UNIVERSITY OF CALIFORNIA

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MECHANISTIC ANALYSIS OF CLASS A β-LACTAMASES THROUGH SITE SPECIFIC MUTAGENESIS

A dissertation submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY AND BIOCHEMISTRY

by

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December 1992

The dissertation of Walter Alexander Escobar is approved:

Dean of Graduate Studies and Research

TABLE OF CONTENTS

ABSTRACT

We have specifically mutated several residues within the Class A *B. licheniformis* β-lactamase sequence. These residues are believed to play an important role in either catalysis or in the structural stability of these enzymes. The main body of this thesis addresses site-directed mutagenesis studies of glutamate 166 and tyrosine 105 in the *B. licheniformis* β-lactamase enzyme.

Site-specific mutation of Glu-166 to Ala in β-lactamase causes a million-fold reduction in catalytic activity toward both penicillin and cephalosporin substrates, and results in the stoichiometric accumulation of a normally transient acyl-enzyme intermediate. Kinetic analysis indicated that substitution of Glu-166 by Ala leads to negligible effect on the acylation half of the reaction but effectively eliminates the deacylation reaction. Such differential effects on the rates of formation and breakdown of an enzyme-substrate intermediate have not been previously reported. Thus, unlike the situation for most transfer enzymes, e.g. the serine proteases, acylation and deacylation in β-lactamase catalysis are not "mirror" images, and must involve different mechanisms. The results suggest an explanation for the different catalytic activities between the β-lactamases and the penicillin-binding proteins involved in bacterial cell-wall synthesis.

Moreover, glutamate 166 was specifically mutated to aspartate and cysteine in order to probe the function of this residue in catalysis. In both cases a large decrease in activity (~1.2×10^5 for E166C and ~1.7×10^3 for E166D) was observed although the kinetics for the two mutants were very different. The pH profiles for E166D and E166C reflected the ionization

characteristics of the new residue at site 166. We have interpreted this result to indicate that the deprotonation of glutamate 166 is in part responsible for the acidic limb of the W.T. activity-pH profiles. A finding which is in agreement with the hypothesized function of glutamate 166 being that of a general base. In addition, E166D was used to probe the postulated mobility of the Ω-loop upon which site 166 resides. If this region is fairly mobile then the displacement of the carboxyl functional group by 1.5 A should not significantly alter the activity. As noted above, however, the enzyme's activity is sensitive to the movement of the carboxyl functional group. A result which argues against the postulated mobility of this region.

Tyrosine 105 is highly conserved throughout the Class A β-lactamase family. In order to probe the function of the hydroxyl group of this residue we mutated Tyr-105 to phenylalanine. Contrary to our expectations, the structure and activity of Y105F appeared to be unaltered when compared to the wild-type enzyme. This result may indicate that the importance of tyrosine at this site is related to the phenyl group (to a greater extent) rather than the hydroxyl group of the tyrosine residue.

ACKNOWLEDGMENTS

I wish to thank my advisor Anthony Fink for his help throughout my graduate career. I appreciate the room he gave me to learn and grow at my own pace as well as his insight which helped guide my efforts.

I also wish to thank all those in the Fink lab whose help was instrumental in this endeavor. Tony Tan whose good humor and insight into chemistry were invaluable. Keith Oberg whose knowledge of instrumentation (especially the computers) was a great support. Daniel Palleros for his help with kinetics. Linda DeYoung for the discussions on physical chemistry as well as her friendship. Jeff Miller for sharing his knowledge in protein chemistry and helping me keep things in perspective. I thank all the others in the Fink lab for your support.

ABBREVIATIONS

CD, circular dichroism

W.T., wild-type

FAP, 6-((2-furylacryloyl)amido)penicillin

T_m, midpoint of thermal denaturation transition

PADAC, 7-(thienyl-2-acetamido)-3-[2-(4-N,N-dimethylaminophenylazo)

CHES, 2-(cyclohexylaminoethanesulfonic acid)

TRIS, tris-(hydroxymethyl)aminomethane

CAPS, 3-(cyclohexylamino)propanesulfonic acid

TCA, trichloroacetate

SDS, sodium dodecyl sulfate

DEAE, diethylamino ethyl

CM, carboxymethyl

HPLC, high pressure liquid chromatography

This thesis is dedicated to my mother Alicia Oliva whose unwavering appreciation for knowledge and education has been a constant inspiration to me.

Chapter I

Introduction

Background

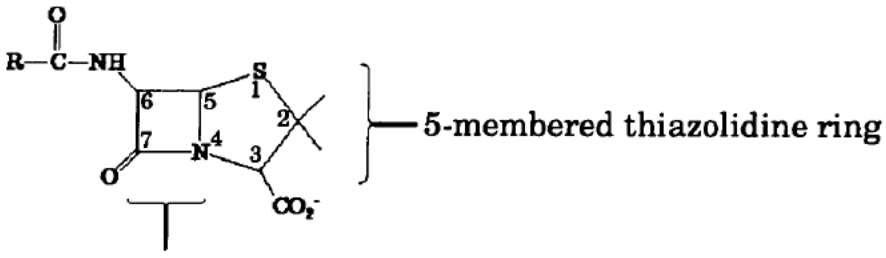
No where is the process of evolution more apparent than in the microbial world. Microorganisms because of their size and physiology are able to reproduce at rapid rates and thus are able to quickly adapt to new selective pressures. A clinically important example of this is the response we have seen from bacteria to treatment with antibiotics. Alexander Fleming noted back in 1929 that *Penicillium notatum* was capable of inhibiting the growth of *Staphylococci* in culture. He postulated the presence of an antimicrobial agent and began a cursory investigation for this material. Unfortunately, Fleming's attempts to isolate this substance, which he termed penicillin, proved ineffectual due to the rapid loss of activity throughout his isolation process (Abraham, 1983).

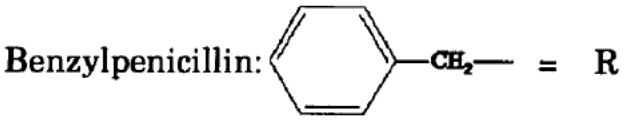
The clinical potential of penicillin was not realized until the late 1930's when H. W. Florey and E. B. Chaney began what was originally an academic survey (Abraham, 1983) of natural products demonstrating antimicrobial activity. Their investigations led to the first clinical trails with benzylpenicillin and the eventual commercialization of this novel drug (see Fig. 1).

Since the end of World War II penicillins, cephalosporins and other antibiotics have received heavy use in treating a large number of bacterial infections. As a consequence, we now find that many bacterial species are becoming resistant to the most commonly used antibiotics. For several years after the war the *Staphylococci* that were encountered remained sensitive to benzylpenicillin. Soon afterwards, resistant strains became common as sensitive strains were eliminated through the application of this bactericide

(Abraham, 1983). Furthermore, we find there has been a rapid increase in the number of sexually transmitted pathogens which are resistant to penicillin. Until 1976 all cases of gonorrhea in the U.S. could be treated with penicillin. Since then the number of resistant *Gonococci* has increased to 2 percent in 1987 and to five percent of all reported cases in 1989 (Aral & Holmes, 1991).

Figure 1. Penicillins are characterized by a beta-lactam ring which is fused to a five membered thiazolidine ring. Cephalosporins differ in that they contain a six membered dihydrothiazine ring fused to the lactam center.





Why Study β-lactamases ?

It is well known that the production of β–lactamases is the principle means by which bacteria achieve antibiotic resistance. These enzymes catalyze the hydrolysis of penicillins and cephalosporins to their biologically inactive products and thus render therapies utilizing these drugs ineffective.

Moreover, since the genes that code for these enzymes are frequently located on plasmids, which are readily shared amongst bacteria, the resistant phenotype is swiftly passed from one cell to another. In light of these increasingly important issues we have chosen to study the specific interactions which occur between these enzymes and their respective substrates. It is believed that a rigorous investigation of the mechanism by which β-lactamases hydrolyze β-lactam antibiotics will lead to the design of penicillin or cephalosporin derivatives which are resistant to the action of β-lactamases.

To fully understand the function of these enzymes it is important to present them in context of their biological melieu. From this we gain insight into the proposed mechanism of β-lactamases and an understanding of their role in the bacterial cell.

Mode of Action and Resistance to β-lactam Antibiotics

A distinguishing feature of prokaryotes is the cell wall. This structure provides rigid mechanical support and prevents the cell's internal osmotic pressure from rupturing the cell's membrane. Although some cell wall components vary, the basic building blocks of the cell wall matrix are evolutionarily conserved. The cell wall is composed of peptidoglycan, a large saccharide polymer which, in many species, is covalently cross linked by tetrapeptides (see Fig. 2). This glycan chain consists of alternating residues of two acylated amino sugars N-acetylglucoseamine (NAG) and N-acetylmuramic acid (NAM) in which only the NAM residues are substituted with the peptide moiety.

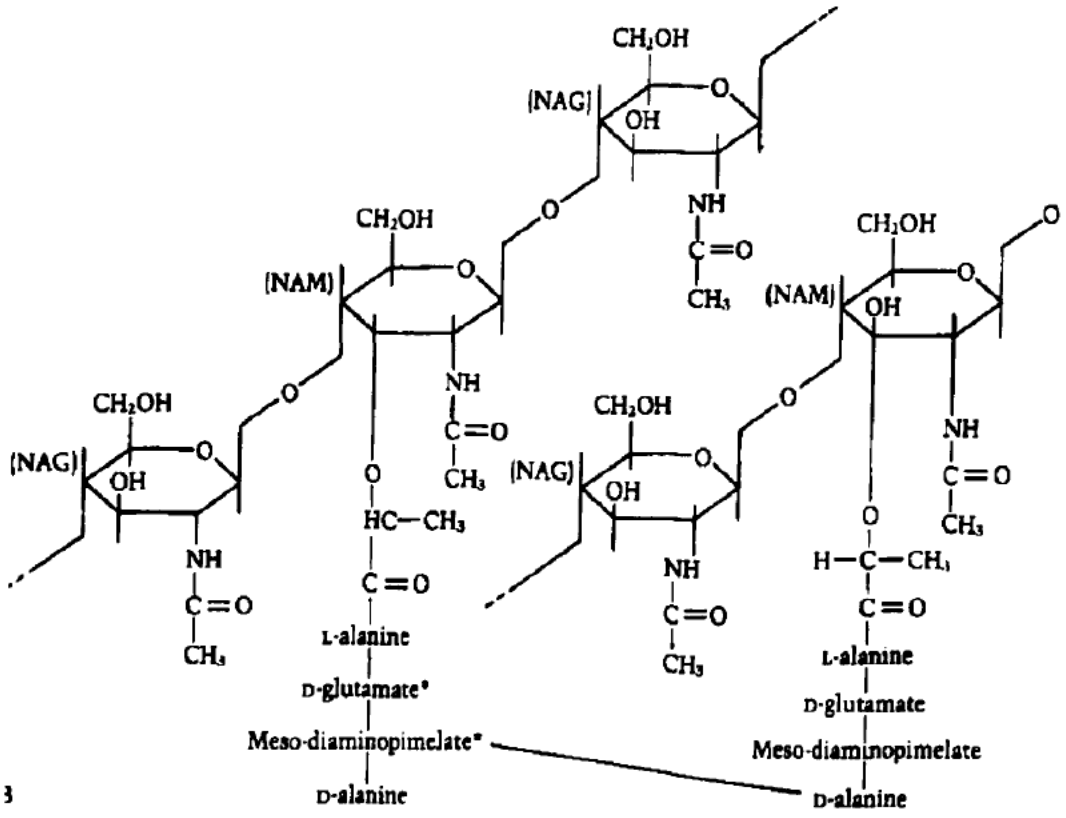


Figure 2. The arrangement of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) residues linked by beta-1,4 glycosidic bonds. Note the alternating L- and D- configuration of the residues which compose the peptide. (Ingraham et al., 1983)

Initially the nascent glycan strands are substituted with pentapeptides comprised of alternating L- and D-amino acid residues (see Fig. 2). The terminal D-alanine is cleaved off in the ensuing cross linking and hydrolysis reactions which occur as the glycan strands are incorporated into the growing cell wall.

As can be surmised, the complex structure of the cell wall requires the action of several enzymes for synthesis. Among these is a group of catalysts

(Ghuysen, 1979) which have been classified as the peptidoglycan cross linking system (PGCS). These enzymes have the unique property of covalently binding penicillin. Consequently, they are also referred to as penicillin binding proteins (PBPs) (for the purposes of this thesis these two terms will be used interchangeably).

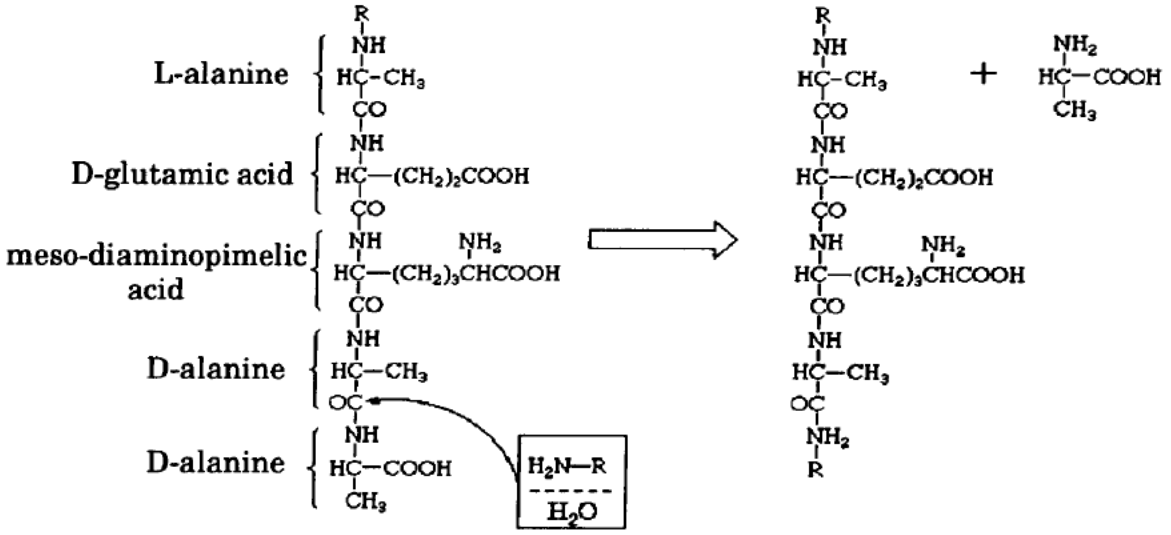
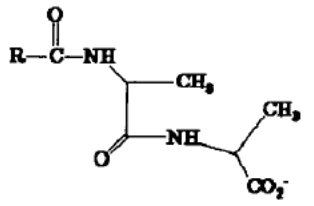


Figure 3. Nucleophilic attack onto the penultimate D-ala residue of the highly conserved D-ala-D-ala carboxy terminus results in either hydrolysis or the formation of a cross link. In this case the primary amine of a meso-diaminopimelic acid residue from another peptide is the nucleophile and the reaction results in the formation of a cross-link. R = peptidoglycan

The bactericidal effects of β-lactam antibiotics are believed to arise from the inhibition of the PGCS. Indeed, it has been demonstrated that bacterial cells treated with penicillins lose the structural integrity afforded them by the cell wall and rapidly convert to spheroid protoplasts (Lederberg, 1956). A function of the PGCS is to catalyze the nucleophilic attack of pentapeptides,

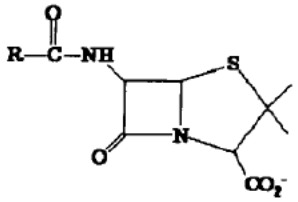
tetrapeptides or H_2O onto the carbonyl carbon of the penultimate D-ala residue of the growing peptidoglycan chain (see Fig. 3). Thus we find that the C-terminal D-ala-D-ala dimer of this peptide is a natural substrate of the PGCS.

Figure 4. The structural similarity of β-lactam antibiotics to natural substrates for PBPs suggests these drugs may act as mechanism based inhibitors.



R = peptidoglycan peptide

Terminal D-ala-D-ala residues



R = defined by specific penicillin

Penicillin substrate

As shown in Figure 4 there is a definite structural similarity between the D-ala-D-ala dimer and β-lactam antibiotics. Consequently, it has been postulated that β-lactam antibiotics may be structural analogs of this dimer and that they act as mechanism based inhibitors of the PGCS. Furthermore, it can be theorized that the structural differences between the D-ala-D-ala carboxyl terminus of the peptidoglycan peptides and β-lactam antibiotics result in the successful transpeptidation or hydrolysis of the former substrate

and in the accumulation of a stable acyl-enzyme complex with the members of PGCS in the latter.

Classification of β-lactamases

Matthew and Harris (1976) have demonstrated that β-lactamase activity is a ubiquitous feature amongst microorganisms. This is supported by investigations (Ghuysen, 1979) which indicate that the β-lactamase function may be an intrinsic property of some PBPs. This is not an unreasonable finding since "β-lactamases" besides sharing structural similarities with PBPs also share some of the same catalytic machinery (Joris et al., 1988). The β-lactamase family of enzymes, however, is distinguished from PBPs by the high rates at which β-lactamases can catalyze the hydrolysis of β-lactams such as penicillins and cephalosporins. The division between these two families becomes increasingly conspicuous in the presence of β-lactam antibiotics since the rates at which PBPs turn over these substrates/inhibitors are often too slow to allow the bacterial cell to survive in their presence.

β-lactamases which do confer antibiotic resistance have been divided and classified to emphasize shared homologies and any differences in modes of catalysis. According to the current classification scheme (Ambler, 1980) at least four different classes of β-lactamases are known to exist: Class A, B, C and D. (Joris et al., 1988; Joris et al; 1991). All of which, except Class B, contain an active site serine residue which is believed to participate as a nucleophilic catalyst in the mechanism. Class B enzymes are characterized

by the presence of Zn^{+2} which is believed to participate as an electrophilic catalyst in the mechanism of these proteins.

We are presently studying the reaction mechanism of the β-lactamase from *Bacillus licheniformis* 749C, a Class A enzyme (Ambler, 1980; Pollock, 1965). The recent publication of the crystal structures for two members of this Class, PC1 from *Staphylococcus aureus* (Herzberg & Moult, 1987) and the enzyme from *B. licheniformis* 749C (Knox & Moews, 1991), have made it an ideal system to analyze through site specific mutagenesis. Knowledge of the three dimensional structure of these enzymes facilitates the interpretation of the kinetic analysis by suggesting possible modes in which groups located within the catalytic site could participate in the mechanism.

Structure of Class A β-lactamases

Five members of this group have been identified: 1) PC1 from *S. aureus*, 2) RTEM from *Escherichia coli*, 3) β-lactamase I from *Bacillus cereus*, 4) the β-lactamase from *Streptomyces Albus* G and 5) the β-lactamase from *B. licheniformis*. The crystal structures for (1) and (5) are virtually superimposible, supporting previous investigations (Joris et al., 1988) indicating that this class of enzymes is closely related.

Class A β-lactamases have in common a two domain structure in which five strands of antiparallel β-pleated sheet are sandwiched between three short α-helices on one side (together these two secondary structural elements make up domain 1) and eight longer α-helices on the other side (domain 2). The catalytic site is located within the cleft created by these two domains of the protein and has the approximate dimensions of 17 by 7 A with the

catalytic site floor positioned 6 A below the protein surface (Herzberg & Moult, 1987; see Fig. 5)

Sequence comparison studies indicate there are several residues of the Class A β-lactamases which are highly conserved. Indeed, the degree of conservation for some of these residues is so high that other classes of β-lactamases and even PBPs contain these same groups (Joris et al., 1988).

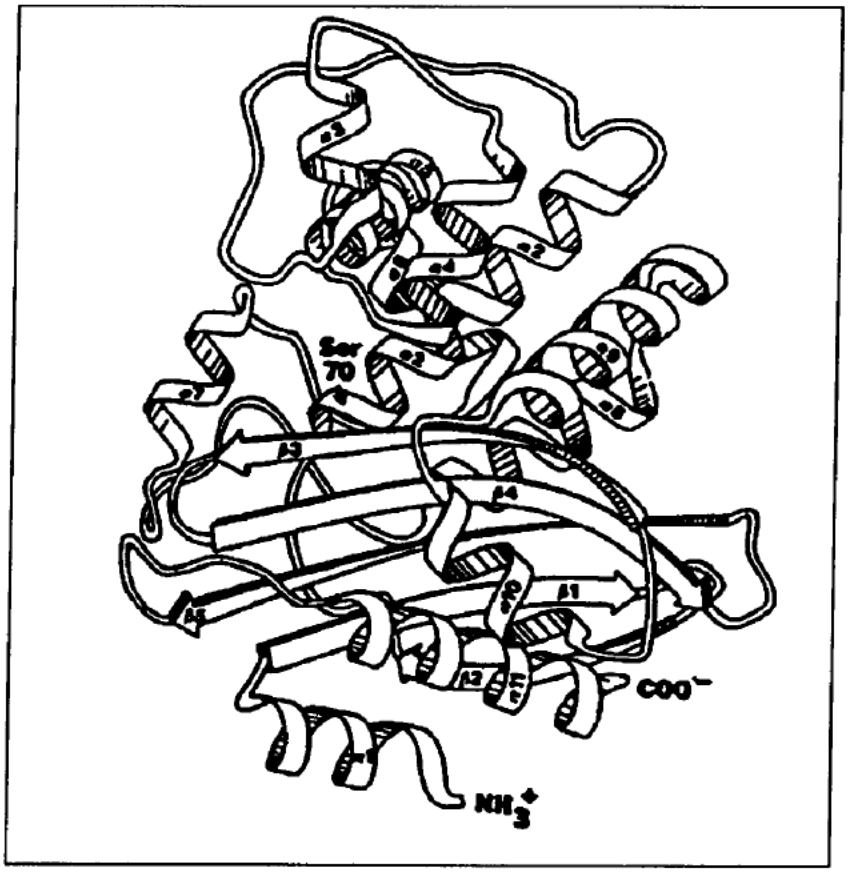


Figure 5. Tertiary structure for the Class A β-lactamase from *S. aureus* (Herzberg & Moult, 1987)

Many of these highly conserved residues are found within the catalytic site of β-lactamase. Among these are serine 70, lysine 234, lysine 73,

glutamate 166, asparagine 170, serine 130 and tyrosine 105 (see Fig 6). Serine 70, glutamate 166, asparagine 170 and lysine 73 are located on the floor of the active site. Serine 70 is located at the N-terminus of an alpha helix (α2) and consequently experiences the positive electrostatic field arising from the helix dipole. Glu-166 and Lys-73 are juxtaposed within the catalytic site and have been postulated to interact through a salt bridge. If this is indeed the case, however, the ionic interaction between these two groups does not contribute significantly to local or global structure since replacement of glutamate with alanine, cysteine or aspartate at site 166 minimally perturbs structure (see chapter 3 and 4).

Substrate specificity is in part determined by a hydrophobic gully which is found at one end of the catalytic site and is lined in the *Staphloccocus aureus* enzyme with residues Val-103 and Ile-239 (Herzberg & Moult, 1987).

Moreover, the main chain NH groups of Ser-70 and Ala-237 stick into the catalytic site (Moews et al., 1990) and are thought to have a role in binding the transition states along the reaction pathway (see below).

Mechanism of β-lactamases

Class A β-lactamases have been shown to proceed through an acyl-intermediate (Cartwright & Coulson., 1980; Cartwright et al., 1989). Thus the reaction catalyzed by these enzymes can be described by the two step mechanism presented in Equation 1. In acylation (k_2) Ser-70 (see Fig. 6) attacks the carbonyl carbon of the substrate lactam ring to form a covalent intermediate (Cartwright et al., 1989). A water molecule is then activated for

nucleophillic attack onto the ester bond of the intermediate which results in the release of the hydrolyzed product (deacylation or k_3).

$$E+S \overset{K_s}{\Longleftrightarrow} ES \underset{k_{-2}}{\overset{k_2}{\Longleftrightarrow}} EA \xrightarrow{k_3} E+P \qquad (1)$$

Similarities between the β-lactamase mechanism and that of serine proteases have led workers to postulate a parallelism in the catalytic strategies employed by these two classes of enzymes. Unfortunately, although the steps in the mechanism appear to be similar, the functional groups capable catalyzing a serine protease reaction are not present in β-lactamases (Herzberg & Moult, 1987).

