephalothin.

**TABLE III. Kinetic Parameters of Alanine 237 Mutants and Wild-Type β-Lactamase on Penam Antibiotics**

| | Benzylpenicillin | | | Ampicillin | | |
|---|---|---|---|---|---|---|
| Amino acid | $K_m$ (μM) | $k_{cat}$ ($sec^{-1}$) | $k_{cat}/K_m$ ($M^{-1}sec^{-1}$) | $K_m$ (μM) | $k_{cat}$ ($sec^{-1}$) | $k_{cat}/K_m$ ($M^{-1}sec^{-1}$) |
| Alanine (wt) | 23 ± 2 | 2224 ± 120 | $9.5 \times 10^7$ | 65 ± 1 | 1670 ± 103 | $2.6 \times 10^7$ |
| Asparagine | 144 ± 3 | 840 ± 30 | $5.8 \times 10^6$ | 634 ± 2 | 2670 ± 86 | $4.3 \times 10^6$ |
| Threonine | 54 ± 1 | 315 ± 4 | $5.8 \times 10^6$ | 45 ± 1 | 225 ± 9 | $5.6 \times 10^6$ |

**TABLE IV. Kinetic Parameters of Alanine 237 Mutants and Wild-Type β-Lactamase on Cephem Antibiotics**

| | Cephalothin | | | Cephalosporin C | | |
|---|---|---|---|---|---|---|
| Amino acid | $K_m$ (μM) | $k_{cat}$ ($sec^{-1}$) | $k_{cat}/K_m$ ($M^{-1}sec^{-1}$) | $K_m$ (μM) | $k_{cat}$ ($sec^{-1}$) | $k_{cat}/K_m$ ($M^{-1}sec^{-1}$) |
| Alanine (wt) | 207 ± 15 | 128 ± 7 | $6.1 \times 10^5$ | 687 ± 6 | 57 ± 1 | $8.2 \times 10^4$ |
| Asparagine | 101 ± 2 | 230 ± 11 | $2.3 \times 10^6$ | 956 ± 28 | 299 ± 21 | $3.1 \times 10^5$ |
| Threonine | 66 ± 1 | 50 ± 3 | $7.5 \times 10^5$ | 199 ± 6 | 29 ± 3 | $1.5 \times 10^5$ |

**TABLE V. Comparison of Relative $k_{cat}/K_M$ of Alanine-237 Mutants**

| Amino acid | Benzylpenicillin | Ampicillin | Cephalothin | Cephalosporin 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 |

lactamase and shows that both mutants are markedly more active than wild-type β-lactamase in catalyzing the hydrolysis of cephem antibiotics. Ala-237 Asn is even better than the previously discovered Ala-237 Thr mutant and it shows almost a 4-fold increase over wild-type in differential activity. 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 mutants Ala-237 Asn and Ala-237 Thr. Whereas wild-type enzyme shows long-term stability as high as 55°C, Ala-237 Thr denatures at 55°C quickly and Ala-237 Asn is somewhat unstable above 35°C and denatures rapidly at 55°C; this accounts for its dramatic decrease in activity at this temperature (see Fig. 3). The pH optimum for Ala-237 Asn is 7.0

## DISCUSSION

Site saturation mutagenesis[4] was performed on alanine-237 of RTEM-1 β-lactamase based on the results showing that a mutation (A237T) at that site showed a substantially increased resistance to cephem antibiotics.[1,2] Although Ala-237 is not conserved among the class A β-lactamases,[27] the crystal structure of the enzyme from *S. aureus*[7] shows that the amide backbone of this residue may form part of the oxyanion hole which stabilizes the intermediate. (In *S. aureus* β-lactamase, residue 237 is glutamine.[28]) The goal of producing all mutations at this site was to discover if any change besides threonine would produce an increased resistance to cephalosporins.

All 20 residues (and the amber mutant) were found within the first 42 plasmids sequenced. Enzymatic activity of a mutant was approximately determined by screening a colony of *E. coli* producing the appropriate mutant for resistance to penam and cephem antibiotics. Such an assessment of activity depends on a number of factors in addition to the inherent enzymatic activity of the particular mutant. The amount of the enzyme actually present in the periplasm importantly influences the antibiotic resistance of the colony and this in turn depends on the stability of the mutant in the proteolytically hostile environment.