Several residues within the catalytic site have been implicated in the mechanism of these enzymes (Madgewick & Waley, 1987; Gibson et al., 1990; Adachi et al., 1991; Ellerby et al., 1990; Escobar et al. 1991). Among those residues believed to play a central role in catalysis are Ser-70, Lys-73, Glu-166 and Lys-234. Although serine 70 is known to participate as a nucleophillic catalyst (Cartwright & Coulson., 1980; Kemal & Knowles, 1981; Cartwright et al., 1989; Dalbadie et al., 1986; Sigal et al., 1982) the function of these other residues is not as clearly defined.

Crystallographic data for the acyl-intermediate of the DD-peptidase of *Streptomyces* R61 (Kelly et al., 1986; Knox et all., 1987) combined with model building studies (Herzberg & Moult, 1987) highlight various points concerning the mechanism of Class A β-lactamases. The required juxtaposition of the Ser-70 Oγ next to the lactam ring carbonyl carbon of the substrate as well as the fairly rigid structure of the β-lactam bicyclic system

orients the C3 carboxylate (see Fig.1 and Fig. 6) of the substrate toward lysine 234 (Herzberg & Moult). Thus we might expect the role of this residue to be that of an electrostatic anchor in binding the ground state of the substrate. In such a case we would anticipate that perturbations at this site would manifest themselves as changes in K_s, the dissociation constant of the substrate with enzyme.

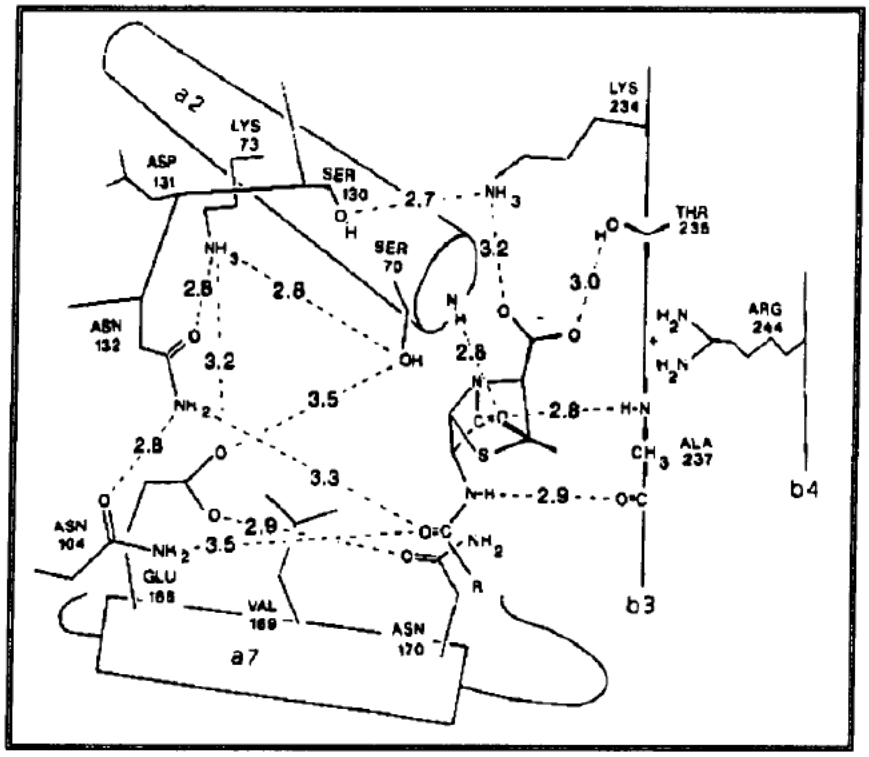


Figure 6. Catalytic site of the *B. licheniformis* β-lactamase depicting groups thought to participate in catalysis (Moews et al., 1990).

Nevertheless, we have demonstrated (Ellerby et al., 1990) that mutations of lysine 234 not only alter the binding affinity of the enzyme for

good substrates but decrease k_{cat}, the first order rate constant which refers to the properties and reactions of the enzyme-substrate, enzyme-intermediate, and enzyme-product complexes. These results indicate that Lys-234 is playing a role in binding the transition state as well as the ground state. This finding is substantiated by similar results observed for the binding subsites of serine proteases. Although they are distal to the catalytic site it has been observed that mutation at these sites affects the k_{cat} of the reaction (Fersht, 1985). Therefore these sites are playing a dual role and partitioning their function between binding the ground state and the transition state of the reaction.

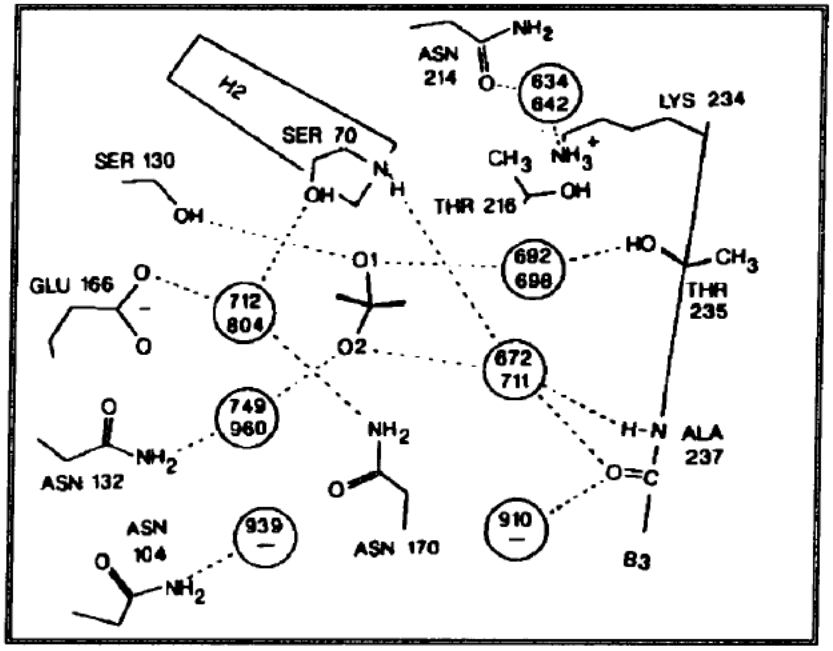


Figure 7. Catalytic site of *B. licheniformis* β-lactamase showing the water molecules which are present in the crystal structure (Knox & Moews, 1991)

Model building studies also indicate that the oxygen of the lactam ring carbonyl is positioned to interact through hydrogen bond formation with the main chain NH groups of Ser-70 and Ala-237 (see Fig. 6). This interaction would stabilize the formation of a negative charge on the carbonyl oxygen of the tetrahedral intermediate and suggests a function for this site analogous to that of the oxyanion hole of the serine proteases.

Lys-73 is highly conserved throughout the Class A β–lactamase family (Joris et al., 1988). Moreover, since replacement of lysine 73 with arginine results in a large decrease in activity (Gibson et al., 1990; Madgewick & Waley, 1987) Lys-73 is thought to participate in the mechanism of these enzymes. Despite the evidence implicating this residue in the mechanism, however, the function of Lys-73 is still uncertain. Its postulated role will be discussed further in chapter 4.

Glutamate 166 has been proposed to participate as a general base and/or a general acid in the mechanism. However new evidence (Escobar et al., 1991, Chapter 4) indicates this residue may be acting as a general base with its function limited to deacylation. Glutamate 166 is believed to activate a water molecule, possibly H_20 molecule 712/804 as in Figure 7, for attack onto the acyl-intermediate. This will be discussed further in Chapters 3 and 4).

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Chapter II

Molecular Genetics

Introduction

Recent advances in the field of molecular biology have ushered in a new era in protein chemistry. Through site-directed mutagenesis it is now possible to uniquely replace any residue within a protein sequence. Consequently, protein structure-function relationships can now be investigated with a resolution previously not available through chemical modification or random mutagenesis.

The study of β-lactamases is one area of research which is taking advantage of this novel methodology. Sequence comparison (Joris et al., 1988) as well as chemical modification (Little et al., 1986; Bristow & Virden, 1978) studies indicate there are several groups within the catalytic site which could participate in catalyzing the reaction of these enzymes. It is believed that the specific mutation of these highly conserved residues will allow for a more detailed understanding of the mechanism of β-lactamases. We have begun a site directed mutagenesis program on the Class A β-lactamase from *Bacillus licheniformis*. The following is a description of the work and techniques used to produce these mutants. It is hoped that this information will help others who plan to apply these techniques to β-lactamases or other systems requiring the high specificity afforded by this technology.

Expression vector

The expression vector used to produce W.T. and mutant β-lactamases was a derivative of the shuttle vector pSS.5 (Ellerby et al., 1990). pSS.5

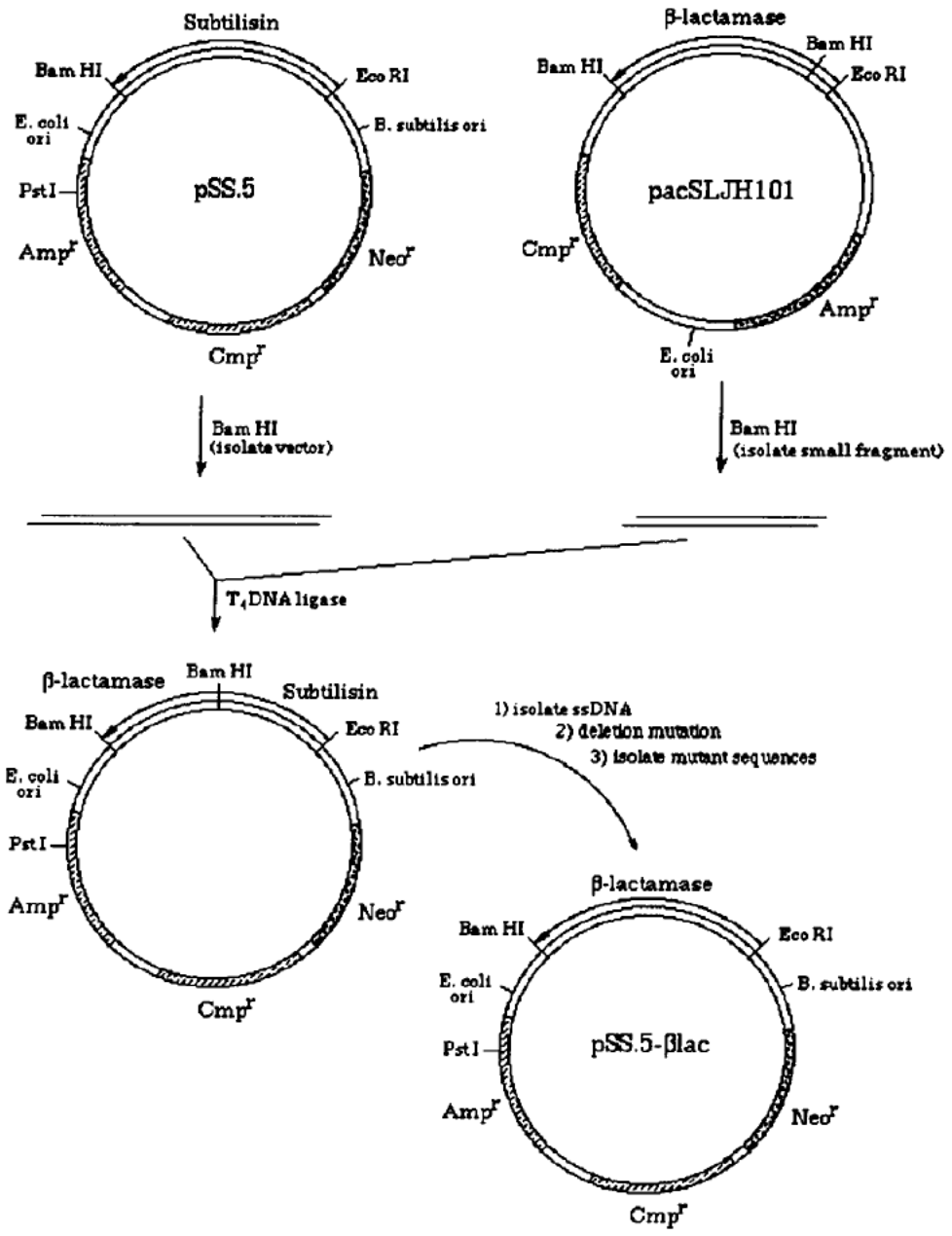


Figure 2. Flowchart for the design of expression vector pSS.5-βlac

contains the origins of replication for *Escherichia coli* and *Bacillus subtilis* and thus can be propagated in both of these organisms. This was a desired

property since it would allow us to carry out the molecular genetics in *E. coli* and express the β-lactamase proteins in *B. subtilis*. pSS.5 also contains the *bla* gene from pBR322 (which codes for the RTEM β-lactamase from *E. coli*), the chloramphenicol acetyl transferase gene (which confers resistance to chloramphenicol) and the neomycin resistance gene all of which allow for the positive selection of organisms carrying this plasmid. In addition, the pSS.5 vector contains the bacteriophage F1 origin of replication (Ellerby et al., 1990), a genetic element which allows pSS.5 to be packaged up as single stranded DNA (Vieira and Messing, 1987). The single stranded plasmid can be used for site specific mutation or sequencing.

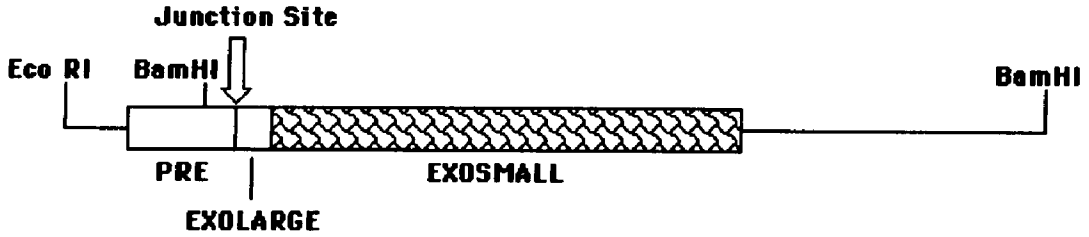
We postulated that expression of the *B. licheniformis* β-lactamase could best be achieved in the industrial strain *B. subtilis* I168-2036 (a proprietary strain loaned to us by J. Wells, Genentech, Inc.) under the control of the *B. amyloliquefaciens* subtilisin promoter. This system has been successfully used for the high level expression of subtilisin. The Bam H1 fragment from pSLJH101 (Fig. 1 and 2A) was cloned into the Bam H1 site of pSS.5 (see Fig. 1). Although cloning the *B. licheniformis* gene in this fashion would eliminate both the β-lactamase promoter and signal sequence (Eco RI-Bam HI fragment of pacSLJH101), no essential code was lost since we were planning to use the corresponding sequences from the subtilisin gene (Fig. 1 and 3A). As indicated in Figure 1, this construct placed the coding sequence for the *B. licheniformis* β-lactamase at the 3' end of the Eco R1- Bam H1 subtilisin fragment. Clones with the correct β-lactamase fragment orientation were identified through restriction analysis with Pvu II and Bgl II (Fig. 2B and 3B for the location of these sites). Deletion mutagenesis was used to

juxtapose the subtilisin signal sequence next to the sequence coding for the secreted form of the *B. licheniformis* β-lactamase. A thirty-one base pair primer (see Table I-A) was utilized for this mutation. The primer was designed so that its 5' half would be complementary to the DNA sequence coding for the last six amino acid residues of the B. amyloliquefaciens subtilisin signal sequence (terminating at codon -78 : underlined in Fig. 3B) and the 3' half would be complementary to the first few residues (starting at codon +35 : underlined in Fig. 2B) of the DNA sequence coding for the exolarge form of the *B. licheniformis* β-lactamase (the exolarge form (Lampen et al., 1980) is the secreted form of this enzyme). Furthermore, this primer introduces a silent mutation which creates a unique Hind III site. Consequently, only mutant plasmids will be digested by the Hind III restriction endonuclease.

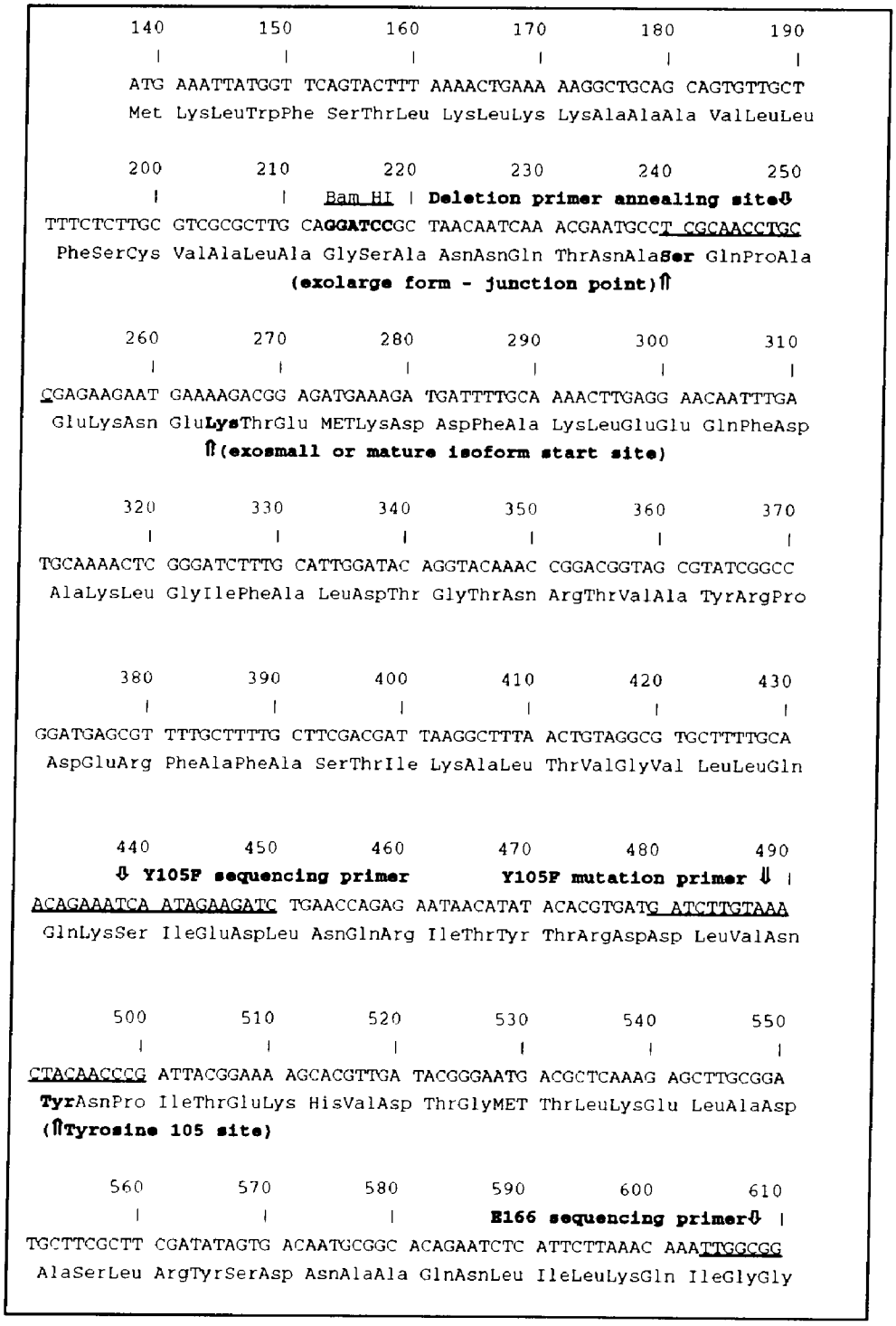
Single stranded DNA was prepared with the K07 helper phage in JM101 cells (Vieira and Messing, 1987; see appendix B). The primer was annealed and used for second strand synthesis.

Figure 2. A) Map of the *B. licheniformis* β-lactamase gene. PRE - signal sequence; EXOLARGE - secreted form of the enzyme; EXOSMALL- mature isoform. B) The coding sequence for the *B. licheniformis* β-lactamase (Neugebauer et al., 1981) Underlined sequences correspond to primer annealing sites.

(A)



(B)


140 150 160 170 180 190
ATG AAATTATGGT TCAGTACTTT AAAACTGAAA AAGGCTGCAG CAGTGTTGCT
Met LysLeuTrpPhe SerThrLeu LysLeuLys LysAlaAlaAla ValLeuLeu
200 210 220 230 240 250
Bam HI
Deletion primer annealing site
TTTCTCTTGC GTCGCGCTTG CAGGATCCGC TAACAATCAA ACGAATGCCT CGCAACCTGC
PheSerCys ValAlaLeuAla GlySerAla AsnAsnGln ThrAsnAlaSer GlnProAla
(exolarge form - junction point)
260 270 280 290 300 310
CGAGAAGAAT GAAAAGACGG AGATGAAAGA TGATTTTGCA AAACTTGAGG AACAATTTGA
GluLysAsn GluLysThrGlu METLysAsp AspPheAla LysLeuGluGlu GlnPheAsp
(exosmall or mature isoform start site)
320 330 340 350 360 370
TGCAAAACTC GGGATCTTTG CATTGGATAC AGGTACAAAC CGGACGGTAG CGTATCGGCC
AlaLysLeu GlyIlePheAla LeuAspThr GlyThrAsn ArgThrValAla TyrArgPro
380 390 400 410 420 430
GGATGAGCGT TTTGCTTTTG CTTCGACGAT TAAGGCTTTA ACTGTAGGCG TGCTTTTGCA
AspGluArg PheAlaPheAla SerThrIle LysAlaLeu ThrValGlyVal LeuLeuGln
440 450 460 470 480 490
Y105F sequencing primer
Y105F mutation primer
ACAGAAATCA ATAGAAGATC TGAACCAGAG AATAACATAT ACACGTGATG ATCTTGTAAA
GlnLysSer IleGluAspLeu AsnGlnArg IleThrTyr ThrArgAspAsp LeuValAsn
500 510 520 530 540 550
CTACAACCCG ATTACGGAAA AGCACGTTGA TACGGGAATG ACGCTCAAAG AGCTTGCGGA
TyrAsnPro IleThrGluLys HisValAsp ThrGlyMET ThrLeuLysGlu LeuAlaAsp
(Tyrosine 105 site)
560 570 580 590 600 610
E166 sequencing primer
TGCTTCGCTT CGATATAGTG ACAATGCGGC ACAGAATCTC ATTCTTAAAC AAATTGGCGG
AlaSerLeu ArgTyrSerAsp AsnAlaAla GlnAsnLeu IleLeuLysGln IleGlyGly

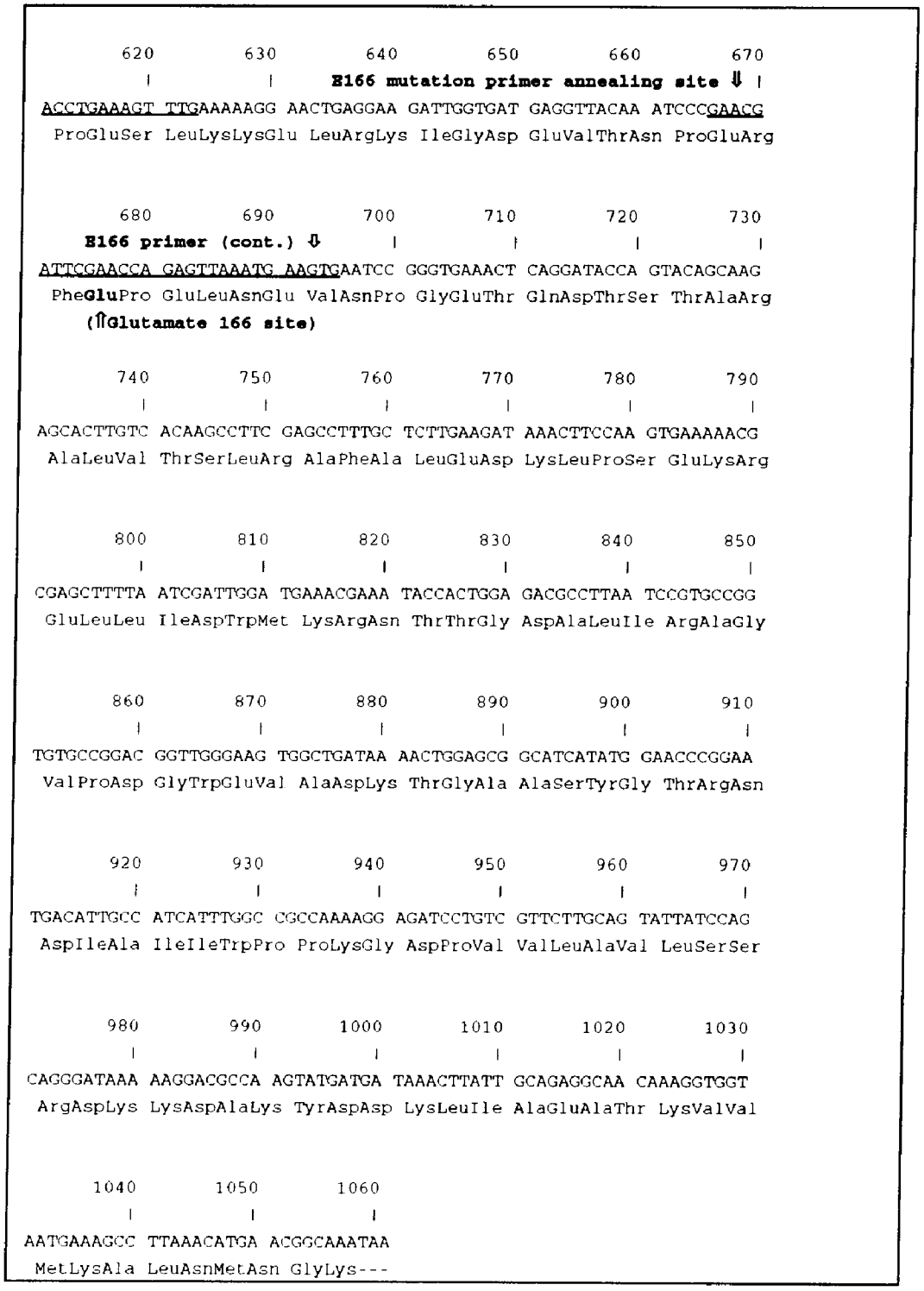
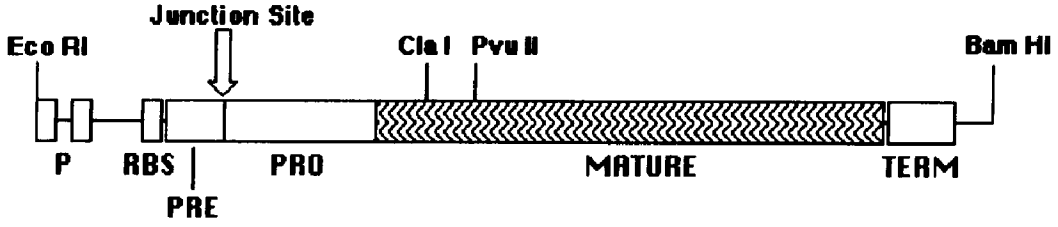

620 630 640 650 660 670
E166 mutation primer annealing site ⇓
ACCTGAAAGT TTGAAAAAGG AACTGAGGAA GATTGGTGAT GAGGTTACAA ATCCCGAACG
ProGluSer LeuLysLysGlu LeuArgLys IleGlyAsp GluValThrAsn ProGluArg
680 690 700 710 720 730
E166 primer (cont.) ⇓
ATTCGAACCA GAGTTAAATG AAGTGAATCC GGGTGAAACT CAGGATACCA GTACAGCAAG
PheGluPro GluLeuAsnGlu ValAsnPro GlyGluThr GlnAspThrSer ThrAlaArg
(⇑Glutamate 166 site)
740 750 760 770 780 790
AGCACTTGTC ACAAGCCTTC GAGCCTTTGC TCTTGAAGAT AAACTTCCAA GTGAAAAACG
AlaLeuVal ThrSerLeuArg AlaPheAla LeuGluAsp LysLeuProSer GluLysArg
800 810 820 830 840 850
CGAGCTTTTA ATCGATTGGA TGAAACGAAA TACCACTGGA GACGCCTTAA TCCGTGCCGG
GluLeuLeu IleAspTrpMet LysArgAsn ThrThrGly AspAlaLeuIle ArgAlaGly
860 870 880 890 900 910
TGTGCCGGAC GGTTGGGAAG TGGCTGATAA AACTGGAGCG GCATCATATG GAACCCGGAA
ValProAsp GlyTrpGluVal AlaAspLys ThrGlyAla AlaSerTyrGly ThrArgAsn
920 930 940 950 960 970
TGACATTGCC ATCATTTGGC CGCCAAAAGG AGATCCTGTC GTTCTTGCAG TATTATCCAG
AspIleAla IleIleTrpPro ProLysGly AspProVal ValLeuAlaVal LeuSerSer
980 990 1000 1010 1020 1030
CAGGGATAAA AAGGACGCCA AGTATGATGA TAAACTTATT GCAGAGGCAA CAAAGGTGGT
ArgAspLys LysAspAlaLys TyrAspAsp LysLeuIle AlaGluAlaThr LysValVal
1040 1050 1060
AATGAAAGCC TTAAACATGA ACGGCAAATAA
MetLysAla LeuAsnMetAsn GlyLys---

Figure 3. A) The gene map for *B. amyloliquefaciens* subtilisin B) The coding sequence for the subtilisin gene (Wells et al., 1983) up to the unique Pvu II site. The junction site is indicated by an arrow. Underlined sequences correspond to primer annealing sites.

(A)



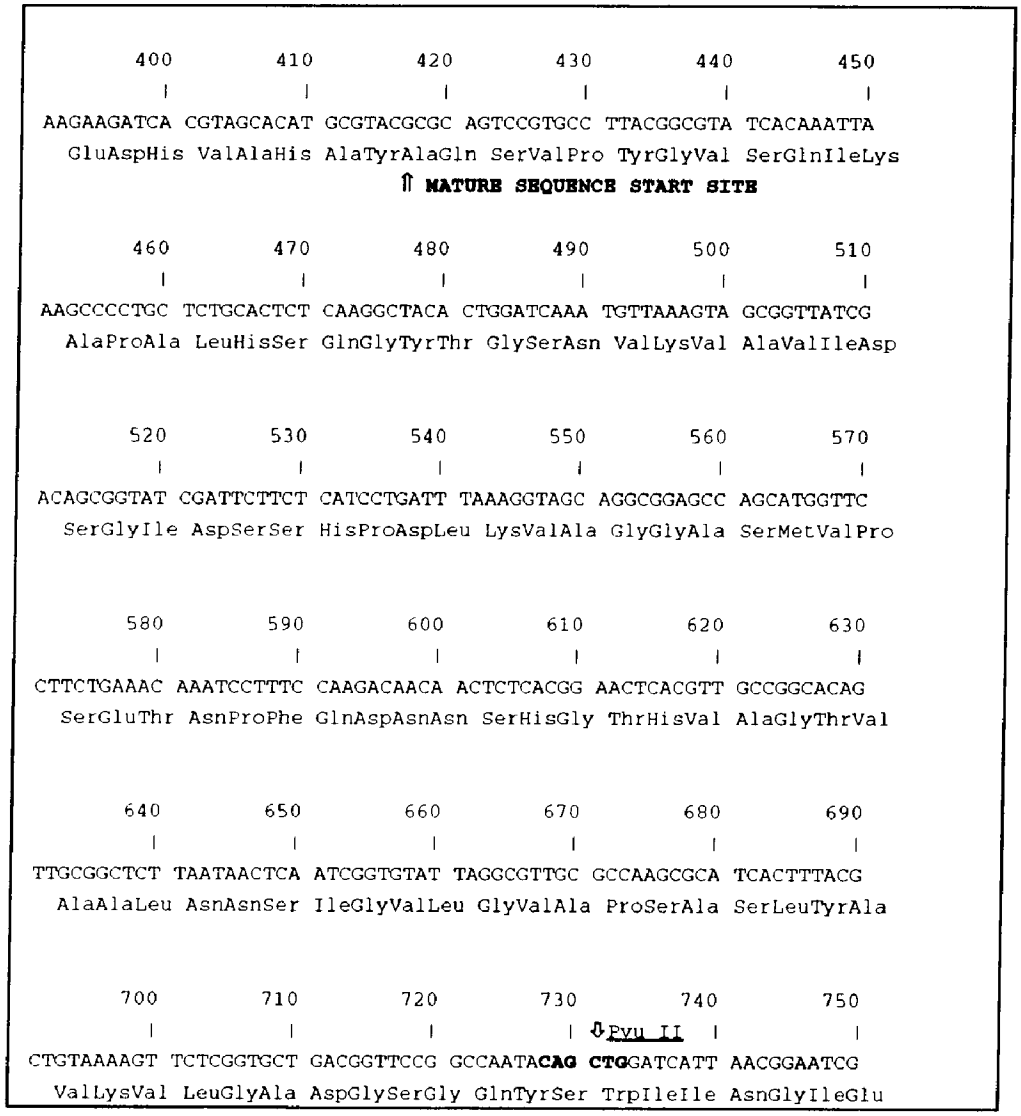
(B)

```
      100         110         120         130         140         150
       |          ⇩ SSP3 primer annealing site        |           |
    GTGAG AGGCAAAAAA GTATGGATCA GTTTGCTGTT TGCTTTAGCG TTAATCTTTA
  fMetArg GlyLysLys ValTrpIleSer LeuLeuPhe AlaLeuAla LeuIlePheThr
                    PRE⇒

      160         170         180         190         200         210
       |           |⇩ Deletion primer annealing site             |
CGATGGCGTT CGGCAGCACA TCCTCTGCCC AGGCGGCAGG GAAATCAAAC GGGGAAAAGA
  MetAlaPhe GlySerThr SerSerAlaGln AlaAlaGly LysSerAsn GlyGluLysLys
                                  ⇑(Sequence junction point)
                                  (Codon -78)
      220         230         240         250         260         270
       |           |           |           |           |           |
AATATATTGT CGGGTTTAAA CAGACAATGA GCACGATGAG CGCCGCTAAG AAGAAAGATG
  TyrIleVal GlyPheLys GlnThrMetSer ThrMetSer AlaAlaLys LysLysAspVal
                      PRO SEQUENCE

      280         290         300         310         320         330
       |           |           |           |           |           |
TCATTTCTGA AAAAGGCGGG AAAGTGCAAA AGCAATTCAA ATATGTAGAC GCAGCTTCAG
  IleSerGlu LysGlyGly LysValGlnLys GlnPheLys TyrValAsp AlaAlaSerAla


      340         350         360         370         380         390
       |           |           |           |           |           |
CTACATTAAA CGAAAAAGCT GTAAAAGAAT TGAAAAAAGA CCCGAGCGTC GCTTACGTTG
  ThrLeuAsn GluLysAla ValLysGluLeu LysLysAsp ProSerVal AlaTyrValGlu
```



This heteroduplex was then transformed into *E. coli* MM294 cells (all *E. coli* genotypes are listed in appendix B) and the plasmid isolated from the transformant cells as a pool. The plasmid pool was digested with Hind III and electrophoresed into a 5% polyacrylamide gel. Linearized DNA enters

polyacrylamide gels much more efficiently than circular uncut DNA and thus we would expect that only plasmids containing the Hind III site should enter

Table I. Primers used for the synthesis of the expression vector and mutants of β-lactamase are listed below. The mutations in the sequence are shown in bold and the unique restriction sites introduced by the primer are underlined. Parenthesis indicate mutations resulting in an amino acid replacement.

OLIGO NAME	SEQUENCE
A) Deletion Primer (31mer)	5'-C-ACA-TCC-TCT-GCC-C**AA-GCT-T**CG-CAA-CCT-GCC-3' Hind III
B) SSP3 (18mer) Deletion sequencing primer	5'-AGA-GGC-AAA-AAA-GTA-TGG
C) E166A (30mer)	5'-GAA-CGA-TTC-(G**CG**)-CCA-**GAG-CTC**-AAT-GAA-GTG-3' Sac I
D) E166C (30mer)	5'-GAA-CGA-TTC-(**TGT**)-CCA-**GAG-CTC**-AAT-GAA-GTG-3' Sac I
E) E166D (30mer)	5'-GAA-CGA-TTC-(GA**C**)-CCA-**GAG-CTC**-AAT-GAA-GTG-3' Sac I
F) E166 SEQ PRIMER (20mer)	5'-TT-GGC-GGA-CCT-GAA-AGT-TTG-3'
G) N170D (29mer)	5'-TC-GAA-CCA-**GAG-CTC**-(**GAC**)-GAA-GTG-AAT-CCG-3' Sac I
H) N170L (29mer)	5'-TC-GAA-CCA-**GAG-CTC**-(**TTA**)-GAA-GTG-AAT-CCG-3' Sac I
I) N170H (29mer)	5'-TC-GAA-CCA-**GAG-CTC**-(**CAC**)-GAA-GTG-AAT-CCG-3 Sac I
J) Y105F (27mer)	5'-GAT-CTT-**GTT-AAC**-(TTC)-AAC-CCG-ATT-ACG-3' Hpa I
K) SEQ PRIMER Y105F (20 mer)	5'-ACA-GAA-ATC-AAT-AGA-AGA-TC-3'

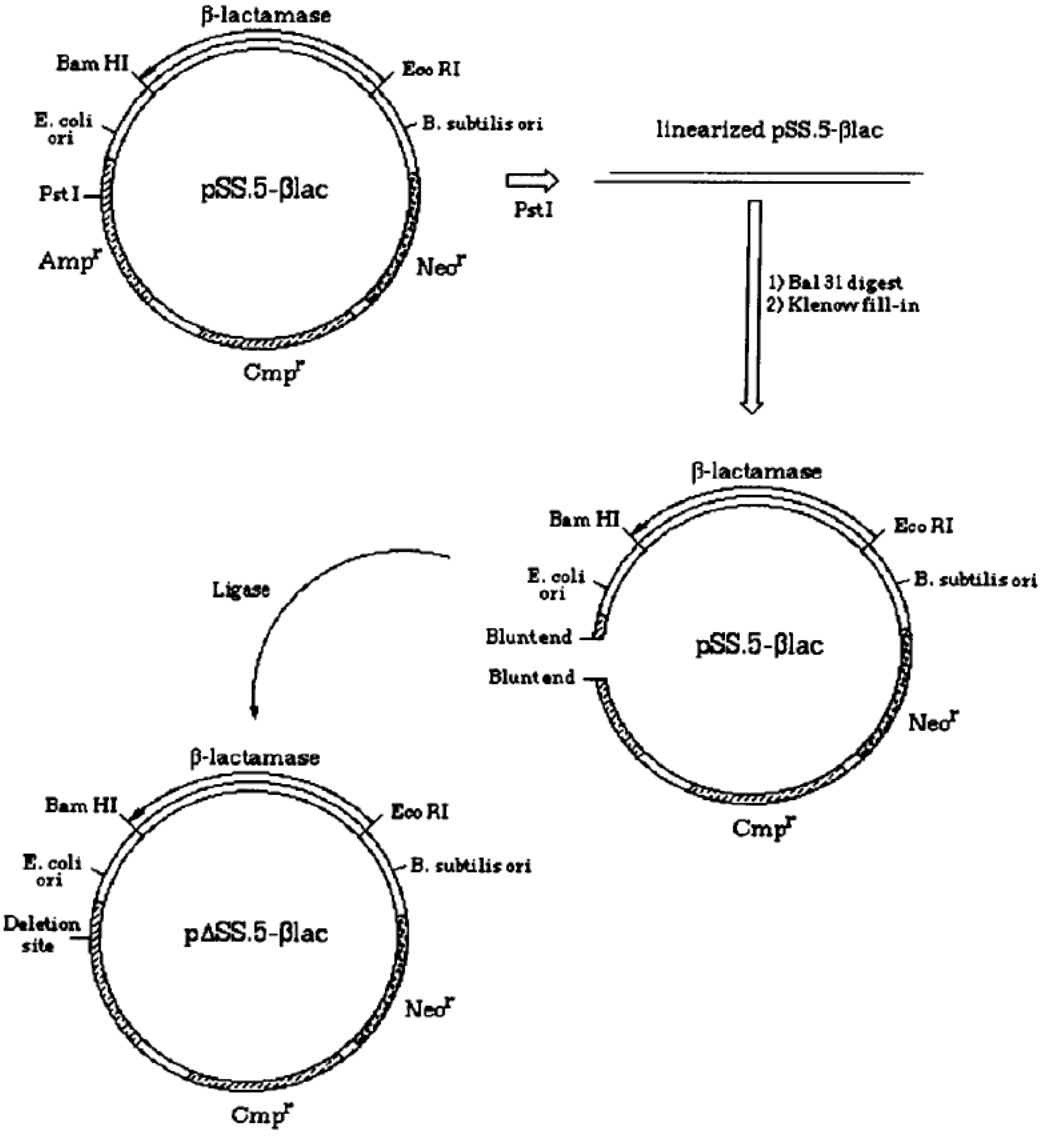
the gel. This process is termed restriction purification (Wells et al., 1986). The resultant band was cut out of the gel and electroeluted to isolate the DNA. The plasmid was then religated to recircularize the plasmid and then transformed into *E. coli* MM 294 cells. Transformants (selected on L.B. + 50 μg/ml ampicillin plates) were restriction analyzed and several were found to demonstrate the correct restriction pattern. Two of these positive sequences were transformed into *E. coli* JM101 cells to isolate single stranded DNA and into I168 *B. subtilis* to check for expression. The mutation was sequenced using the primer SSP3 (Table I and Fig. 3B) which was designed to sit ~ 70 bp upstream of the mutation site. The sequence was confirmed through dideoxy sequencing (Sanger et al., 1977) using Sequenase (U.S. Biochemicals). Wild-type β-lactamase was produced at high levels as determined through time-course activity assays and SDS-PAGE.

Deleting the E. coli β-lactamase gene (bla gene)

Due to the large decrease in activity seen with several of the mutant β-lactamases we decided to inactivate the *E. coli*. ampicillin resistance gene on pSS.5-βlac. Although, we expected the *E. coli* gene would not be expressed effectively in *B. subtilis* (Ehrlich, 1978) the small amounts of activity observed for the *B. licheniformis* β-lactamase mutants made the study of their kinetics extremely sensitive to any contamination which might arise from the *bla* gene. The *bla* gene was inactivated by an internal deletion (see Fig. 5). This was accomplished by cutting the plasmid at the unique Pst I site which is centrally located in the *bla* gene. The ends were then digested back with the exonuclease Bal 31. The vector's digested ends were blunt ended

and then religated to recircularize the plasmid. That this deletion did inactivate the gene was shown by plating the *bla* inactivated plasmid (ΔpSS.5-βlac) onto L.B. plates containing either 50 μg/ml carbenicillin or 20 μg/ml chloramphenicol. It was observed that although the ΔpSS.5-βlac

Figure 5. Flowchart for the design of expression vector ΔpSS.5-βlac



plasmid transformed the cells to chloramphenicol resistance, they were carbenicillin sensitive. Note that the pSS.5-βlac plasmid used to inactivate the *bla* gene was one which contained the E166A *B. licheniformis* β-lactamase gene. This was important since it was observed that the W.T. *B. licheniformis* gene conferred resistance to carbenicillin in *E. coli.* (Ehrlich, 1978). Consequently if the pSS.5-βlac used to make the deletion contained an active form of the *B. licheniformis* β-lactamase it would not have been possible to distinguish the deleted from the undeleted plasmids.

Mutations

L234A: Jim Wells (Genentech, Inc.) mutated the Lys-234 site of *B. licheniformis* to an alanine while concomitantly introducing a silent mutation which would create a Nhe I site. This unique site was used to restriction purify mutants from the transformation pool (Wells et al., 1986). The gel purified mutant sequences were religated and transformed into JM101. Transformants were selected on L.B. + 50 μg/ml carbenicillin plates. Several colonies were grown and their plasmid DNA isolated (see appendix A). The plasmids were restriction analyzed with Eco R1 and Nhe I. About one third of the vectors demonstrated the correct restriction pattern of which four were sequenced through dideoxy-sequencing. One of the vectors demonstrating the correct sequence was transformed into *B. subtilis* I168 which was used to express this mutant.

Site 166: It was decided to mutate site 166 of *B. licheniformis* β-lactamase to cysteine, aspartate and alanine. The reasons behind this choice

are elaborated in chapters three and four. The expression vector ΔpSS.5-β-lac was used to create these mutants via the Kunkle method (Kunkle et al., 1985). The primers used are shown in Table I (C,D and E). The Kunkle method requires that the ssDNA be initially prepared in an *E. coli dut*$^-$, *ung*$^-$ strain; *E. coli* strain CJ236 was used for this purpose (Template prep. as in Appendix A). The *dut* gene produces dUTPase and therefore a strain deficient in this enzyme will have an increased intracellular pool of dUTP. The higher concentrations of dUTP compete with dTTP for incorporation into replicating DNA. The incorporated uracil residues cannot be removed in the ung$^-$ strain because it is lacking the uracil glycosylase gene product which removes the uracil bases creating abasic sites. Consequently, 20 - 30 uracil residues are incorporated per genome. When a second strand is synthesized in vitro using a site-directed primer we find that one strand of our heteroduplex (the W.T. strand) contains uracils while the newly synthesized mutant strand does not. When this heteroduplex is transformed into a *dut*$^+$, *ung*$^+$ *E. coli* cell line the wild type strand is inactivated, generating mutagenesis efficiencies of 50% - 60%. Mutants were constructed so as to contain a silent mutation which introduced a Sac I site. This site was used to restriction analyze transformants to determine which of these carried the mutant allele. Plasmids which contained the Sac I site were further restriction analyzed to insure the sequences had not suffered deletions or insertions. Finally, mutants were sequenced using Sequenase (U.S. Biochemicals) and the primer E166 SEQ PRIMER (Table I -F, annealing site as in Fig. 2B).

Site 170: Due to its central location within the catalytic site as well as its highly conserved nature throughout the Class A β-lactamase family we have chosen to investigate the function of asparagine 170 through site-directed mutagenesis. As with site 166, the mutations at site 170 were accomplished via the Kunkle method. Asparagine 170 was replaced with aspartate, leucine and histidine using the primers listed in Table I (G, H and I respectively). Again we were able to introduce a unique Sac I site which was later used to identify mutant sequences. The mutants were further restriction analyzed to insure there had been no anomalous alterations in the sequence and the mutation site was sequenced using primer E166 SEQ PRIMER (Table I - F, annealing site as in Fig. 2B).