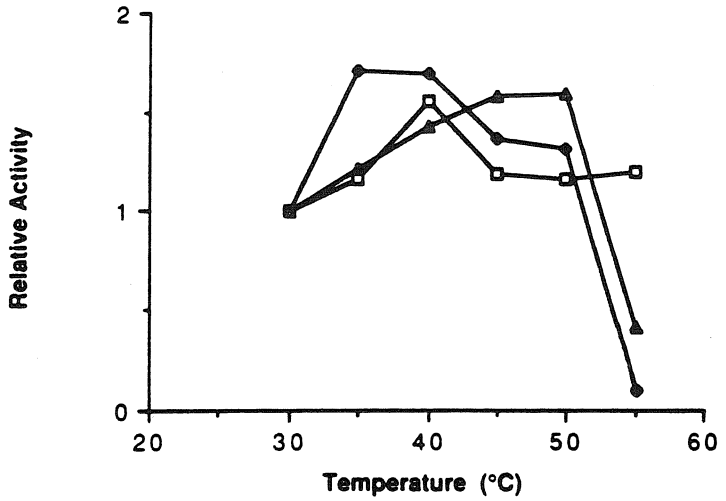


Fig. 3. Plot of relative activity ($k_{cat}/K_M$) versus assay temperature (°C) for wild-type β-lactamse (◇), A237T (—), and A237N (◆). Activity measured at 30°C, pH 7.0 is designated as 1.0.

As measured by the phenotype of *E. coli* colonies, only the mutant Ala-237 Pro was completely inactive; mutants with Ala-237 Arg, Ala-237 Glu, Ala-237 Gln, Ala-237 Ile, Ala-237 Phe, and Ala-237 Val conferred no resistance to cephems, but did provide appreciable resistance to penams, indeed only colonies to Ala-237 Arg and Ala-237 Trp (as well, of course, as Ala-237 Pro) were unable to grow on concentrations of benzylpenicillin of 2000 μg/ml.

Of the more active mutants, Ala-237 Thr and Ala-237 Asn stood out from the others in their abilities to confer resistance to cephems. Kinetic analysis of these mutants showed them to be somewhat less active than wild-type on penams such as benzylpenicillin (6% for both Ala-237 Thr and Ala-237 Asn) and ampicillin (16% for Ala-237 Asn, 22% for Ala-237 Thr) but significantly more active than wild-type on cephems such as cephalothin (380% for Ala-237 Asn, 130% for Ala-237 Thr) and cephalosporin C (380% for Ala-237 Asn, 180% for Ala-237 Thr). Thus, the Ala-237 Asn mutant is very similar to the Ala-237 Thr mutant in its activity toward representative penams but about twice as active toward cephems. In other terms, the RTEM enzyme shows a penam/cephem preference that depends, of course, on the particular substrates being compared and ranges from about 50 to 2000 whereas in the Ala-237 Thr mutant this preference is reduced to about 4 to 40 and in the Ala-237 Asn mutant to about 2 to 20. Thus, for the Ala-237 Asn mutant the ratio has been altered by about twice as much as for the Ala-237 Thr mutant but in neither mutant has a quantitative preference for a cephem substrate been observed. Such a clear preference for cephems is, indeed, one of the important distinctions between class A and class C β-lactamases.[27,29,30] Why if these mutations create better catalysts of cephem inactivation have they not been incorporated more generally into class A β-lactamases? First, because though these mutants are better cephemases they are considerably less active than the wild-type RTEM enzyme as penamases which are the substrates they have likely evolved to inactivate. Second, the thermal stabilities of the two mutants Ala-237 Thr and Ala-237 Asn have been reduced somewhat relative to the wild-type in a structural background that features a stabilizing disulfide bond. Analogous mutations in other β-lactamases that lack this disulfide might be sufficiently more destabilizing as to be significantly disadvantageous.[31]

The most interesting aspect of these relative activities relates less to the somewhat greater activity of Ala-237 Asn than of Ala-237 Thr toward cephems than to the very similar behavior of two mutants that involve such disparate substitutions as Asn and Thr. This suggests that a particular interaction between the substrate (or, more significantly the transition state) and the amino acid side chain is not the dominant feature in the behavior of these mutants, but rather subtle reorganizations of the wall of the active site including the conserved triad from residue 234 to 236.[32] Therefore to understand these significant changes in activities toward various substrates really requires highly accurate three-dimensional information about the structure of the complex between the enzyme (or mutant) and the substrate or, ideally, the transition state of the reaction.

Nevertheless these results dramatize the unex-

pected insights that arise when one uses an approach that assesses a number of mutations produced, for example, by site saturation for those that confer a particular function, in this case an increased activity toward cephems, and focuses those mutations to regions of the protein that more general mutagenesis has shown to be important in determining that aspect of the protein's function.

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