Y105F: Chemical modification studies (Bristow & Virden, 1978) as well as sequence comparison studies indicate that tyrosine 105 may be playing an important role in either maintaining the structure of β-lactamase or perhaps in the mechanism of these enzymes. Indeed we have postulated that the phenolic hydroxyl group of this residue may be responsible for the alkaline limb of the rate pH profiles seen with Class A β-lactamases. To investigate the function of the tyrosine 105 hydroxyl group this site was specifically mutated to phenylalanine. A detailed description of the kinetic analysis for this mutant is given in chapter 5. Mutagenesis was carried out via the Kunkle method (as described above) using primer Y105F (Table I - J; annealing site is shown in Fig. 2B). This primer introduces a Hpa I site into the sequence of the mutant which can be used to screen transformants for the presence of the mutation. The mutant sequences where isolated and

restriction analyzed. Those displaying the correct restriction pattern were sequenced with Sequenase (U.S. Biochemicals) and primer SEQ PRIMER Y105F (Table I - K; annealing site is shown in Fig. 2B).

Expression in Bacillus subtilis

The transformation process in *B. subtilis* is slightly more complex than in *E. coli.* The uptake of plasmid DNA in *B. subtilis* is believed to proceed

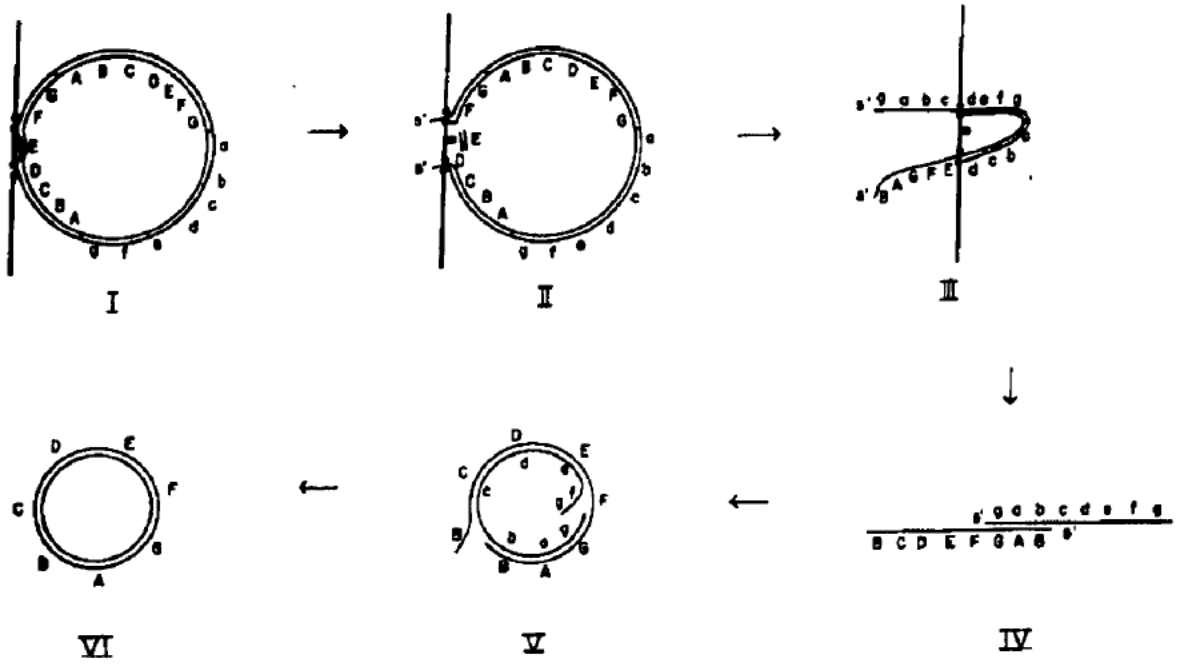


Figure 6. Transformation in *B. subtilis.* I) Trimeric concatamer binds to the surface of the cell at the receptor site (solid rectangle). II) The plasmid is cleaved by endonucleses at two nearby sites (open circles). III) Transport of the DNA into the cell begins. Only single strands of a specific polarity are imported, however, while the lagging strands are partially degraded. IV)Complementary strands anneal V) Circularization occurs as a result of redundant single-stranded termini. VI) Repair of the monomeric plasmid to a covalently continuous circular (CCC) form. (Dubnau, 1982)

through a specific mechanism requiring the action of a nucleic acid receptor and membrane bound nucleases (see Fig. 6).

As indicated in the Figure above, due to the partial loss of genetic information which occurs during the uptake of DNA it is necessary to have redundant sequences or concatamers (multimers) of the plasmid. Concatamers are readily isolated from *E. coli* strains which contain the *rec A* gene (Lewin, 1990). The *rec A* gene product allows for the homologous recombination of monomers to form higher multimers. Competent cells were prepared and transformed as described in appendix A. The cells were plated out on L.B. plates containing 5 μg/ml chloramphenicol to select for transformants.

The *B. amyloliquefaciens* subtilisin promoter was chosen to regulate the expression of the *B. licheniformis* β-lactamase in our expression vector. One advantage of this system is that the subtilisin promoter is not recognized until the culture has reached stationary phase. When over-expression of a heterologous gene product is deleterious to the cell it is preferred to put off the expression of the cloned gene until the culture reaches a maximal cell density. In this manner deleterious effects on the growth of the cells can be avoided and large amounts of protein can be produced since ~10^9 cells/ml will produce the cloned protein simultaneously. The subtilisin promoter becomes active only after the cells have entered the stationary phase because this promoter is recognized by an RNA polymerase holoenzyme containing a subunit (sigma factor) produced only in response to nutrient depletion (Lewin, 1990).

One disadvantage of using a *Bacillus* species to express our protein is that *Bacilli* undergo sporulation. The sporulation process results in the production of a small cell encased in a hard proteinaceous coat. This endospore is released into the environment during autolysis of the mother

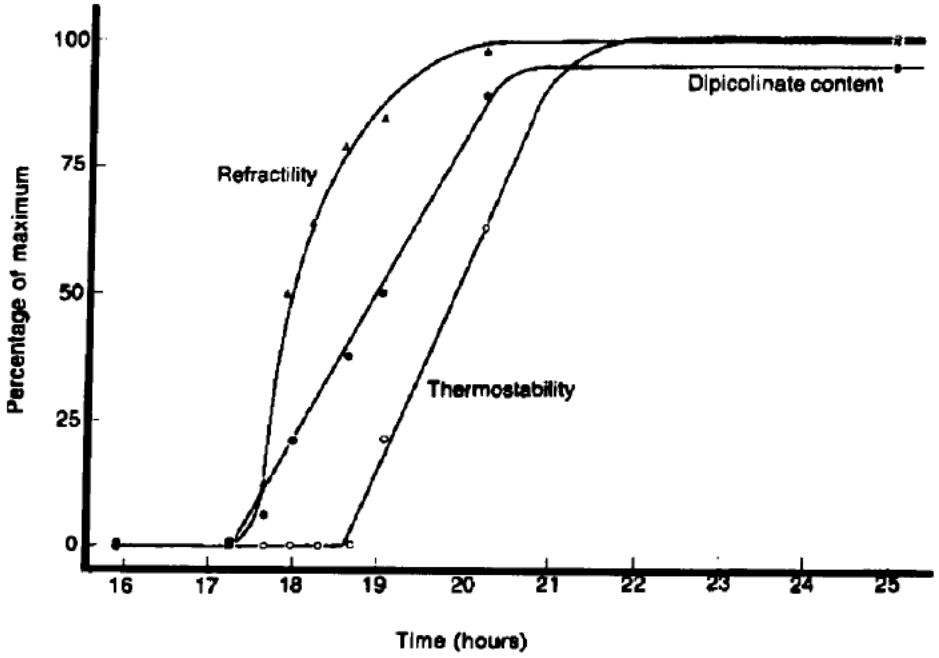


Figure 7. The production of endospores in a *B. cereus* culture monitored by refractility, thermostability and dipicolinate content. Time indicates the age of the culture. Endospores display refractile properties and are characterized by their thermostability (resistance to heat strerilization). Moreover, dipicolinate is a major component of the endospore coat (Stanier et al., 1979).

cell (Stanier et al., 1979). Therefore, if one is isolating extracellular protein from *Bacillus* it is advantageous to stop protein production before the cells begin to sporulate and release large amounts of contaminating proteins into the media. As shown in Figure 7 this process begins for *Bacillus cereus* approximately 17 hours after the cells are inoculated into a rich media.

Under the conditions we used to grow our cultures (chapter 4), lysis began 16 hours after the culture was initiated. In order to avoid the high degree of protein contamination seen upon sporulation we routinely stopped culture growth at 13 - 15 hours after inoculation.

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Chapter III

Site Specific Mutation At Site 166 Leading To The Accumulation Of A Kinetic Intermediate

Introduction

The β-lactamase from *Bacillus licheniformis* 749C is a Class A β-lactamase, exhibiting both penicillinase and cephalosporinase activities, and characterized by the presence of an active site serine residue (Ambler, 1980: Pollock, 1965: Joris et al., 1988). As mentioned in chapter 1, various studies indicate there are several groups within the catalytic site which are known to play a role in the mechanism. However, the function for many of these residues remains unclear. Glu-166 is one such residue.

Glu-166 has been postulated to act as a general base in catalysis (Herzberg and Moult,1987; Moews et al., 1990), possibly in a manner analogous to histidine in the catalytic triad of serine proteases, or, perhaps in a more limited fashion, participating only in acylation or deacylation (Herzberg and Moult, 1987). Madgwick and Waley (1987) have shown that conversion of Glu-166 to Gln leads to a dramatic reduction in the ampicillin resistance of an *E. coli* strain in which the gene for the *B. cereus* β-lactamase had been inserted. To investigate the role of this residue in catalysis we have specifically replaced Glu-166 with alanine and characterized the kinetic properties of the resulting mutant.

EXPERIMENTAL PROCEDURES

Mutagenesis

As described in chapter 2 the *Bacillus amyloliquefaciens* subtilisin promoter and signal sequence were used to express the *B. licheniformis* β-lactamase in *B. subtilis*, (using the shuttle vector pSS5-βLac) (Ellerby et al., 1990; chapter 2). Mutagenesis was accomplished via the Kunkle method

(Kunkle,1985), in conjunction with restriction purification (Wells et al., 1986) to enhance mutation efficiency. The sequence of the oligonucleotide used to generate E166A was: 5'-GAA-CGA-TTC-(GCG)-CCA-GAG-CTC-AAT-GAA-GTG-3'. This sequence replaces Glu 166 with Ala (in parenthesis) and introduces a Sac I site (underlined). Thus, digestion of mutant sequence with Sac I allows for restriction purification. The E166A sequence was confirmed by dideoxy-sequencing (Sanger et al., 1977), with the aid of Sequenase (U.S. Biochemicals). The primer sequence used was 5'-TT-GGC-GGA-CCT-GAA-AGT-TTG-3', and was designed to sit 50 bp upstream from the mutagenesis site.

Nitrocefin burst experiment

The acyl-enzyme burst reaction of E166A β-lactamase with nitrocefin under steady-state conditions was monitored at 482 nm. A 10 μM nitrocefin solution (0.05 M potassium phosphate, 0.5 M KCl, pH 7.0) was prepared and incubated at 30 °C. E166A was added to give final concentrations of 2.0 or 4.5 μM.

HPLC analysis

HPLC of furylacryloylpenicillin- and nitrocefin-labeled E166A β-lactamase was carried out as follows. Substrates and enzyme were mixed for 5 sec at 0 °C. The reaction mixture was immediately loaded onto a minigel column (DEAE-Sephadex G25, 1.3 x 5 cm) and spun at 4000 rpm at 0 °C for 1 min. to remove excess substrate. The column had previously been equilibrated with 0.05 M potassium phosphate, pH 7.0 buffer. The eluent was

collected in an Eppendorf vial and placed on ice. The enzyme-substrate mixture was analyzed using a Beckman gradient HPLC with a model 165 detector and a C3 ultrapore RPSC column (4.6 mm diameter x 7.5 cm) operated at a flow rate of 1 ml/min. Before use the column was equilibrated with 5 % (v/v) acetonitrile. The following program was used: (a) 5 % acetonitrile, 2 min; (b) 5 % - 45 % (v/v) acetonitrile, 15 min; (c) 45 % (v/v) acetonitrile, 8 min; (d) reversal of gradient, 15 min. The FAP-E166A mixture was monitored at 305 nm and 280 nm to assay for the presence of FAP and E166A respectively. Both traces show a single peak with coincident retention times of 14.7 min. Similar results were seen for the nitrocefin experiment monitored at 385 nm (nitrocefin) and 280 nm (E166A). The coincident peaks had a retention time of 15 min. Controls were done to check for E166A contribution to signal at 305 nm and 385 nm. Both controls indicated negligible contributions to absorbance at wavelengths used to assay for substrates.

Acylation rate determination

Stopped-flow kinetics experiments were performed with a Biologic SFM-2 instrument. The reaction of β-lactamase (final concentration 4.0 μM) and nitrocefin (final concentration 160 μM) was monitored at 482 nm, at pH 7.0 (50 mM phosphate buffer), 20 °C.

Investigation of substrate induced conformation changes

Proteolysis experiments were carried out as follows. All solutions were prepared in sodium phosphate buffer, 0.05 M, pH 7.0. Stock solutions of

nafcillin (252 mM), nitrocefin (500 μM) and FAP (1 mM) were prepared. Nine microliters of E166A (45 μM) were pre-incubated with nafcillin (7 μL) for 4 min. at 25 °C, and with nitrocefin (7 μL) and FAP (7 μL) for 30 sec. A Control involved pre-incubation with 7 μL of buffer alone. After the prescribed times α-chymotrypsin (Worthington Biochemical Corp.) (4 μL of 2.5 μM) was added to the solutions, mixed (final concentrations were thus: β-lactamase 20 μM, α-chymotrypsin 0.5 μM), and the digestion allowed to proceed for 4 min and 20 min, at which time the solutions were centrifuged in DEAE-Sephadex minigel tubes for 1 min, the eluant being quenched in 5 μL of 50% TCA. The precipitated protein was then taken up in SDS running buffer and loaded onto a 10-15% Phastsystem SDS polyacrylamide gel. Both Coomassie blue and silver staining were used to visualize the samples.

RESULTS AND DISCUSSION

Structural effects of the mutation.

The close proximity of Lys-73 and Glu-166 (3.2 A) in β-lactamase suggests the presence of an ionic interaction between these residues. Since ion pairs often contribute to the stability of proteins we investigated whether the structure of E166A was perturbed from that of the wild-type. The thermal stability of wild-type and E166A mutant were determined at pH 7.0 by tryptophan fluorescence. Both proteins had identical T_m's of 64 ± 1 °C. As shown in Fig. 1, the far-UV circular dichroism spectra are superimposible within experimental error. In addition, the tryptophan fluorescence spectra (not shown) were superimposible for the wild-type and the E166A mutant,

confirming negligible effects of the mutation on secondary and tertiary structure. Further corroboration came in the form of experiments with α-chymotrypsin, which indicated similar slow rates of proteolysis for both wild type and E166A β-lactamases.

Kinetic effects of the mutation.

The kinetic characteristics of the wild-type and mutant enzymes with both penicillin and cephalosporin substrates were investigated. Nitrocefin was chosen as a cephalosporin substrate because of the very large Δε associated with cleavage of its β-lactam bond, which facilitated monitoring

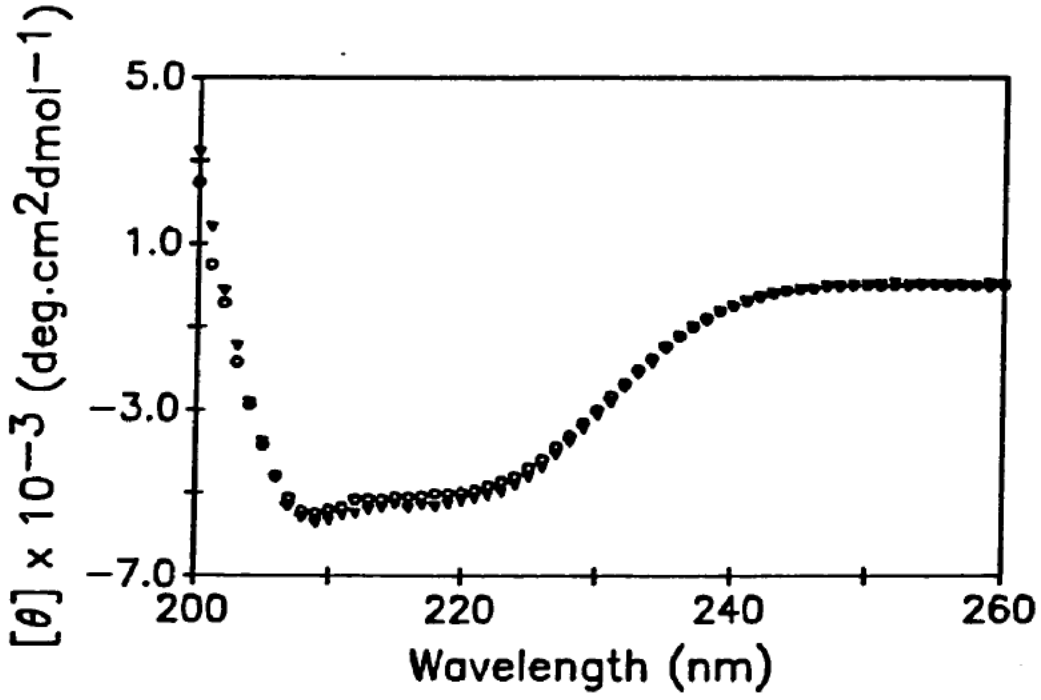

Fig. 1. Far-UV circular dichroism spectra of E166A (circles) and wild-type β-lactamase (inverse triangles) in 0.05 M potassium phosphate, pH 7.0, 30 °C. Data courtesy Tan (1990).

the catalytic reaction. FAP, 6-(2-furylacryloylamido)-penicillin, was chosen as a representative penicillin substrate since it has a distinctive chromophore in the 300 - 350 nm region. For both substrates the value of k_{cat} was reduced at least a million-fold in the mutant compared to wild-type (Table I). Because the values of K_m for the mutant were so low (<<1 μM) it was not possible to determine their values due to limitations on the sensitivity of the assay Similarly, it was not possible to accurately determine the value of k_{cat} for nitrocefin because the rate was so slow. Thus, only an upper limit could be measured. A greater decrease in the cephalosporinase activity was noted. Corroboration of the very low values of K_m was obtained in experiments measuring the concentration of acyl-enzyme with the mutant enzyme (see below); stoichiometric amounts of acyl-enzyme were produced with enzyme and substrate concentrations of the order of 1 μM. The decrease in k_{cat} is of the same order of magnitude as that seen with the replacement by alanine of serine or histidine from the active site of subtilisin (Carter and Wells, 1988) and clearly indicates a central role for Glu-166 in the chemistry of β-lactam catalysis.

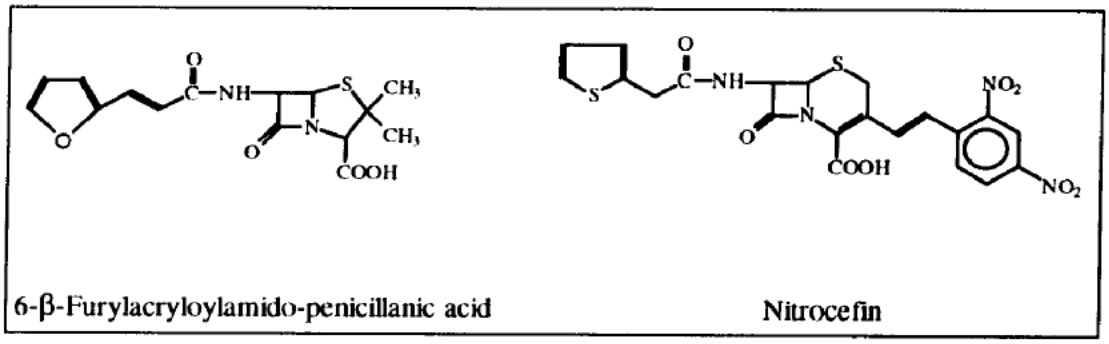

Figure 2. Structure of substrates used to analyze E166A

Accumulation of the acyl-enzyme.

There have been several reports (Virden et al, 1990; Cartwright et al., 1989; Pratt et al., 1988) of the formation of an acyl-enzyme intermediate by Class A β-lactamases, indicating that the catalytic mechanism may be represented by a two-step mechanism (equation 1), where EA represents the covalent acyl-enzyme intermediate.

$$E + S \xleftrightarrow{K_s} ES \xrightarrow{k_2} EA \xrightarrow{k_3[H_2O]} E + P \qquad (1)$$

When the E166A mutant enzyme was added to a solution of nitrocefin at pH 7.0, 30 °C, an immediate increase in absorbance was observed at 482 nm. The intensity indicated a 1:1 stoichiometry between nitrocefin and the enzyme (assuming the same extinction coefficient for the acyl-enzyme as for the product cephalosporinoic acid) (Fig. 2). This suggests that rapid formation of the acyl-enzyme occurred, followed by very slow turnover. The spectrum of the putative acyl-enzyme is also shown in Fig. 2, and is very similar to that of hydrolyzed nitrocefin in the visible region. With FAP a burst was also seen corresponding to formation of the acyl-enzyme, with essentially a 1:1 stoichiometry assuming similar extinction coefficients for acyl-enzyme and product at 330 nm.

Corroboration that the acyl-enzyme intermediate accumulated rapidly was obtained in HPLC experiments. Analysis of mixtures of E166A β-lactamase and a small excess of substrate at pH 7.0 and 0 °C showed the substrate chromophore co-eluting with the enzyme in a 1:1 stoichiometry

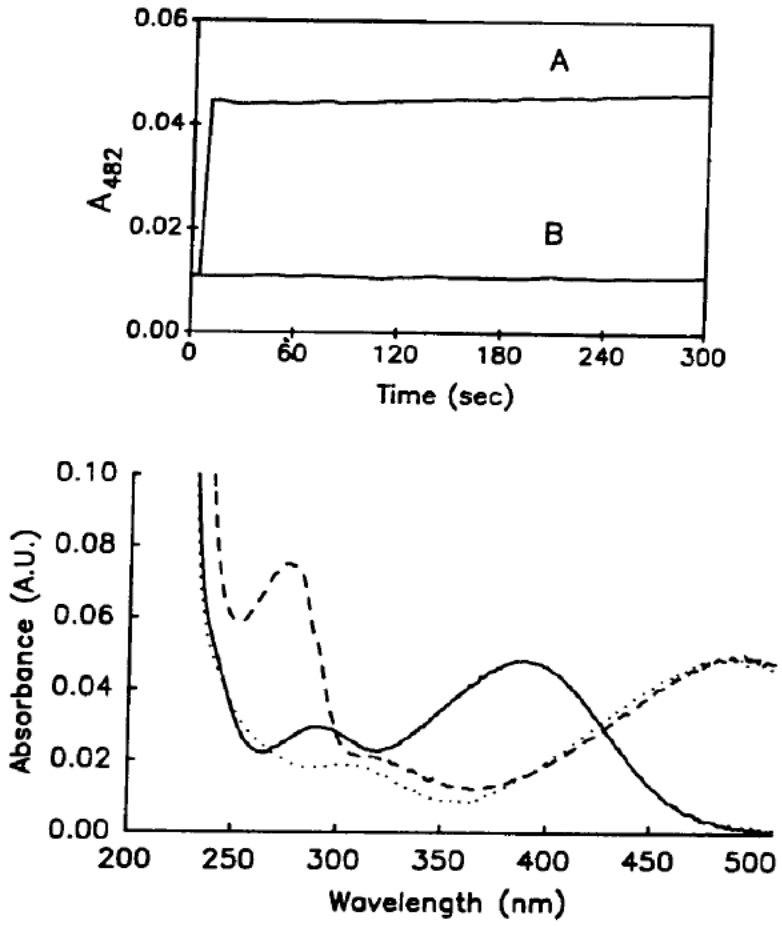

Fig. 2. The reaction of E166A β-lactamase with nitrocefin under steady-state conditions. The upper panel shows the time-dependent changes monitored at 482 nm. Curve A shows the reaction of E166A with nitrocefin: the immediate increase of the signal at 482 nm is followed by slow turnover of substrate. The burst is equivalent to the formation of 2 μM product indicating the reaction is stoichiometric (1:1). Curve B shows the rate of spontaneous hydrolysis of nitrocefin solution alone. A control in which E166A was added to the same buffer without nitrocefin gave a negligible change in signal. The lower panel shows the spectra of nitrocefin (-), the acyl-enzyme formed as in curve A (- - -), and hydrolyzed nitrocefin (....). Nitrocefin concentrations were 2 μM, temperature was 1 °C, pH 7.0.

(Fig. 3). Similar behavior indicative of the accumulation of the acyl-enzyme was observed with both FAP and nitrocefin. Stopped-flow experiments were carried out in order to directly measure the rate of acylation of E166A β-lactamase with nitrocefin. These experiments indicated that the acylation reaction was essentially complete in the dead-time of the apparatus, allowing estimation of a minimum first-order rate constant of 1000 s^{-1} for the acylation reaction at 20 °C, pH 7.0. Confirmation of the long-lived nature of the acyl-intermediate was obtained from experiments in which the stability of the acyl-enzyme was investigated by HPLC. Analysis by HPLC at several time-points indicated the gel-filtration-purified acyl-enzymes underwent hydrolysis to release the penicilloic acid product on a time-scale of days at 4 °C.

The accumulation of the acyl-enzyme in the present study indicates that deacylation is rate-limiting for E166A β-lactamase for both substrates. Thus k_{cat} represents the value of k_3, the deacylation rate constant. The dramatic decrease in the rate of deacylation with the E166A mutant is consistent with Glu-166 acting as a general base catalyst to activate water in the deacylation step.

Values of k_2, the acylation rate constant, for good substrates of the Class A β-lactamases are typically of the order of (1 - 6) x 10^3 s^{-1} (Martin and Waley, 1988). For rate-limiting deacylation

$$K_m = k_3K_s/(k_2 + k_3)$$

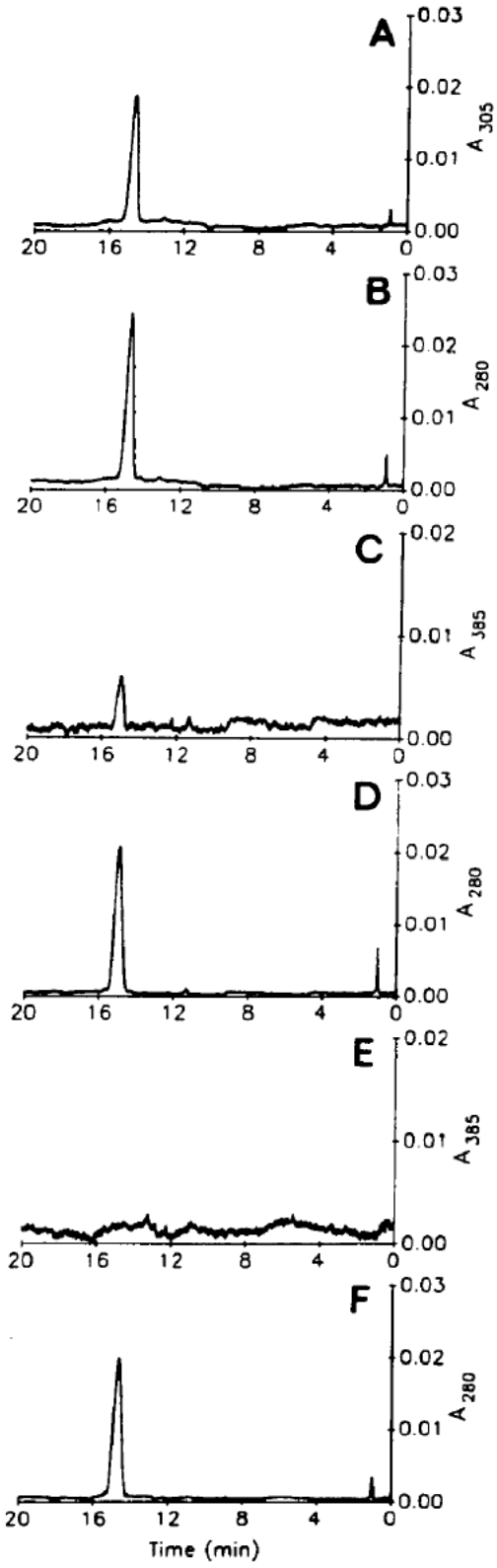

Fig. 3. HPLC traces of furylacryloylpenicillin- and nitrocefin-labeled E166A β-lactamase. The FAP-E166A mixture was monitored at A) 305 nm and B) 280 nm to assay for the presence of FAP and E166A respectively. Both traces show a single peak with coincident retention times of 14.7 min. Similar results were seen for the nitrocefin experiment monitored at C) 385 nm (nitrocefin) and D) 280 nm (E166A). Enzyme only control is shown in E) at 385 nm and F) at 280 nm. Frame A and B contributed by A. Tan.

It has previously been shown for other Class A β-lactamases with FAP as substrate (Virden et al., 1990; Cartwright et al., 1989) that the rate-limiting step is deacylation. We estimate that for the wild-type enzyme K_s is of the order of 10^{-4} M. The results from the stopped-flow experiments indicate that K_s is similar or larger for the mutant, and that value of k_2 for the mutant must be similar to that of the wild-type. Thus, there is relatively little effect of the mutation on the rate of acylation, and the substitution of Ala for Glu-166 leads to a very large differential effect on the rates of acylation and deacylation. This behavior by the E166A enzyme is in contrast to that of the corresponding aspartate mutant (E166D) in which the acylation rate appears to be comparable to that of deacylation, since no accumulation of the acyl-enzyme occurs (Gibson et al., 1990; chapter 4). It is likely that the replacement of Glu-166 by aspartate leads to some of the catalytic function of Glu-166 in deacylation remaining (either by positioning of the aspartate carboxylate via a conformational change or via a bridging water molecule). For benzylpenicillin both acylation and deacylation are reduced about 2000-fold with E166D (Gibson et al., 1990); it is possible that nitrocefin is an unusual substrate, due to its highly activated β-lactam bond, and for less activated substrates Glu-166 also participates as a catalytic group for acylation. Recently, Adachi et al. (1991) have reported that mutants of the RTEM-1 β-lactamase in which Glu-166 is replaced by Asn, Gln or Ala have drastically reduced deacylation rates and thus accumulate covalent acyl-enzyme intermediates with bezylpenicillin. They also observe that with E166D the rate of deacylation is significant, although much slower than for the wild-type.

The possibility that the E166A mutation resulted in the substrates acting as Type A substrates (Citri et al., 1976) and that the lack of deacylation resulted from substrate-induced deactivation was investigated as follows. We have previously shown that inactivation by Type A substrates leads to conformational changes resulting in a stabilized acyl-enzyme which is more susceptible to proteolysis (Fink et al., 1987). Thus the susceptibility of E166A, nafcillin-inactivated E166A (nafcillin is a Type A substrate) and the acyl-enzymes of E166A with nitrocefin and FAP, to proteolysis by chymotrypsin was studied. Only the nafcillin-inactivated enzyme showed enhanced proteolysis, indicating that the formation of the stable acyl-enzyme between E166A and nitrocefin and FAP was not due to their acting as Type A substrates. This is in contrast to a recent report in which hydrolysis of cephalosporin C by the K73R mutant of *B. cereus* β-lactamase exhibited Type A behavior, although the corresponding E166D mutant showed normal behavior (Gibson et al., 1990).

Mechanistic implications.

The stoichiometric accumulation of an enzyme-substrate intermediate is a novel result. This stable acyl-enzyme offers an excellent opportunity for structural studies on what is normally a transient, and productive, catalytic intermediate. The data indicate that Glu-166 is an essential catalytic residue only in the deacylation step of the catalytic reaction, and that its function in acylation appears to be minimal. This is in marked contrast to the serine proteases, which catalyze a similar reaction but in which acylation and deacylation involve essentially the same catalytic mechanism with His-57

functioning as a general base catalyst in both steps. In the E166A mutant it is likely that solvent catalysis HO^- is responsible for the observed very slow deacylation.

If Glu-166 is not involved in acylation the question arises as to whether there is another active-site group which may act as a general base to activate the hydroxyl of Ser-70 for nucleophilic attack on the lactam carbonyl. Examination of the active-site structure of B. licheniformis β-lactamase (Moews et al., 1990) reveals that in the free enzyme there are no side-chains sufficiently close to Ser-70 to act in this fashion. Thus, either major conformational changes occur on binding of substrate to reposition a suitable group or the nucleophilic attack by Ser-70 is unassisted. Interestingly, Glu-166 would be the most likely candidate for the former proposal, but is ruled out by the present results. However, there is evidence to suggest that general base-catalyzed assistance of the nucleophilic attack by Ser-70 on the substrate lactam carbonyl might not be necessary. For example, the reactivity of penicillin β-lactam ring is intrinsically greater than that of normal amides (Page, 1984; 1987). Structural studies, combined with model-building of the substrate in the active-site, suggest that the β-lactam carbonyl will be polarized by hydrogen bonding to two backbone amides (Ser-70, Ala-237), increasing the reactivity of the group to nucleophilic attack (Herzberg and Moult, 1987; Moews et al., 1990).In addition the hydroxyl of Ser-70 will be activated by the effect of the helix dipole moment from helix a2 (Moews et al.,1990).

The present results support the proposal of Moult and Herzberg (1987) that Glu-166 is involved in activating water in deacylation and not in the

acylation reaction. It is likely that the factors mentioned above are sufficient to permit the nucleophilic attack by serine in the acylation reaction. It has been suggested that Lys-73 is involved in facilitating proton transfer to the β-lactam nitrogen (Herzberg and Moult, 1987), however, it is also possible that the proton comes from a solvent molecule. The replacement of Glu-166 by Ala will also disrupt the salt bridge between Glu-166 and Lys-73. Thus it is possible that the mutation may have indirect effects due to changes in the position and pK of the side-chain of Lys-73. However, the present results rule out such possibilities if Lys-73 acts as a catalyst in the acylation half of the reaction, and it is difficult to conceive reasonable roles for Lys-73 which involve only the deacylation step.

The pH-rate profile of β-lactamase catalysis shows an acidic pK in the vicinity of 5, which is also detected by titration/modification of the thiol group of the S70C mutant (Knap and Pratt, 1991). The most likely residue responsible for this pK is Glu-166. It is possible thatGlu-166 plays a variable role in catalyzing acylation, depending on the nature of the substrate, nitrocefin, for example is known to be a highly activated β-lactam, and might require little assistance in formation of the acyl-enzyme. Less activated substrates may benefit from potential catalysis by Glu-166.

Comparison of the structures of β-lactamase from B. licheniformis and the penicillin-binding protein DD-peptidase from *Streptomyces* R61 reveals that the residue in the equivalent position to Glu-166 of the β-lactamase is Phe-164 (Knox and Kelly, 1989). Thus a simple explanation why a stable acyl-enzyme is formed by the DD-peptidases and related penicillin-binding proteins involved in bacterial cell-wall synthesis is that the Phe-164 prevents

the positioning and general base catalysis of the water molecule necessary for deacylation. Consequently it is the potential for catalysis by Glu-166 which leads to the breakdown of the acyl-enzyme in the β-lactamases. This is supported by the findings in the present study in which replacing Glu-166 by alanine prevents deacylation.

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Chapter IV

The Role of Glutamate-166 in β-Lactamase Catalysis Probed by Site Directed Mutagenesis

Introduction

As a first approximation to describing the mechanism for β-lactamases an attempt has been made to compare their mechanism to other well studied systems. For example, several lines of evidence indicated that the catalytic reaction of β-lactamases was similar to that of serine proteases. The active site serine residue (Ser-70 in the numbering system of Ambler (1980)) undergoes nucleophilic attack on the β-lactam amide bond leading to the formation of an acyl-enzyme intermediate. If β-lactamases do have a reaction pathway analogous to that of the serine proteases then one would also expect to find the catalytic triad characteristic of these proteases. Herzberg and Moult (1987) compared the structure of the catalytic site for these two classes of enzymes by a least squares fit algorithm. They found that although a lysine was approximately positioned to substitute for histidine of the triad there was no appropriately placed carboxyl group to take the role of aspartate. This raised the question of how serine was activated for attack on the carbonyl carbon of the lactam ring, and the related question of how water was activated to attack the acyl-intermediate in the process of deacylation. From the active-site structure two likely candidates for increasing the nucleophilic reactivity of Ser-70 are lysine 73 and glutamate 166.

Glutamate 166 has been implicated in the mechanism of Class A β-lactamases. It is highly conserved and is found in several penicillin binding proteins outside of the Class A family. X-ray crystallography (Herzberg and Moult, 1987; Moews et al., 1991) has demonstrated that the carboxyl group of this residue is located within the catalytic site close to Ser-70 (3.5 A) and

Lys-73 (3.2 A). Furthermore site-directed mutagenesis studies (Madgewick and Waley, 1987; Gibson et al., 1990; Adachi et al., 1991; Escobar et al., 1991, Delaire et al., 1991) have directly implicated this group in the mechanism since specific mutations at site 166 result in a large decrease in activity.

Could Glu-166 be participating as a general base in the reaction? The possibility that this residue is responsible for the acidic limb of the pH profile of these enzymes would support this hypothesis. To act as a general base the enzyme would become active as the carboxyl group ionized with increasing pH. Work by Cartwright et al. (1989) indicates this may be the case. It was noted that the acidic pK_a of the pH profile for β-lactamase was insensitive to changes in temperature. This is characteristic of groups displaying small heats of ionization such as the carboxyl group.

We have previously shown (chapter 3) that conversion of Glu-166 to Ala leads to more than a million-fold decrease in catalytic activity: however, this mutant enzyme is so inactive it was not technically feasible to determine its pH-dependence. To further explore its role in the catalytic mechanism we have specifically mutated Glu-166 to Cys and Asp. The E166D mutant should have a pH-rate profile analogous to that of the W.T. since the pK_a of glutamate (4.1) and aspartate (3.9) are similar. However, if Glu-166 is responsible for the acidic limb of the pH profile then replacing Glu-166 with Cys should dramatically alter the pH profile. That is, we would expect the acidic pK_a to reflect the ionization properties of the incorporated cysteine residue with an anticipated pK in the vicinity of 9. Furthermore it has been

suggested that the omega loop which contains Glu-166 may be relatively mobile (Herzberg and Moult, 1987). If this site is mobile then one might expect that shifting the carboxyl group of Glu-166 by one methylene group, ~ 1.5 A, as in the E166D mutant, should not appreciably alter catalysis.

EXPERIMENTAL PROCEDURES

Materials

Benzylpenicillin and phenoxymethylpenicillin were purchased from Sigma. Nitrocefin was a gift from Glaxo. Sequenase used for sequencing mutants was purchased from U. S. Biochemicals. Restriction enzymes, Klenow fragment and ligase used for mutagenesis were obtained from New England Biolabs.

Mutagenesis

E166C and E166D mutant β-lactamases where made as described in chapter 2. Mutations were introduced via the Kunkle method (Kunkle, 1985) and sequenced with Sequenase (U.S. Biochemicals). The primers used for mutagenesis were 5'-GAA-CGA-TTC-(XXX)-CCA-GAG-CTC-AAT-GAA-GTG-3' where (XXX) was (TGT) for the cysteine mutant and (GAC) for the aspartate mutant. These primers were designed to introduce a Sac I site (underlined) to allow for restriction purification (Wells et al., 1986).

Purification procedure

The following purification protocol is a modified version of the protocol described by Tan (1990). The wild-type and mutant enzymes were purified as follows. *Bacillus licheniformis* β-lactamase (W.T., E166C and E166D) was expressed and purified from *Bacillus subtilis*. Fresh streaks were inoculated into 6 liters of rich media (Ellerby et al., 1990) and grown on a 37 °C shaker for 12 to 14 hours. Cells were removed by centrifugation (8000 rpm for 30 min.) and the cell-free supernatant dialyzed against 0.02 M sodium acetate pH 4.8 (exchanged twice in 30 liters of buffer). CM-Sepharose matrix (CL-6B from Sigma) was pre-equilibrated with the same buffer. The protein was equilibrated with CM-Sepharose matrix (15 ml of gel) for 1 hour by gently swirling on a shaker at room temperature. This enzyme-matrix mixture was batch loaded onto a CM-Sepharose column (50 ml of gel in 2.5 x 19.5 cm column) and eluted with a linear salt gradient of 0 - 0.25 M NaCl. Fractions containing β-lactamase activity were pooled and exchanged via ultrafiltration with an Amicon YM10 filter into 0.02 M TRIS pH 7.2 buffer. This was equilibrated with DEAE-Sepharose (0.02 M TRIS pH 7.2 for 1 hour - 10 ml of gel), batch loaded onto a DEAE-Sepharose column (same size as above) and eluted with a linear salt gradient of 0 - 0.25 M NaCl. Fractions containing β-lactamase activity were pooled and exchanged into 0.05 M potassium phosphate pH 7.0 buffer by ultrafiltration with a YM10 filter. All proteins were purified to homogeneity as determined by SDS-PAGE using the Pharmacia PhastSystem

Kinetics

The pH-rate profiles for E166C were determined by initial velocities using Hanes-Woolf analysis (Segal, 1975). Above pH 9 the relatively high rate of spontaneous hydrolysis and exceedingly low K_m for Nitrocefin permitted only the determination of k_{cat}. The Nitrocefin-W.T. and the phenoxymethyl-E166D pH profiles were obtained from complete progress curves and analyzed by the method of Koerber and Fink (1987). All points were determined in triplicate. Substrate hydrolysis was measured at 30°C with a Perkin-Elmer 320 spectrophotometer using a 1 cm path length cell. All buffers contained 0.5 M KCl and were brought to a final concentration of 50 mM buffer. The buffers used for the pH profiles were sodium acetate for pH 4.0 - 5.5, potassium phosphate for pH 6.0 - 7.5, TRIS-HCl for pH 8.0 - 8.5, CHES for pH 9.0 - 9.5 and CAPS for pH 10.0 - 11.00.

Burst kinetics with E166C

Nitrocefin (45 μM in 50 mM potassium phosphate, 0.5 M KCl at pH 7.0) was preequilibrated to 30 °C. E166C was added to a final concentration of 2.41 μM. The reaction was monitored at 482 nm with a Perkin-Elmer model 320 spectrophotometer.

RESULTS AND DISCUSSION

Structure of the mutant proteins

Circular dichroism studies as well as thermal stability measurements indicate that removal of the carboxyl group at site 166, as in the E166A

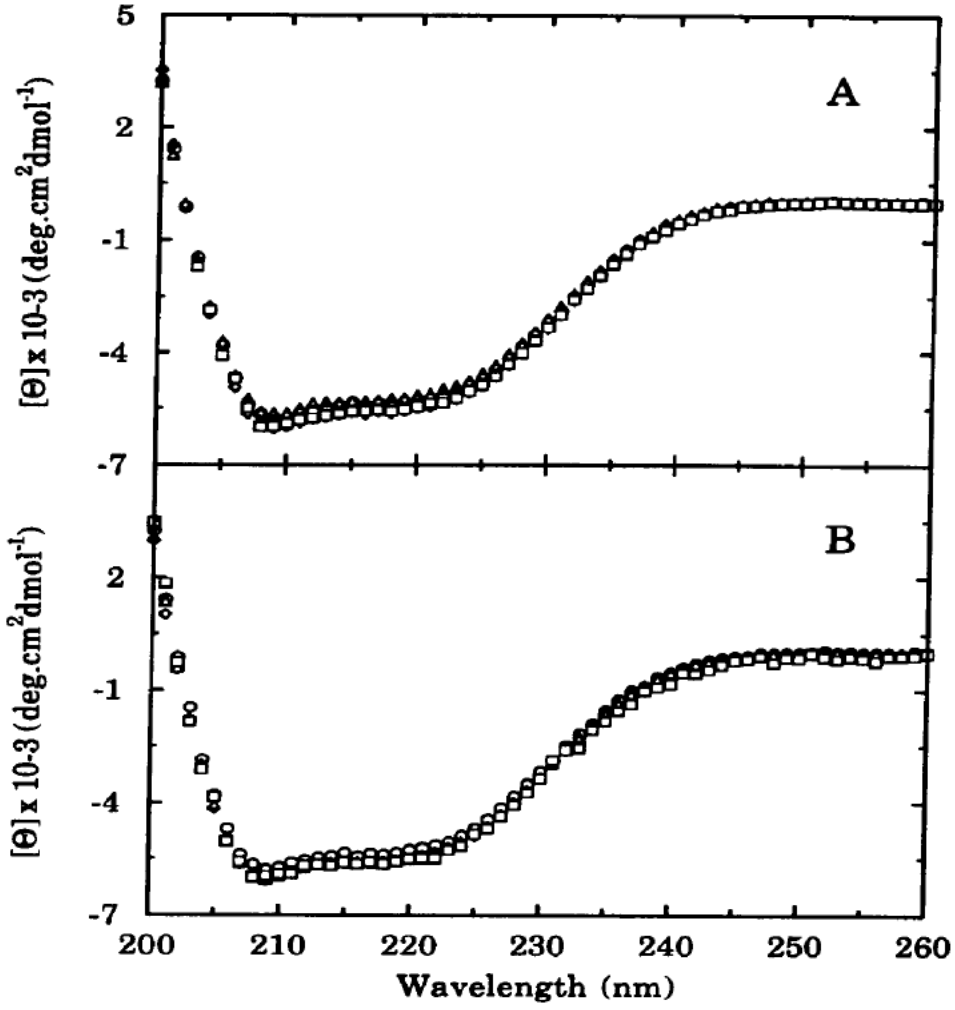

Fig. 1. Comparison of the structures of wild-type and the E166C and E166D β–lactamase mutants as a function of pH by far-UV CD spectra. Panel A shows the spectra for E166C , and Panel B those for E166D. The symbols are as follows: circles, native wild-type at pH 7.0; diamonds, pH 4.0, E166C or E166D; triangles, pH 7.0, E166C or E166D; squares, pH 9.0, E166C or E166D. Data courtesy of Tan (1990).

enzyme, does not significantly perturb the secondary or tertiary structure of this enzyme (Escobar et al., 1991, chapter 3). This is supported by the high resolution crystallographic map obtained for E166A which deviates by 0.25 A (r.m.s.) in backbone atoms from the W.T. structure (Knox et al., 1992). Similarly, mutation of Glu-166 to cysteine or aspartate has minimal effects on secondary structure as evidenced by their CD spectra (see Fig. 1). CD spectra for the mutants at pH 4, 7 and 9 (Fig. 1 A and B) were all superimposible on that of the native W.T. spectrum (pH 7.0) within experimental error.

Thermal denaturation of the mutant proteins was also carried out to measure their relative stability. The results are given in Table I. At pH 7.0, the E166C mutant had the same thermostability as the wild-type, but the T_m of E166D was somewhat less stable. At pH 9.0, the E166D mutant was as stable as the wild-type, and the E166C mutant was more stable. At pH 4.0, both mutants were less stable than the W.T. enzyme, the E166D mutant showed a markedly biphasic unfolding transition.

Kinetics

The pH-rate profile for the W.T. enzyme was determined with phenoxymethylpenicillin and Nitrocefin. These are both good substrates for this enzyme with turnover numbers in the thousands per second for the W.T. (Ellerby et al., 1990). Nitrocefin was chosen because of the large $\Delta\varepsilon$

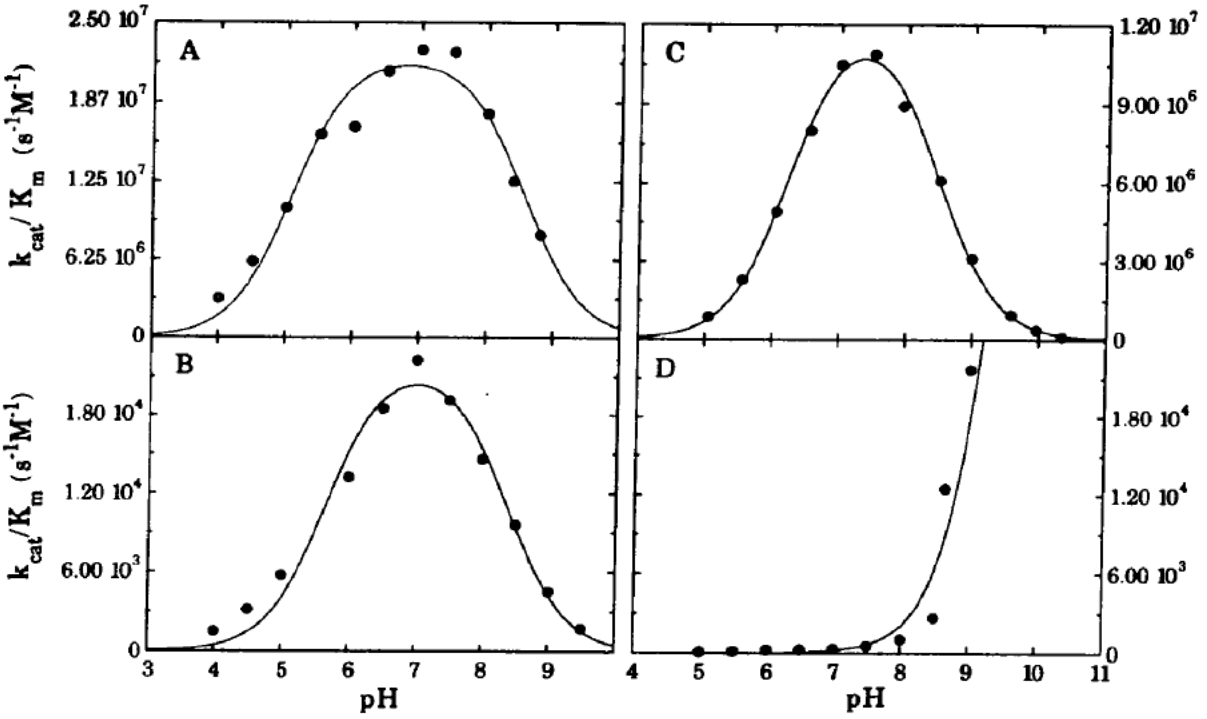

Fig. 2. k_{cat}/K_m vs. pH profiles for the mutants and wild-type β–lactamase. Panel A, W.T. with phenoxymethylpenicillin; Panel B, E166D with phenoxymethylpenicillin; Panel C, W.T. with Nitrocefin; Panel D, E166C with Nitrocefin. All reactions monitored at 30 °C. E166D kinetic data courtesy of Tan (1990). W.T.-phenoxymethylpenicillin data courtesy of Ellerby et al. (1990).

associated with the hydrolysis of this cephalosporin. This is ideal for studying the kinetics of mutants displaying little activity such as E166C. Phenoxymethylpenicillin is relatively stable at acidic pH and is thus suitable for studies at low pH values. As can be seen in Fig. 2 the k_{cat}/K_m pH-profiles for both of these substrates with the wild-type enzyme have the characteristic bell-shaped curve seen with many enzyme systems. The common interpretation for such curves is that the deprotonation of a residue within the protein results in the activation of the enzyme (at acidic pH) while the deprotonation of a second group results in the decreased catalytic efficiency of the enzyme at basic pH. Values from these curves were fit to equation 1

$$k_{obs} = k_{lim}/(1 + [H^+]/K_1 + K_2/[H^+]) \qquad (1)$$

in order to determine K_1 and K_2, the ionization constants corresponding to the acidic and basic limbs respectively. The pK_a's measured for the wild-type and mutant enzymes are given in Table II. The values of pK_1 and pK_2 for k_{cat}/K_m correspond to the pK's of the key ionizing groups in the *free* enzyme. The unusual dependence of k_{cat}/K_m on pH for E166C is discussed in detail below. Interestingly pK_1 for wild-type enzyme with Nitrocefin is higher than that observed with most substrates.

The reaction catalyzed by Class A β-lactamases has been shown to proceed through an acyl-intermediate. Thus, as with other acyl-transfer systems (e.g., the serine proteases), the reaction can be described by a two step mechanism, eq. 2, where EA represents the acyl-enzyme, and k_2 and k_3

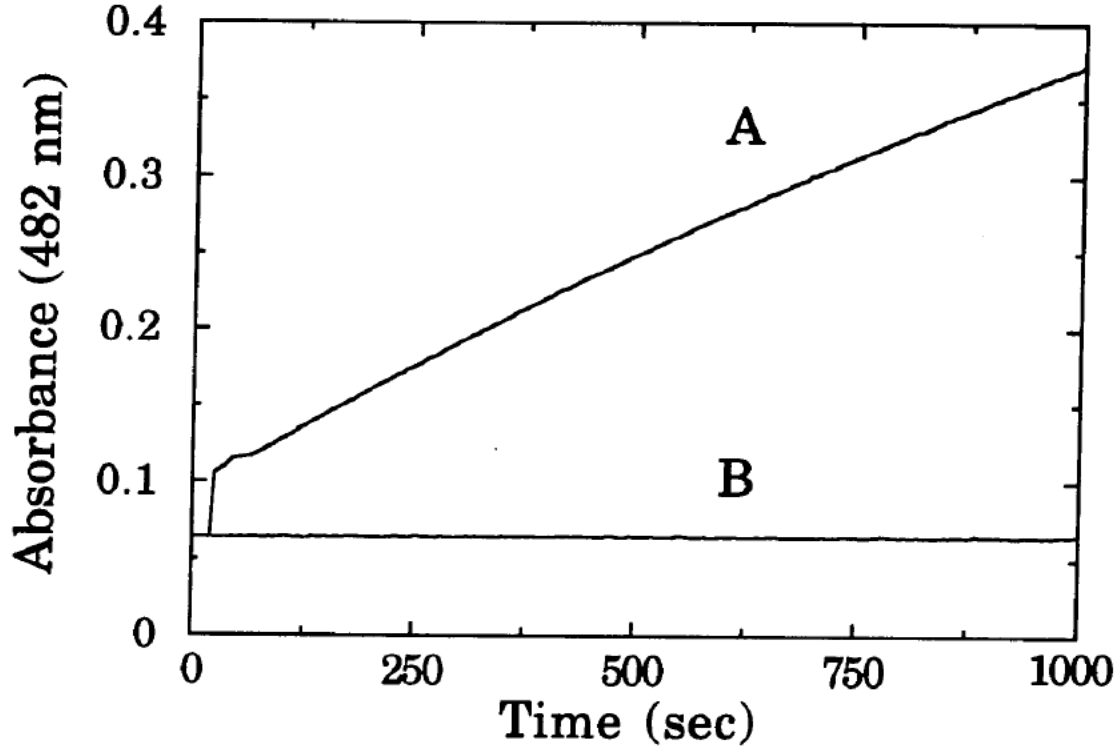

Fig. 3. Burst seen upon incubation of E166C with Nitrocefin. The reaction was monitored at 482 nm and 30 °C. The concentrations of E166C and Nitrocefin were 2.41 μM and 45 μM respectively. The rapid increase in absorbance (curve A) corresponds to a stoichiometric production of acyl-enzyme (2.41 μM) indicating that acylation is rapid and that it is followed by a much slower deacylation step. Line B represents the baseline in the absence of enzyme.

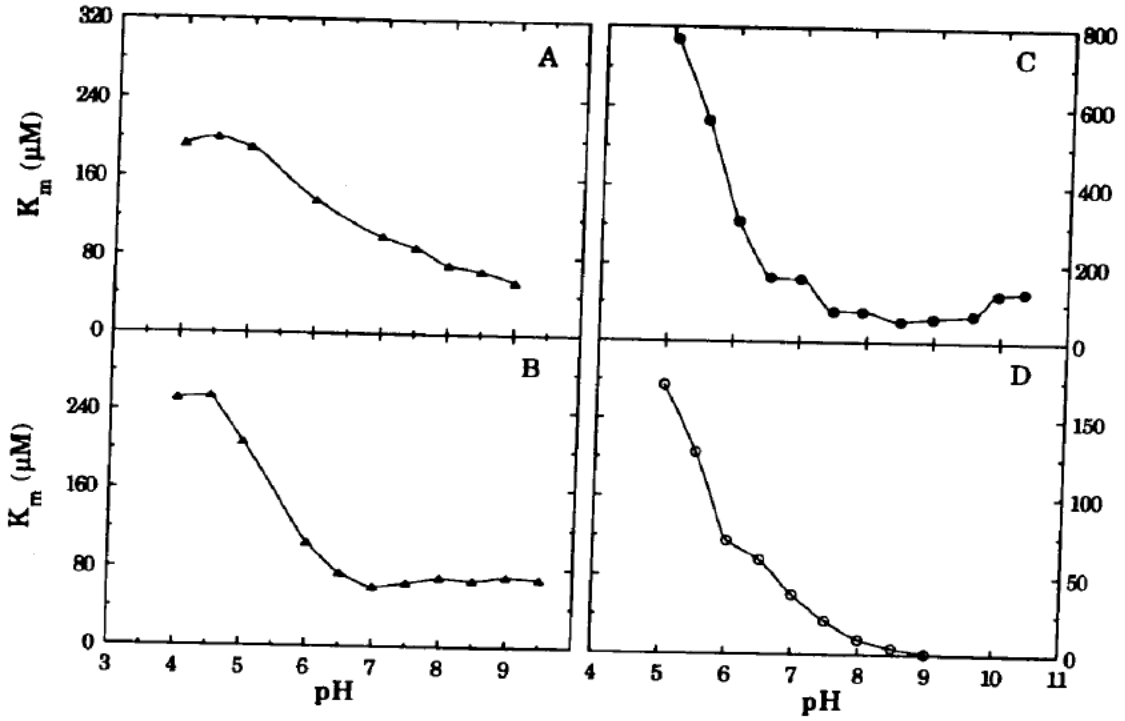

Fig. 4. K_m-pH profiles for the mutants and W.T. β–lactamase. Panel A, W.T. with phenoxymethylpenicillin; Panel B, E166D with phenoxymethylpenicillin; Panel C, W.T. with Nitrocefin; Panel D, E166C with Nitrocefin. All reactions monitored at 30 °C. E166D kinetic data courtesy of Tan (1990). W.T.-phenoxymethylpenicillin data courtesy of Ellerby et al. (1990).

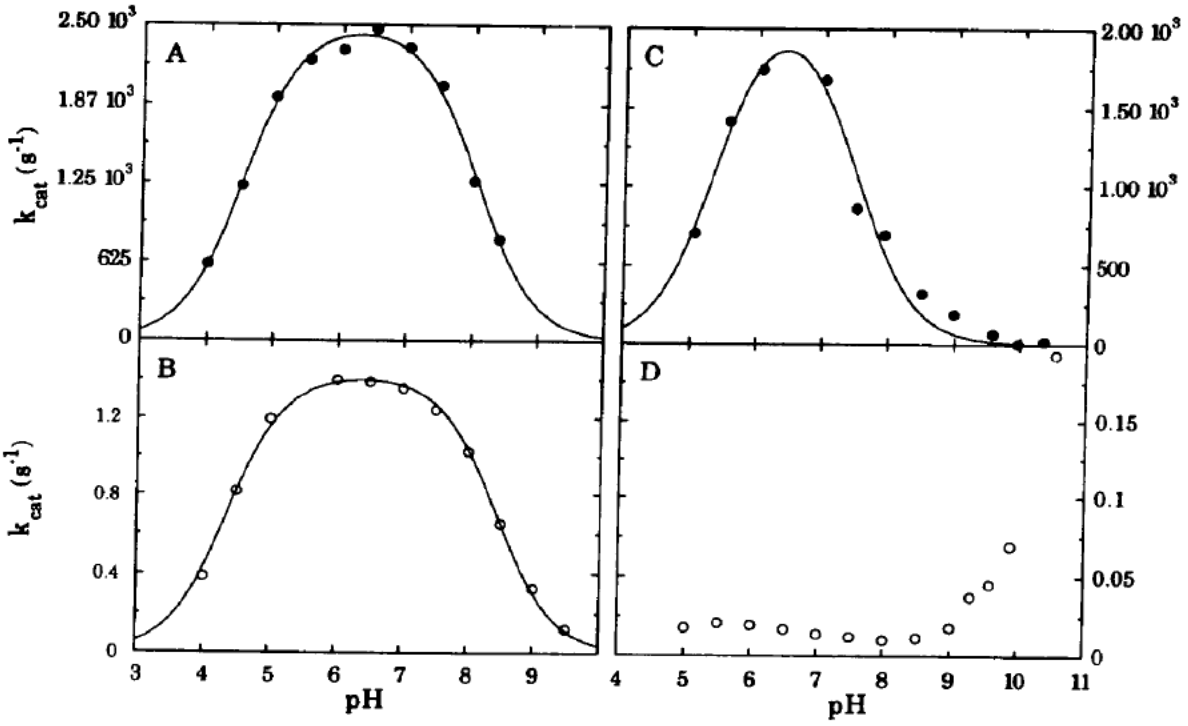

Fig. 5. k_{cat}-pH profiles for the mutants and W.T. β-lactamase. Panel A, W.T. with phenoxymethylpenicillin; Panel B, E166D with phenoxymethylpenicillin; Panel C, W.T. with Nitrocefin; Panel D, E166C with Nitrocefin. All reactions monitored at 30 °C. The E166C data (Panel D) was not fit to a standard ionization curve since the shape of the profile was not appropriate for this type of analysis. E166D kinetic data courtesy of Tan (1990). W.T.-phenoxymethylpenicillin data courtesy of Ellerby et al. (1990).

Table I: Thermal denaturation midpoints (T_m) for Wild-Type, E166C and E166D β-lacatamase

	Wild Type	E166C	E166D
pH	T_m (°C)	T_m (°C)	T_m (°C)
4.0	52.2 ± 1	46.4 ± 1	43,60[a] ± 1
7.0	65.0 ± 1	64.0 ± 1	60.0 ± 1
9.0	56.5 ± 1	60.2 ± 1	55.6 ± 1

[a] Biphasic transition.

Table II: Ionization constants (pK_a) for Wild-Type, E166C and E166D β-lactamases

	Substrate : Nitrocefin				Substrate: Phenoxmethylpenicillin			
	Wild-Type		E166C		Wild-type		E166D	
	k_{cat}/K_m	k_{cat}	k_{cat}/K_m	k_{cat}	k_{cat}/K_m	k_{cat}	k_{cat}/K_m	k_{cat}
pK_{a1}	6.22	5.33	9.68	N.D.	5.0	4.5	5.7	4.4
pK_{a2}	8.49	7.49	N.D.	N.D.	8.6	8.1	8.4	8.4

All pKa values < ± 0.1

Table III: Kinetic constants for Wild-Type, E166C and E166D β-lactamases at pH 7.0, 30 °C

	Substrate : Nitrocefin			Substrate: Phenoxmethylpenicillin		
	Wild-Type	E166C	k_{mut}/k_{wt}[a]	Wild-type	E166D	k_{mut}/k_{wt}[a]
k_{cat} (s^{-1})	1685	1.41×10^{-2}	8.4×10^{-6}	2.33×10^{3}	1.35	5.8×10^{-4}
K_m (M)	1.61×10^{-4}	3.83×10^{-5}	0.24	1.0×10^{-4}	6.1×10^{-5}	0.61
k_{cat}/K_m ($M^{-1}s^{-1}$)	1.05×10^{7}	3.68×10^{2}	3.5×10^{-5}	2.3×10^{7}	2.23×10^{4}	9.7×10^{-4}

Standard deviation for above values < ± 10 %

[a] Ratio of mutant/wild-type value.

the acylation and deacylation rate constants, respectively. During acylation Ser-70 attacks the carbonyl carbon of the lactam ring to form a covalent penicilloyl-enzyme intermediate. Water then attacks the ester bond of the intermediate and releases the hydrolyzed product in the deacylation step.

$$E + S \xleftrightarrow{K_s} ES \xrightarrow{k_2} EA \xrightarrow{k_3[H_2O]} E + P \qquad (2)$$

It has been suggested by Escobar et al. (1991) that Glu-166 is essential in only the deacylation half of this mechanism, in which it activates a key bound water molecule for nucleophilic attack on the acyl-enzyme (Knox and Moews, 1991). If this is the case then one might expect that the decrease in activity seen with the cysteine and aspartate mutants results from a large decrease in k_3, the deacylation rate constant, in which case k_{cat} should approximate k_3 in these mutants. This assumption is borne out by the observed rapid increase in absorbance seen upon incubation of E166C with Nitrocefin (Fig. 3). This burst indicates a relatively rapid acylation rate (complete within the dead time of manual mixing) followed by a slower deacylation process. Assuming that the extinction coefficient for the acyl-enzyme is the same as that for the product of Nitrocefin hydrolysis, we calculate a 1 : 1 stoichiometry for enzyme and substrate at zero time in this reaction.

E166D Kinetics

The effect of mutating Glu-166 to Asp on the kinetic parameters over the pH range 4.0 to 9.5 was examined using phenoxymethylpenicillin. The

K_m values were similar to those for the W.T. (Table III) in the pH range studied (Fig. 4). The k_{cat} values for E166D were significantly lower (at least 1,500 fold lower, see Table III) than those of the W.T. in the observed pH range. However, the shape of the k_{cat} vs. pH profile was very close to the one obtained for the W.T. (Fig. 5). The pK_1 and pK_2 for E166D from the k_{cat} pH profile were comparable to the values for the wild-type enzyme with this substrate (Table II). The plot of k_{cat}/K_m vs. pH (Fig. 2) reflects the pK value(s) of the essential residue(s) of the free enzyme involved in catalysis. The k_{cat}/K_m values were approximately 1,000 fold lower than those obtained for the W.T. (Table II). The pK_1 value obtained from the acidic limb of Figure 2 is 5.7 which is 0.7 pH units higher than the W.T. value (5.0). The basic limb, pK_2 = 8.4, approximates the W.T. value of 8.6. The small changes in the K_m values indicate the mutation does not greatly perturb the binding of substrate. The conservative nature of this mutation should not significantly alter the electrostatic potential of the catalytic site and therefore E166D should maintain the binding properties which are dependent on this potential (Ellerby et al., 1990). Decreased values of k_{cat} and k_{cat}/K_m indicate the catalytic efficiency of E166D has been disrupted. Figure 5 (k_{cat} vs. pH) and Figure 2 (k_{cat}/K_m vs. pH) demonstrate that it is predominantly the acidic limb which is affected by the mutation. The higher pK_1 values for E166D reflect the environment in which the carboxyl group resides. Since the side chain of Asp is one methylene group shorter than that of Glu it is likely the carboxyl group finds itself in a region of decreased polarity which would increase the pK_a.

Mobility of the Ω loop in β-lactamase

The Ω loop which contains residue 166 has been postulated to be relatively mobile (Herzberg & Moult, 1987). The location of this loop over a large internal cavity of the enzyme suggests a relatively loose packing around the loop. Given that β-lactamases are thought to possess high internal mobile (Robson & Pain, 1976; Carrey & Pain, 1978) there is the possibility that any conformational flexibility afforded by loose packing would allow this region to breath more extensively. To investigate this possibility we compared the activity of E166D to that of W.T.. As discussed above there is a decrease in activity corresponding to three orders of magnitude. In the aspartate mutant the carboxyl functional group is withdrawn by one methylene group (~ 1.5 A) compared to that of the W.T. . Thus, we find that enzyme activity is very sensitive to the change of position of the carboxyl functional group, a result which argues against flexibility at this site.

E166C Kinetics

In contrast to the bell-shaped pH profiles for the W.T. and E166D β-lactamases, the k_{cat} pH-profile of the E166C mutant is very different (Fig. 5). At neutral pH the value of k_{cat} is 10^{-5} that of the W.T.. Values of k_{cat} are very low below pH 9.0 and appear to have a slight increase around pH 5.5. The value of k_{cat} begins to increase dramatically above pH 9.0. As discussed previously the acidic pK_a of the pH-profiles for these enzymes is thought to involve the ionization of Glu-166. Thus we would expect that if this

functional group was replaced with one of a higher pK_a then the acidic limb should adjust itself to reflect this new pK_a. The rise in activity which we see above pH 8.0 supports this belief, given the anticipated pK of the Cys being 9 or above. If, as indicated by the burst experiment (see Fig. 3), k_3 is rate-limiting throughout the pH profile then it is clear that the residue at position 166 plays a role in catalyzing the deacylation step.

The kinetic parameter k_{cat}/K_m reflects the apparent second order rate constant for the reaction of enzyme and substrate, and reflects the pK_a of key ionizing groups in the free enzyme. As seen for the E166C mutant the k_{cat}/K_m profile shows an increase above pH 8.0. We believe that this increase reflects the ionization of cysteine 166 in E166C and implies that ionization at this site is important for activity. If we fit the E166C k_{cat}/K_m pH-profile with equation 3:

$$k_{obs} = k_{lim}/(1 + [H^+]/K_1) \qquad (3)$$

we find there is an ionization constant of pK_a 9.7 (for k_{lim} of 1×10^5) for the free enzyme (Fig. 2). This pK_a value correlates well with that seen for thiols of polar aliphatic groups, (Friedman, 1973) and supports the hypothesis that the ionization of Cys-166 activates the enzyme. Attempts to directly determine the ionization constant of Cys-166 were unsuccessful due to technical problems.

An alternative explanation for the activation which occurs above pH 8.5 is that there is hydroxide ion-assisted catalysis. To investigate this possibility we examined the activity of the enzyme as a function of hydroxide ion

concentration. Technical difficulties prevented acquisition of data above pH 10.5. Although there is a linear dependence on the hydroxide ion concentration above pH 8.5 (see Fig. 6), the slope is 0.6, not 1, as would be expected if hydroxide ion were responsible for the catalysis. Thus we believe the increase in activity seen between pH 8.5 and 10.5 arises from the ionization of the thiol group of Cys-166.

Conclusion and Proposed Catalytic Mechanism

Glutamate 166 of Class A β-lactamases has been postulated to participate in the mechanism of these enzymes as a general base, and to be responsible for the ionization reflected in the acidic limb of the pH profile for catalysis. Mutations at this site to cysteine and aspartate resulted in a large decrease in activity, a result previously seen for other mutations at this site by several workers (Adachi et al., 1991; Gibson et al., 1990; Madgwick & Waley, 1987), demonstrating the central function of this residue in catalysis. Indeed, mutation of Glu-166 to Ala generates a mutant that is too inactive for practical kinetic measurements (Escobar et al., 1991). Evidence indicating that this loss of activity did not result from conformational changes comes from the CD and thermal stability studies, and is supported by previous investigations with E166A (Escobar et al., 1991, Knox et al., (1992).

Although E166D activity is down three orders of magnitude, the shape of the pH profiles for this mutant approximate those of the wild type, an expected result, given the similarities in the intrinsic pK_a's of aspartate and

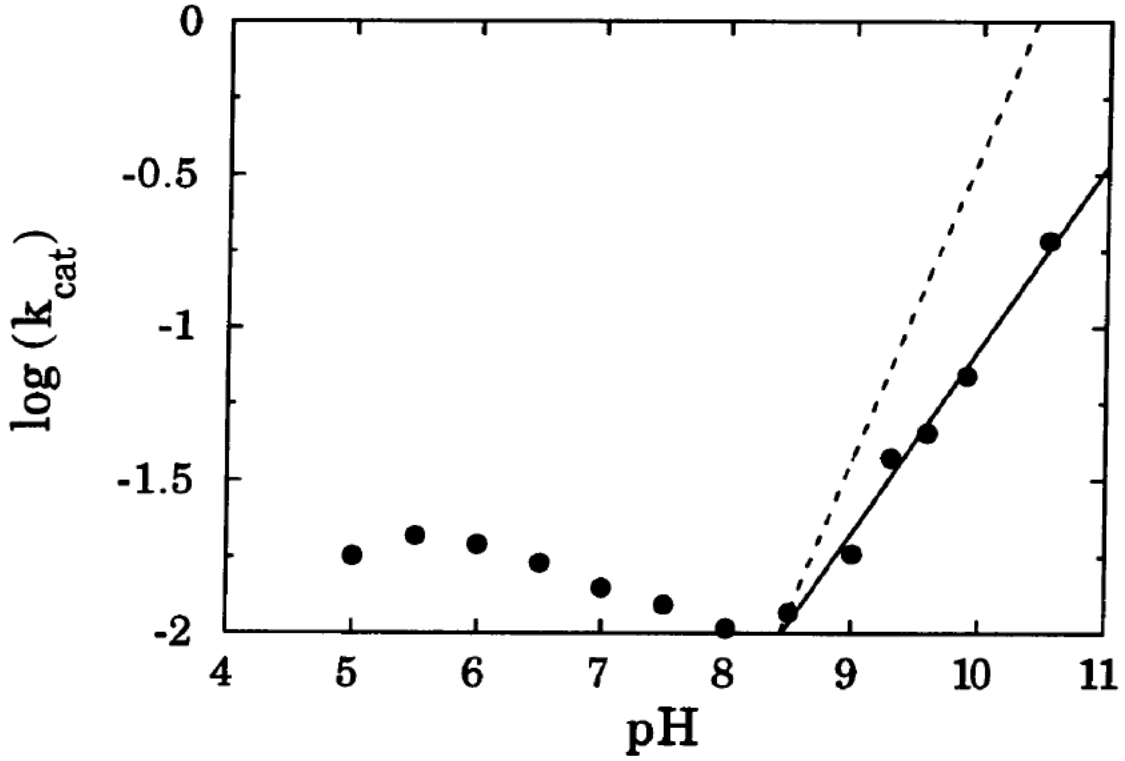

Fig. 6. The dependence of k_{cat} on hydroxide ion concentration. The slope of the log k_{cat} vs. pH plot for the data above pH 8.5 has a slope of 0.6. The broken line shows a slope of 1, which would be expected if the reaction was hydroxide-catalyzed.

glutamate. In contrast, the pH profiles for E166C are very different than those of the W.T.. Moreover, we observe a large decrease in activity with this mutant (at pH 7.0, the optimum for the W.T., k_{cat}/K_m is decreased by 10^{-5} with Nitrocefin). In the only other report concerning pH-dependence of a Glu-166 mutant Delaire et al. (1991) also noted that the pH profiles for the E166Y mutant of RTEM β-lactamase also have an appreciably modified shape in comparison to the W.T. profiles, as well as decreased activity. The stoichiometric burst observed with E166C and Nitrocefin indicates that the acylation rate is relatively rapid compared to the deacylation step, a finding which suggests that the role of Glu-166 is largely limited to deacylation. Given the observed value of k_{cat} for the wild-type enzyme with Nitrocefin, compared to that for E166C, coupled with the rate of formation of the acyl-enzyme in the "burst" experiment, we estimate that the decrease in acylation rate constant, k_2, for the mutant can be no less than 200 x that for k_3 (deacylation). In other words replacement of Glu-166 by Cys leads to a decrease of 10^{-5} in k_3, and less than a decrease of 10^{-3} in k_2. The activation which occurs with E166C under alkaline conditions probably results from the ionization of the cysteine residue, although the dependence on hydroxide ion concentration at high pH makes it difficult to distinguish from catalysis by hydroxide ion. The loss of activity seen with E166D indicates the catalytic mechanism is quite sensitive to the location of the carboxyl group at site 166. This result argues against a mobile environment for this region of β-lactamase.

Lys-73 is highly conserved throughout the Class A β-lactamase family as well as other classes of serine β-lactamases and penicillin binding proteins (Joris et al., 1988). Moreover, since replacement of lysine with arginine results in a large decrease in activity (Gibson et al., 1990; Madgwick & Waley, 1987) Lys-73 is thought to participate in the mechanism of these enzymes. There are several different hypothesis concerning its contribution to the mechanism. It has been suggested that Lys-73 polarizes the Ser-70 hydroxyl group so as to direct transfer of its proton onto the 2° amine produced upon acylation of the lactam carbonyl carbon. (Herzberg and Moult, 1987). It is also possible this lysine is acting as a general base and activating Ser-70 for attack onto the lactam ring. The lysine ammonium group would have to be deprotonated under physiological conditions and its side chain pK_a should appear in the acidic limb, not the alkaline limb, of the W.T. pH profiles. In light of the present results this possibility can be eliminated.

If Lys-73 does not participate as a proton shuttle in acylation and the activity of Glu-166 is limited to deacylation then one is left with the question of how Ser-70 is activated for nucleophilic attack. It has been suggested that Ser-70 may not require activation as a consequence of several factors which make the reaction favorable (Escobar et al., 1991). However, this still leaves open the question of where the Ser-70 hydroxyl proton is located during the formation of the tetrahedral intermediate. If Lys-73 is directing the exchange of the proton from Ser-70 onto the tertiary amine of the lactam ring there must exist a potential gradient which allows this proton to position itself on the nitrogen atom (N4) of the intermediate. However, this tertiary amine is

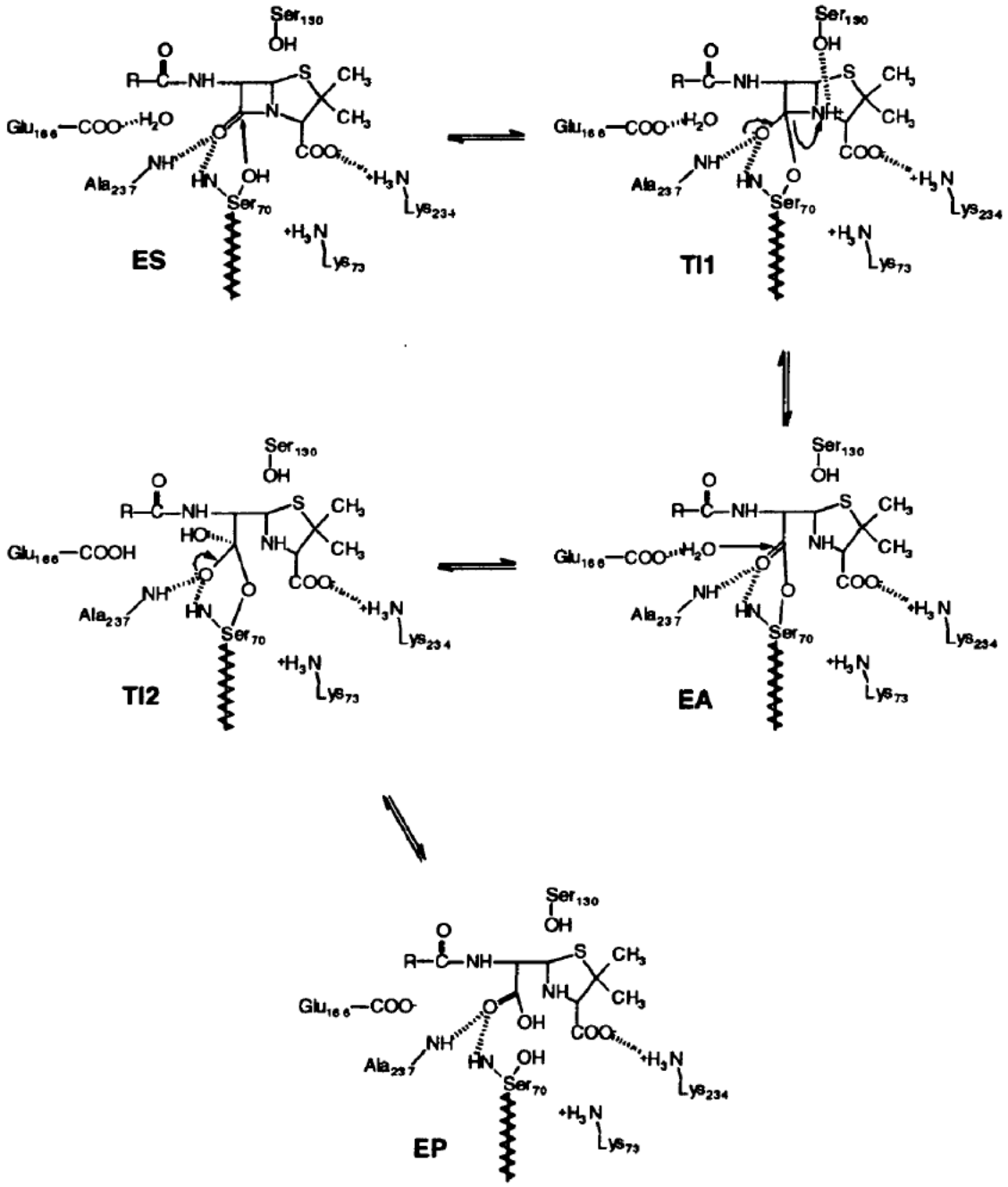

Fig. 7. Postulated mechanism for Class A β–lactamases.

also fairly acidic (Proctor, 1982) and is an unlikely candidate to receive this proton unless some other form of stabilization is taking place. From the crystal structure (Knox & Moews, 1991) we observe that Ser-130 may be appropriately positioned to stabilize a proton on the amine through a hydrogen bond interaction. This proposed role in the mechanism (Fig. 7) might explain why this residue is so highly conserved in Class A β-lactamases. With the collapse of the tetrahedral intermediate, the tertiary amine becomes a secondary amine and its basicity correspondingly increases. The acyl-intermediate that is formed is then attacked by water, which is activated by Glu-166, and the resulting hydrolyzed substrate is released.

An alternative mechanism is that Glu-166 acts as a general base in both acylation and deacylation, but the requirements for catalysis in the acylation step are less stringent for the reasons previously enunciated (Escobar et al., 1991), as a consequence the effect of mutations at Glu-166 are much less drastic on acylation than on deacylation. This differential effect might be further enhanced in highly activated substrates such as Nitrocefin.

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Chapter V

Site Specific Mutagenesis of Tyrosine 105

Introduction

As mentioned in chapter 1 there are several groups both within and near the catalytic site of Class A β-lactamases which are highly conserved (Joris et al., 1988). The degree of conservation observed for these residues often extends past the boundaries of Class A enzymes, frequently including Class C and D β-lactamases and the PBP's. Such a high degree of conservation is generally taken to indicate that a residue is playing a pivotal role in either catalyzing the reaction of the enzyme or maintaining the structure of the enzyme. One such residue is tyrosine 105 (in the numbering system of Ambler (1980)).

In addition to the ubiquitous nature of Tyr-105 there are other lines of evidence which indicate this residue may play an important role in the action of these enzymes. Its proximity to the catalytic site as well as its proximity to valine 103 (Herzberg & Moult, 1987) suggests that perturbations at this site might disrupt activity. Valine 103 lines a gully of the catalytic site where model building studies suggest it may be a key residue in determining binding properties as well as substrate specificity (Herzberg & Moult, 1987)

Possible function of Tyr-105

Tyrosine residues are commonly observed to participate in hydrogen bond formation through their phenyl hydroxyl group. Thus they can participate in maintaining the structural integrity of proteins (intramolecular hydrogen bonding) or in the binding of substrate (intermolecular bonding). One would expect that ionization of the tyrosine side chain hydroxyl would disrupt any hydrogen bond interactions this

residue might have via the hydroxyl group. If the role of a given tyrosine residue is key to maintaining enzyme structure then such an ionization could disrupt the local if not the overall organization of the enzyme resulting in the decreased catalytic efficiency of the enzyme.

Although several workers have postulated that the alkaline limb of the activity-pH profiles for the Class A β-lactamases arises from the deprotonation of lysine 234 or lysine 73 this has been shown not to be the case (Ellerby et al., 1990; Richards, personal communication). Consequently we have chosen to investigate whether deprotonation of Tyr-105 results in the decreased catalysis represented by the alkaline limb of the activity-pH profiles for Class A β-lactamases. The ionization constant (pK_a) for the tyrosine hydroxyl group in solution is 9.11 (Fersht, 1985). Given the proper environment (polar) within the protein this value could very well drop to the observed value for the alkaline limb of the wild-type (W.T.) pH profile. The pK_{a2} of the K_{cat}/K_m-pH profile with phenoxymethylpenicillin is 8.6 (Ellerby et al., 1990). The pKa values derived from K_{cat}/K_m-pH profiles represent ionizations which occur within the free enzyme.

Tyrosine 105 has been mutated to phenylalanine through site-directed mutagenesis. By specifically removing the phenyl hydroxyl group at site 105 we hope to probe what function this group might have in either the structure or function of Class A β-lactamases.

EXPERIMENTAL PROCEDURES

Materials

Benzylpenicillin, phenoxymethylpenicillin were purchased from Sigma. Nitrocefin was a gift from Glaxo. PADAC was purchased from Calbiochem. Sequenase used for sequencing mutants was purchased from U. S. Biochemicals. Restriction enzymes, Klenow fragment and ligase used for mutagenisis were obtained from New England Biolabs. The data analysis application Kaleidagraph™ was purchased from Synergy Software.

Mutagenesis

The Y05F mutant β–lactamase was made as described in chapter 2. Mutations were introduced via the Kunkle method (Kunkle, 1985) and sequenced with Sequenase (U.S. Biochemicals). The primer used for mutagenesis was 5'-GAT-CTT-GTT-AAC-(TTC)-AAC-CCG-ATT-ACG--3' This sequence replaces Tyr-105 with Phe (in parenthesis) and introduces a Hpa I site (underlined). Thus, digestion of mutant sequence with Hpa I allows for restriction purification (Wells et al., 1986). The Y105F sequence was confirmed by dideoxy-sequencing (Sanger et al., 1977), with the aid of Sequenase (U.S. Biochemicals). The primer sequence used was 5'-ACA-GAA-ATC-AAT-AGA-AGA-TC-3, and was designed to sit 40 bp upstream from the mutagenesis site.

Purification

The purification procedure used to isolate Y105F is the same as that for E166C and E166D described in Chapter 4.

Kinetics

The pH profile for Y105F was obtained from complete progress curves and analyzed by the method of Koerber and Fink (1987) via Kaleidagraph™. All points were determined in triplicate. The hydrolysis of phenoxymethylpenicillin was monitored at 240 nm and 30°C with a Hewett-Packard 8452A diode array spectrophotometer using a 1 cm path length cell. All buffers contained 0.5 M KCl and were brought to a final concentration of 50 mM buffer. The buffers used for the pH profile were sodium acetate for pH 4.0 - 5.5, potassium phosphate for pH 6.0 - 7.5, Tris-HCl for pH 8.0 - 8.5 and CHES for pH 9.0 - 9.5. The kinetic properties of the mutant were also analyzed with benzylpenicillin, nitrocefin and PADAC. These studies were done as described above in 50 mM potassium phosphate, 0.5 M KCl pH 7.0. All four of these β-lactams are known to be good substrates of the W.T. enzyme since they are hydrolyzed rapidly in the presence of the *B. licheniformis* β-lactamase (see Table I and Fig.1). The extinction coefficients and wavelengths used are the same as those listed for these substrates in Ellerby et al. (1990).

Circular dichroism measurements of the mutant protein

The far-u.v. CD spectra of the mutant protein was measured at pH 4.0, 7.0 and 9.0 using an AVIV model 60DS instrument at 30 ^{o}C. Protein concentrations (final) were 11.5 μM for the Y105F mutant at all three pHs. The cell path length was 0.1 cm. Spectra are the average of three scans with a slit width of 1.5 nm. The buffers used were: 50 mM potassium phosphate for pH 7.0, 50 mM sodium acetate for pH 4.0 and 50 mM CHES for pH 9.0. All these buffers contained 0.5 M KCl.

RESULTS AND DISCUSSION

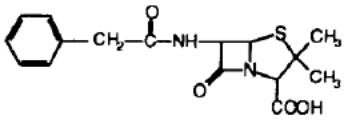
Structure

As demonstrated in Figure 2 the secondary structure of Y105F is not appreciably affected by the mutation. The circular dichroism spectra of Y105F at pHs 4, 7 and 9 are superimposible on that of W.T. under native conditions (pH 7.0). This finding argues against a key role for the hydroxyl group of Tyr-105 in maintaining the structure of the Class A β-lactamases, at least in a global sense. These results, however, do not preclude the possibility that there has been a small structural perturbation of the region neighboring site 105. If this perturbation does not affect the overall secondary structure of the protein it might not be visualized by CD.

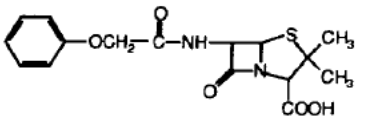
Kinetics

As shown in Table I the k_{cat} values for the reaction of Y105F with the four substrates used in this study (see Fig. 1) approximate those of the W.T. enzyme. This is in comparison with the other mutations of the *B.*

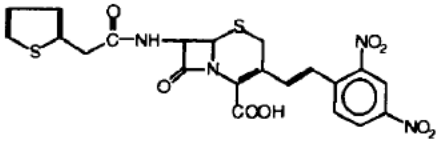
licheniformis β-lactamase at positions 234 and 166 which have a greatly decreased activity. The k_{cat} is reduced by three orders of magnitude for the K234A mutant (Ellerby et al., 1990; chapter 1) and five to six orders of magnitude for the E166A mutant (et al., 1991; chapter 3). Interestingly, although the k_{cat} is slightly decreased for three of the four substrates used in this study (Y105F) the value for benzylpenicillin has increased by ~25%.



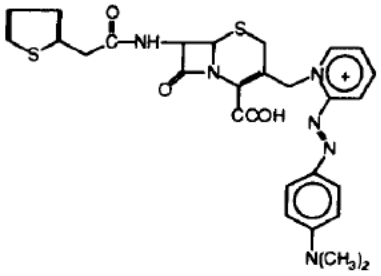
Benzylpenicillin



Phenoxymethylpenicillin



Nitrocefin



PADAC

Figure 3. Substrates used to characterize Y105F β-lactamase. Two representative penicillins (top) and two cephalosporins (bottom) were used to check for differential effects of the mutation on catalysis for these two types of β-lactam antibiotics.

Moreover, as indicated in Table I, it appears that Y105F binds less tightly to all the substrates. The K_m is increased between 2 to 3 fold in the Y105F mutant when compared to the W.T. values. The observed differences in k_{cat} and K_m might indicate that the structure of the catalytic site has been

altered. However, since the detected differences in the kinetic constants are small (within the same order of magnitude) any modifications to the structure of the catalytic site are probably small. Activity-pH profiles were fit to the ionization curve equation described in chapter 4. The k_{cat}-pH profile of the Y105F enzyme is very similar to that of the W.T. enzyme with phenoxymethylpenicillin (Fig. 3). The pK_{a1} with Y105F is 4.5 ± 0.1 which is the same as the W.T. value (Ellerby et al., 1990). The ionization constant for the alkaline limb is slightly different, however, with a value of 8.6 ± 0.1 for Y105F and 8.1 ± 0.1 for the W.T. enzyme (Table II). The absolute values from this profile are close to those seen for the W.T. enzyme. Most importantly, we note that the alkaline limb is still present and that it has not been abolished by the mutation. Thus it seems reasonable to state that the ionization of the Tyr-105 hydroxyl group is not responsible for the alkaline limb of this profile.

By comparing the K_m-pH profiles for the mutant Y105F and W.T. enzymes we can see that there seems to be a slight decrease in the binding affinity of Y105F for phenoxymethylpenicillin (Fig. 4). The K_m is approximately 2 fold greater for Y105F than W.T. across the pH range studied. As stated earlier, this difference in binding could reflect small changes in the catalytic site.

The k_{cat}/K_m-pH profile is also similar to that of the wild-type enzyme (see Fig. 5). The pK_{a1} for Y105F is 5.3 ± 0.1 while that of the W.T. is 5.0 ± 0.1. The alkaline limb is characterized by a pK_{a2} of 8.8 ± 0.1 in Y105F while 8.6 ± 0.1 is the W.T. value. Again we find the alkaline limb is present in the profile although the absolute values of the Y105F profile are approximately

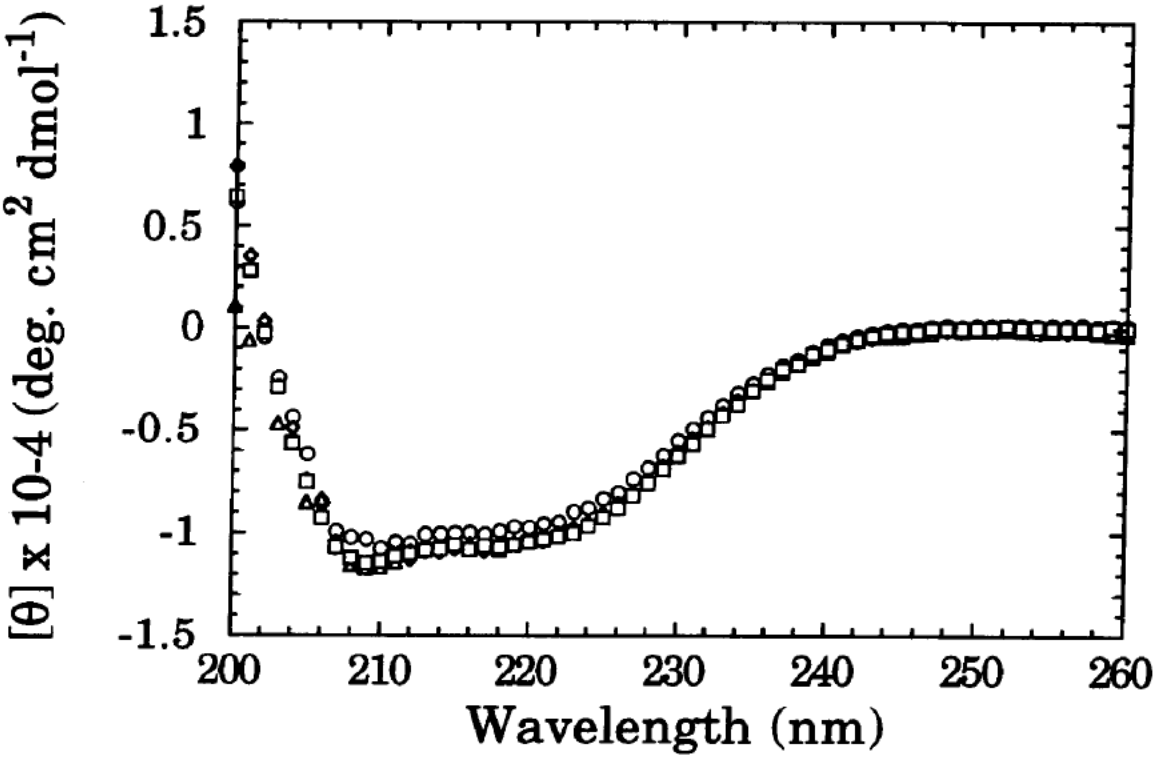


Figure 2. Circular Dichroism spectra of Y105F at pH's 4, 7 and 9. Spectra are superimposible on that of the native wild-type enzyme within experimental error. Studies were done at 30 °C. Diamonds: Y105F at pH 7; triangles: Y105F at pH 4; circles: Y105F at pH 9.0; squares: W.T. enzyme at pH 7.0.

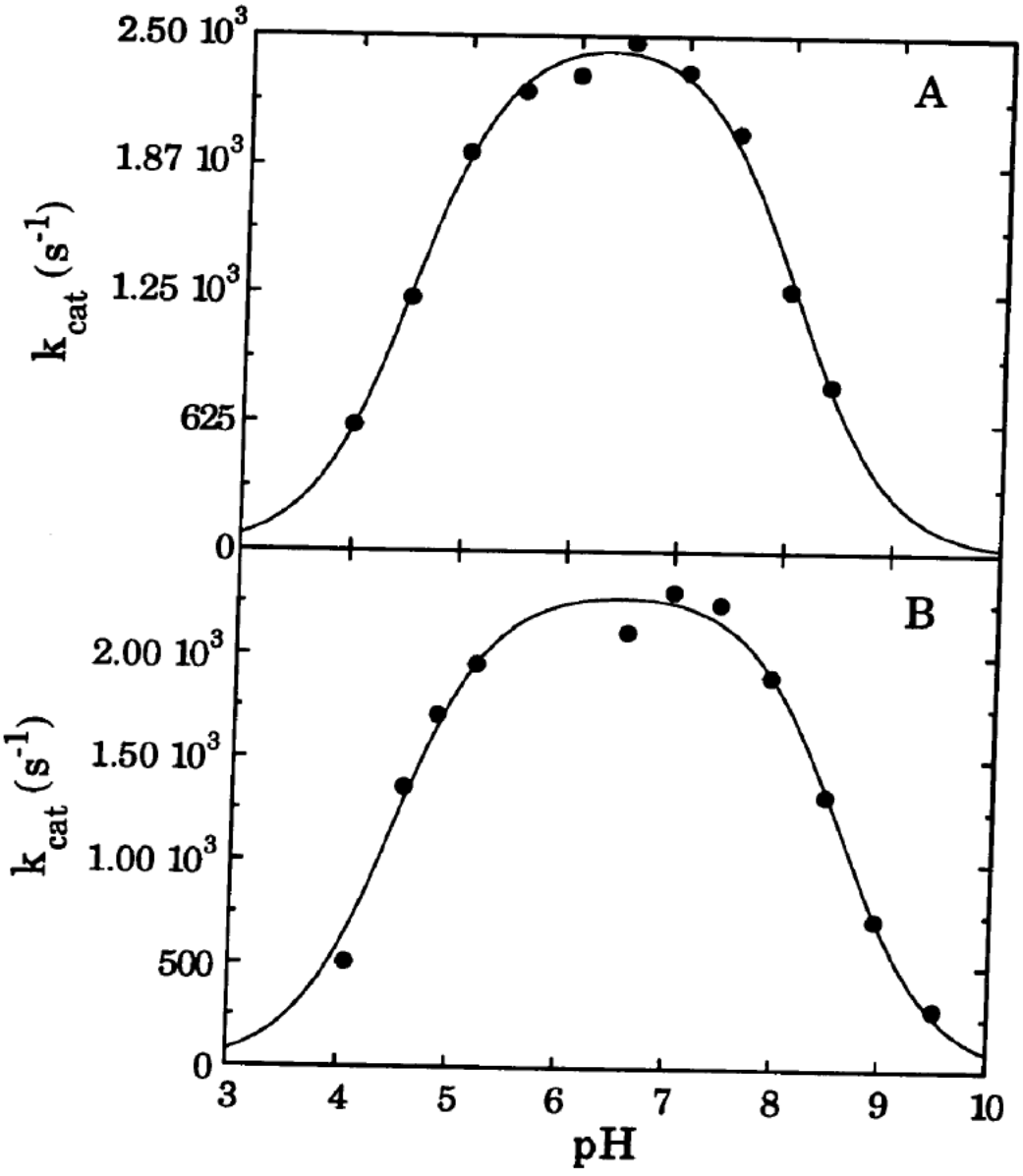


Figure 3. k_{cat}-pH profiles for (A) wild-type and (B) Y105F β-lactamase. Studies done with phenoxymethylpenicillin at 30 °C. pK_a values for the two profiles are listed in Table II. Note that the Y105F values approximate those of the W.T. enzyme.

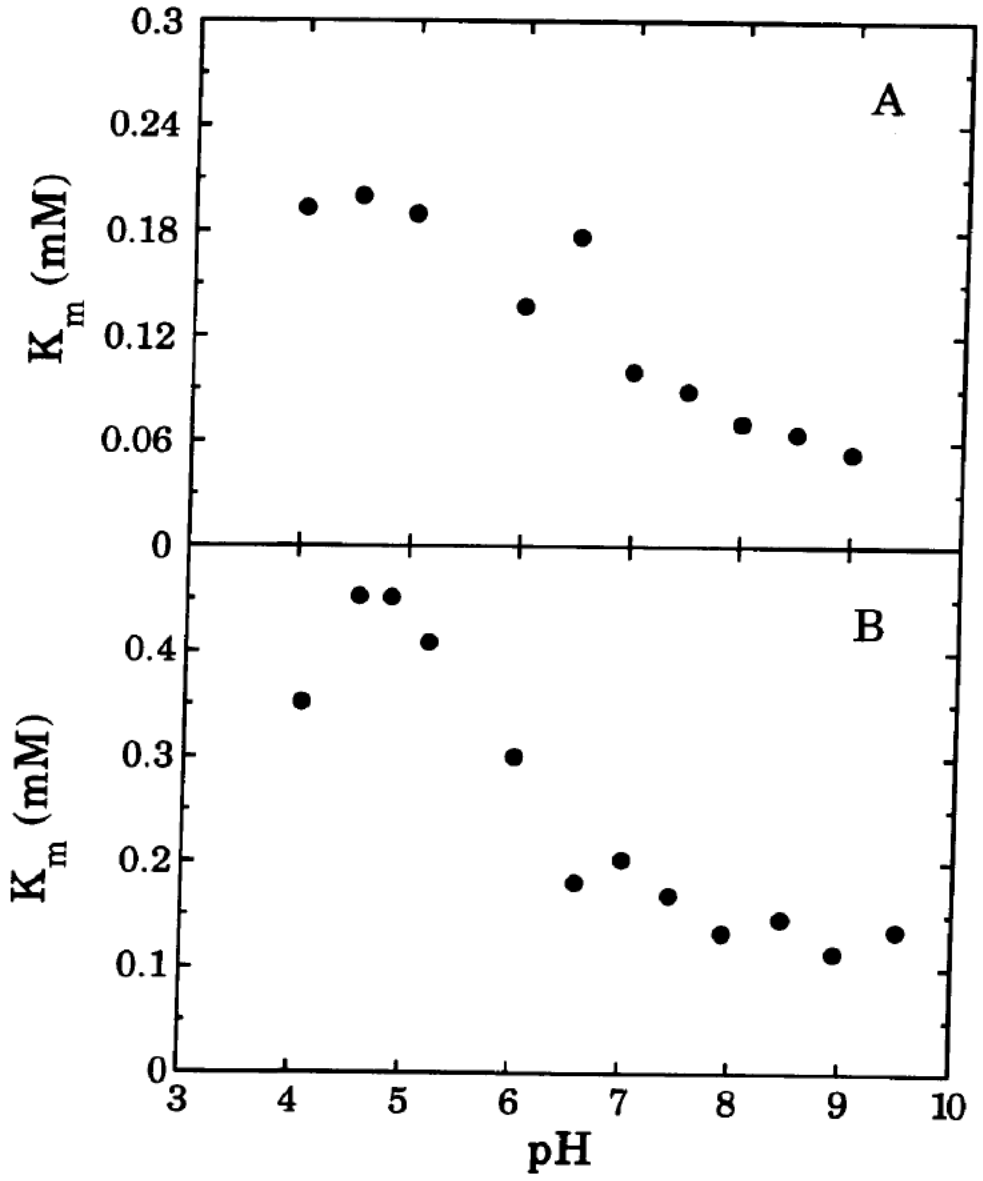


Figure 4. Km-pH profile for (A) wild-type and (B) Y105F β-lactamase with phenoxymethylpenicllin at 30 °C. W.T. values as from Ellerby et al. (1990). The Y105F values are approximately 2 fold greater than those for the W.T. throughout the profile.

Table I: Substrate specificity profile for W.T. and Y105F B. licheniformis β-lactamase

	Wild Type β-lactamase [a]			Y105F β-lactamase			
Substrate	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m	rel [b]
Benzylpenicillin	2650	0.12	2.15×10^7	3760	0.33	1.12×10^7	0.52
Phenoxymethylpenicillin	2552	0.1	2.48×10^7	2305	0.2	1.15×10^7	0.46
Nitrocefin	1088	0.041	2.64×10^7	720	0.19	3.8×10^6	0.14
PADAC	667	0.14	4.63×10^6	457	0.26	1.76×10^6	0.40

[a]Wild type values are from Ellerby et al. (1990). Units for k_{cat}, K_m and k_{cat}/K_m are s^{-1}, mM and $M^{-1}s^{-1}$ respectively. [b] rel values are ratios of k_{cat}/K_m (Y105F)/ k_{cat}/K_m (W.T.). Standard deviation, < ± 6.0 % for W.T. and < ± 10.0 % for Y105F.

Table II: pKa's for W.T. and Y105F β-lactamase with phenoxymethylpenicillin

	Wild Type [a]		Y105F	
	pk_{a1}	pk_{a2}	pk_{a1}	pk_{a2}
k_{cat}	4.5 ± 0.1	8.1 ± 0.1	4.5 ± 0.1	8.6 ± 0.1
k_{cat}/K_m	5.0 ± 0.1	8.6 ± 0.1	5.3 ± 0.1	8.8 ± 0.1

[a] Wild Type values are from Ellerby et al. (1990)

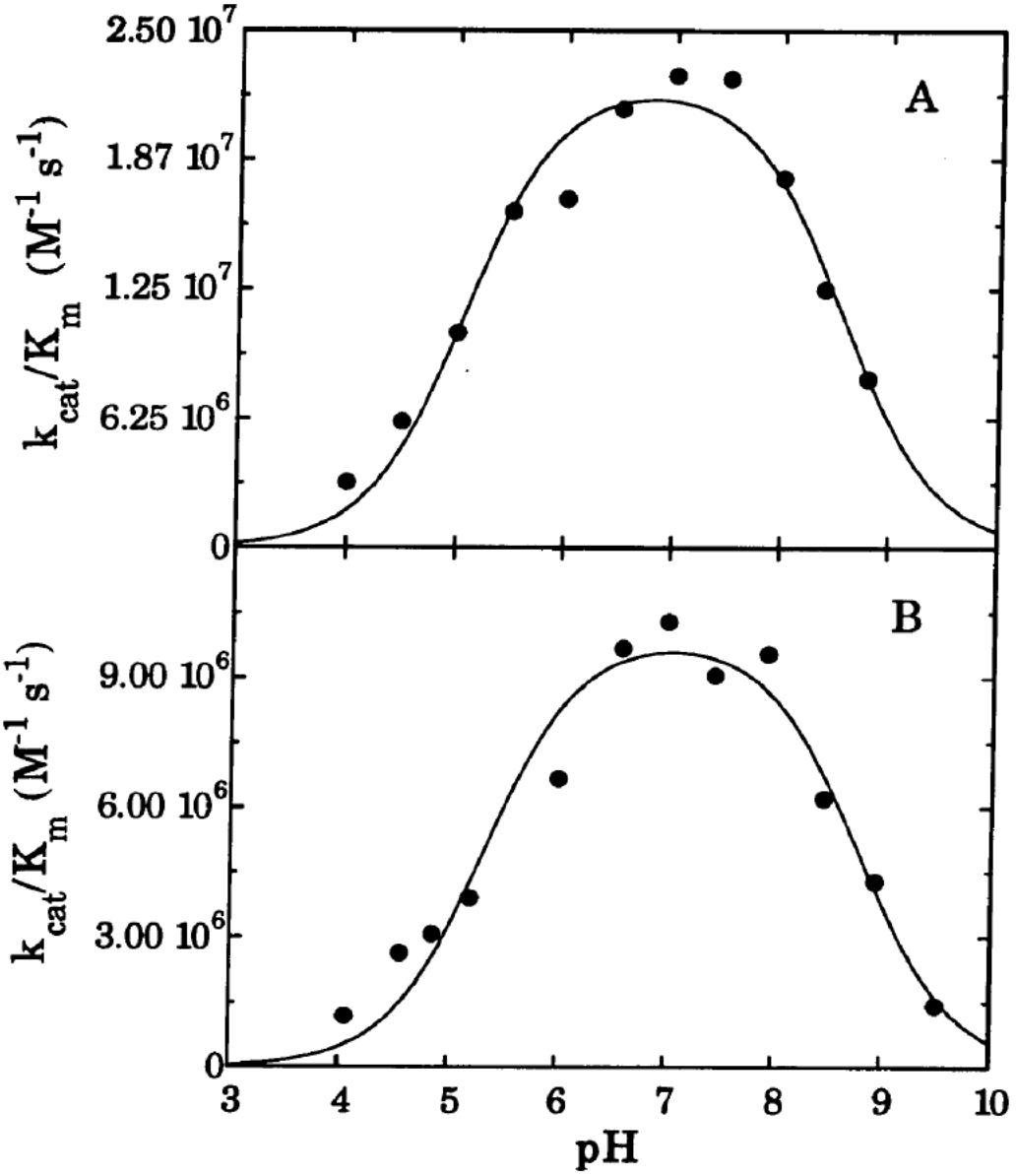


Figure 5. k_{cat}/K_m-pH profile for (A) wild-type and (B) Y105F β-lactamase with phenoxymethylpenicillin at 30 °C. Wild-type values are from Ellerby et al. (1990). The Y105F profile maintains the same shape as that of wild-type but the values are consistently half those of the wild-type. This difference arises from the observed increase in K_m values.

half those of the W.T. profile. This difference arises from the increased K_m values seen in Fig. 3.

Why is Tyr-105 so highly conserved?

The mutation of tyrosine 105 to phenylalanine does not severely disrupt the function of β-lactamase. If the degree to which this residue is conserved throughout the β-lactamase family indicates its importance to the action of these enzymes then we can conclude that the hydroxyl moiety of tyr-105 is not the sole factor determining its importance to the enzyme. Perhaps the hydrophobic phenyl group is playing a larger role in stabilizing the overall structure. This could be investigated by replacing Tyr-105 with alanine, thereby eliminating any hydrophobic interactions this side chain has with its surroundings.

The observed increase in activity with benzylpenicillin is hard to explain given that the structure for this is substrate is very similar to that of phenoxymethylpenicillin (Fig. 1). One possible explanation is that there has been a shift in the structure of the catalytic site which has altered the specificity of the enzyme such that it prefers the difference in structure of benzylpenicillin over that of phenoxymethyl penicillin.

To study whether the catalytic site of the Y105F mutant has been perturbed it will be necessary to employ a high resolution technique such as crystallography. As indicated by the CD studies with Y105F there have been no gross changes to the secondary structure of the enzyme. This is supported by the high catalytic rates seen with Y105F which also indicate the catalytic site is not severely disrupted.

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Appendix A

Appendix A

The following is a listing and discription of the commonly used protocols and solutions relating to the molecular biological techniques discussed in chapter 2.

A. Site-directed mutagenesis

Site-directed mutagenesis requires the synthesis of a mutant primer (DNA oligomer). This primer is designed to complement the region surrounding the mutation site and incorporates the mutation itself into its sequence. The mutagenenic primer is annealed to single stranded template DNA and is used to initiate a second strand synthesis. The resulting heteroduplex can then be transformed into E. coli and the cells containing mutant sequences selected. Mutant selection is achieved by several means including colony filter hybridization and restriction analysis.

1) Kination - Inorganic phophate must be transferred to the 5' hydroxy-terminus of the primer so that the ligation reaction (recircularization of the heteroduplex) can be carried out.

- 1 µl (500 ng) primer, 1 µl 10x kinase buffer (New England Biolabs), 1 µl 10 mM ATP, 6 µl H_2O, 1 µl T4 Polynucleotide Kinase (New England Biolabs)
- incubate at 37 °C for 1 hour

2)Annealing - Annealing primer to kinased primer

- 2 µl of kinased primer (100 ng), 4 µl 10x ligase buffer (New England Biolabs), 1 µl template (of equivalent concentration as that of primer), 33 µl H_2O

- Denature at 95 °C for 10 min. then cool to room temperature (R.T.) and put on ice

3) Second strand synthesis - Primer is used to form the heteroduplex

- 3 µl 2 mM dNTP's, 1 µl 10x ligase buffer, 2µl 10 mM ATP, 2 µl H_2O, 1 µl ligase (New England Biolabs), 1 µl Klenow fragment (New England Biolabs)

- be sure to add ligase first since the 5'→3' exonuclease activity of the Klenow fragment may strip off your mutagenic primer.

- 30 minutes at R.T.

- Add 3 µl dNTP's again and 1 µl Klenow, 30 min. at R.T.

- Add 1 µl ligase, let sit 10 - 15 min. at R.T.

B. Transformation of Escherichia coli

The following is a revised version of the protocol described in Chung & Miller (1988) Nucleic Acid Research 16, 3580.

- Cells are inoculated into 5 ml L.B. media (Maniatis T., Fritsch E. F., Sambrook, J., *Molecular Cloning*, Cold Spring Harbor Laboratory (1982) p. 68) and grown to an optical density (600 nm) of 0.3 - 0.6.

- Resuspend in 1/10 volume of L.B. transformation buffer (L.B. pH 6.1, 10% PEG (3350), 5 % DMSO, 10 mM $MgCl_2$, 10 mM $MgSO_4$) and let sit on ice for 20 minutes.

- Add your DNA, 20 µl 5x KCM buffer (0.5 M KCl, 0.15 M $CaCl_2$, 0.25 M $MgCl_2$) and q.s. to 100 µl with ddH_20

- Mix 100 ml DNA cocktail with 100 ml cells and let sit on ice for 20 min.

- Warm to R.T. for 10 min.

- Add 1.5 ml of L.B. media and grow out for 1/2 hour

- Plate the cells out onto plate containing appropriate drugs.

C. Transformation of Bacillus subtilis

The transformation process in B. subtilis requires that the plasmid DNA is concatamerized (trimers or greater) see Dubnau, Molecular Genetics of Bacillili. Therefore the plasmid to be transformed must be isolated from strains of E. coli which concatamerize their plasmids (ie. rec A^+).

-Two step transformation procedure (Cutting & Vander Horn, 1990)

T base	per litre:	
	$(NH_4)_2SO_4$	2 g
	$K_2HPO_4.3H_2O$	18.3 g
	KH_2PO_4	6 g
	trisodium citrate $2H_2O$	1 g
	Autoclave at 15 lb/in^2 for 15 min. This medium can be made up at 10× working strength.	
SpC	Made fresh on the day of use from the following sterile solutions:	
	T base	20 ml
	50% (w/v) glucose	0.2 ml
	1.2% (w/v) $MgSO_4.7H_2O$	0.3 ml
	10% (w/v) Bacto yeast extract	0.4 ml
	1% (w/v) casamino acids	0.5 ml
	Growth requirements[a]	
SpII	Made fresh on the day of use from the following sterile solutions:	
	T base	200 ml
	50% (w/v) glucose	2 ml
	1.2% (w/v) $MgSO_4.7H_2O$	14 ml
	1% (w/v) casamino acids	2 ml
	10% (w/v) bacto yeast extract	2 ml
	0.1 M $CaCl_2$	1 ml
	Growth requirement[a]	
SpII + EGTA	SpII (200 ml) with 4 ml EGTA (0.1 M, pH 8.0) but without $CaCl_2$. SpII + EGTA can be frozen at −20°C in small aliquots, although repeated freeze-thawing should be avoided.	

Preparation of competent cells

1. Streak out the strain to be made competent on an LB or TBAB agar plate[a] as a large patch and incubate overnight at 30°C.
2. The following morning scrape the cell growth off the plate and use to inoculate fresh, pre-warmed, SpC medium[b] (20 ml) to give an OD_{600} reading of about 0.5.
3. Incubate the culture at 37°C with vigorous aeration and take periodic OD readings (OD_{600}) to assess cell growth.
4. When the rate of cell growth is seen to depart from exponential (i.e. no significant change in cell density over 20–30 min) inoculate 200 ml of pre-warmed, SpII medium[b] with 2 ml of stationary-phase culture and continue incubation at 37°C with slower aeration.
5. After 90 min incubation[c], pellet the cells by centrifugation (8000*g*, 5 min) at **room temperature.**
6. Carefully decant the supernatant into a sterile container and **save**[d].
7. Gently resuspend the cell pellet in 18 ml of the **saved** supernatant and add 2 ml of sterile glycerol; mix gently.
8. Aliquot the competent cells (0.5 ml) in sterile tubes, freeze[e] rapidly in liquid nitrogen or a dry-ice/ethanol bath and store at −70°C.

Transformation

1. Thaw competent cells rapidly by immersing frozen tubes in a 37°C water bath.
2. Immediately, add one volume of SpII + EGTA[f] to the thawed cells; mix gently.
3. In a sterile test tube add competent cells (0.2–0.5 ml) to the DNA solution (<0.1 ml[g]) and incubate in a roller drum at 37°C[h].
4. Dilute the transformed cells as appropriate in T Base[b] containing 0.5% glucose and plate immediately onto selective media[i].

D. Preparing K07 Stock

K07 helper phage is necessary for the production of single stranded DNA used in site-specific mutation and sequencing (Vieira and Messing, 1987).

-Grow JM101 (male strain) on in 2xYT (Maniatis et al., 1980) at 37 °C

-Dilute JM101 1:100 in 2xYT and infect with K07 from a single plaque (5 ml culture).

-Grow up 6 hrs at 37 °C. Spin down cells (7K, 10 min, 4 °C) Sorvall SM24 rotor. Keep supernatent at 4 °C until needed.

- Prepare another overnight culture of JM101 in 2xYT. Dilute 1:100 in 200 ml 2xYT (1 liter flask). Grow up until OD550 ~ 0.5 (~ 2 hours at 37 °C)

- Infect with K07 supernatent from above. Grow up 6 hours at 37 °C.

-Harvest cells (7K, 10 min., 4 °C) Sorvall SS34 rotor. Sterile filter (0.2 μM) and store in aliquots at 4 °C. Should be good for > 1 year with a phage titer > 10^{11}/ml.

E. F1 Origin Plasmid Template Preparation

- Poke fresh colonies(JM101) into 5 ml 2xYT at R.T.
- Place on ferris wheel (or shaker) at 37 °C for ~ 5 hours (mid log)
- Add 50 μl of K07 helper phage.
- Mix and let sit for 30 minutes to allow for thorough infection - 37 °C
- Place on ferris wheel at 37 °C overnight.
- Spin the cells down at 7K for 15 min., decant to fresh tubes.
- Add 1 ml 20 % PEG, 2.5 M NaCl
- Let sit at R.T. for 10 min.
- Pellet phage at 15K for 15 min.
- Aspirate supernatent and respin tubes briefly
- Thoroughly aspirate any remaining PEG.
- Resuspend pellet in 400 μl T.E. (Maniatis et al., 19??) and 5 μl of 10 mg/ ml RNase.
- Transfer to eppendorf tubes and let RNase adigest for 15 min.
- Phenol-$CHCl_3$ extract 2-3 times.
- Add 1/2 volume 7.5 M NH_40Ac and 3 volumes ethanol.

- Place on dry ice 10 min., microfuge 10 min., aspirate, 70% ethanol rinse once.

- Resuspend in 25 µl T.E.

Appendix B

Genotypes of E.coli

Strain	Genotype
1)JM101	Δ(*lac-pro*AB); *thi*$^{-}$, *sup*E, [F',*tra*D36, *pro*AB+,*lac*I^{q}ZΔM15]
2)MM294	F^{-}, *end*A1, *hsd*R17(rK-mK+), supE44, thi-1
3)CJ236	F', *cat*(= pCJ105;M13^{s}Cm^{r}) *dut*, *ung*1, *thi*-1, *rel*A1/pCJ105 (Cm^{r})

Order Number 9312434

Mechanistic analysis of class a β-lactamases through site-specific mutagenesis

Escobar, Walter Alexander, Ph.D.

University of California, Santa Cruz, 1992

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