Generation and Kinetic Characterization

of Hybrid Beta-lactamases

LIU HONGBING

Ph.D.

THE HONGKONG

POLYTECHNIC UNIVERSITY

2003

Generation and Kinetic Characterization of Hybrid Beta-lactamases

Submitted by Liu Hongbing

For the degree of doctor of philosophy in the Hong Kong Polytechnic University

Abstract

The β-lactam antibiotics have been in use for human and animal therapy for more than 50 years. The synthesis of β-lactamases represents the most widespread and the most efficient mechanism devised by the pathogenic bacteria to escape the lethal action of β-lactam antibiotics. Of all β-lactamases, class A enzymes are the most commonly encountered by clinicians and hence, have been extensively studied. However, despite numerous kinetic, structural and site-directed mutagenesis studies, we have not completely succeeded in explaining the diversity of the specificity profiles of β-lactamases and their surprising catalytic power. The solutions to these problems may represent the cornerstones on which better antibiotics and inhibitors of β-lactamases can be designed, hopefully on a rational basis.

The generation and analysis of hybrid enzymes is one of the most powerful tools for understanding structure-function relationships of enzymes and the creation of enzymes with novel properties. Eighteen chimeric β-lactamase genes

(six from *penPC* and *penP*, 12 from *penPC* and *pc1*) were successfully generated in *E. coli* RR1 by *in vivo* intramolecular recombination within the homologous regions between two class A β-lactamase genes [the β-lactamase I gene from *Bacillus cereus* (*penPC*) and the β-lactamase gene from *Bacillus licheniformis* (*penP*); or *penPC* and the β-lactamase gene from *Staphylococcus aureus* (*pc1*)]. These hybrid genes encode novel β-lactamases with their N-terminal moiety derived from PenPC and C-terminal moiety derived from either PenP or PC1.

While PenPC, PenP and their hybrid β-lactamases were highly expressed in *Bacillus subtilis* by the use of a novel expression vector, ϕ105 MU331 prophage, the efforts to express PC1 and its hybrid β-lactamases with the same system were proved to be unsuccessful. The expressed PenPC, HybridB, HybridC, HybridD, HybridE and HybridF were purified to high purity by affinity binding to Celite, but PenP and HybridA were purified by cation–exchange chromatography.

PC1 and its hybrid β-lactamases were successfully expressed in *E. coli* as GST fusion proteins. The expressed GST fusion proteins with PC1, PenPC, Hybrid1, Hybrid2, Hybrid3, and Hybrid4 were highly soluble. However, the expressed GST-Hybrid5 was only partially soluble, and the other GST fusion proteins were found to be expressed as inclusion bodies. Many efforts made to improve the solubility of these fusion proteins were demonstrated to be

unsuccessful, including lowering the growth temperature during induction from 37°C to 20°C, 25°C or 30°C, decreasing IPTG concentration to 0.05 mM, expressing fusion protein in different host stains [BL21, BL21(DE3)] and the addition of ethanol (to a final concentration of 3%) to the growth medium. Nevertheless, when all these hybrids were expressed as maltose binding protein (MBP) fusions in *E. coli*, all the expressed fusion proteins were found to be highly soluble. They were purified by a one-step amylose affinity column.

Detailed kinetic characterizations and stability studies were carried out for these purified β-lactamases. Many conclusions are drawn: (1) The C-terminus of PenPC is essential to its stability and effective catalysis. The replacement of it (42 amino acids) with the corresponding sequence from PC1 causes significant loss of enzyme activity and stability. The identity and similarity of this C-terminal region between PenPC and PC1 are 30% and 62%, respectively, which are quite similar to the identity (34%) and similarity (57%) between the two entire polypeptides; (2) The region in between the crossover points in Hybrid5 and Hybrid6 may be very important for the determination of substrate specificity of Class A β-lactamases, since the K_m values of MBP-Hybid5 (similar to PC1) for many β-lactams are significantly different from those of MBP-Hybrid6 (similar to PenPC). By alignment of protein sequences, the two

hybrids differ at a 43-amino-acid region, covering Ser 70 and Lys 73, which are crucial active site residues for Class A β-lactamases. The identity and similarity of this region between PenPC and PC1 are 41% and 69%, respectively, which are much higher than those of the two entire polypeptides. Some residues in this region may play important role in determining substrate specificity; (3) Compared with their parents, hybrid enzymes lose their stability to different extent; (4) Some hybrids, especially those with crossovers occurred near the middle of the two β-lactamases, such as Hybrid6, Hybrid7 and Hybrid8, dramatically lost their catalytic efficiency in hydrolyzing penicillin G, ampicillin and nitrocefin. In contrary, they have high substrate affinity to other β-lactams with bulky side-chains such as methicillin and oxacillin. This suggests that the generation of hybrid enzymes is similar to the evolution process. In carving out a larger active site to accommodate the β-lactams with bulky side-chains, these new enzymes lost their power to hydrolyze β-lactams with simple side-chains. Also, they lost their internal actions that formerly contributed to their internal integrity, lowering their stability.

Acknowledgement

I am deeply indebted to my chief-supervisor, Dr. Thomas Y. C. Leung, for his support and guidance throughout my graduate program. His patience and enthusiasm have been most encouraging and his dedication to research most inspiring. I am also indebted to Dr. Larry Chow for his help and useful discussion.

Enormous thanks must go to Prof. Jean-Marie Frère, Dr. Moreno Galleni and Dr. Andre Matagne for providing critical comment on my research project during my academic visit to University of Liege, Belgium. Their helpful guidance and suggestions made my research progress much more smooth.

I would like to express my appreciation to the colleagues in the laboratory, especially Christine, Reiko, John, Michael, Huang Jiang, and Dias, for their help and support.

I would also like to express my gratitude to Mr. C. H. Cheng and all the staff and technicians of the Biology section of the Department of Applied Biology and Chemical Technology for their generous assistance.

I would like to thank Drs. Peter Lam, K.Y. Chow, Damu Tang, Wilson Ching and Zhifang Zhang, for the time and effort they had taken to share their technical expertise when I worked at the Hong Kong University of Science and Technology.

Finally, I would like to express my great appreciation to my wife for her endless love, encouragement and support during my prolonged career as a student.

Table of Contents

	Page
Abstract	
Acknowledgements	
Abbreviations	i - iii
Chapter one: Introduction	
1.1 β-Lactams and their target enzymes	1
1.2 β-Lactamases	9
1.3 Fighting the β-lactamases	16
1.4 X-ray crystallographic data	31
1.5 Class A β-lactamases and their catalytic mechanism	25
1.6 Hybrid enzymes	33
1.7 The objective of this study	40
Chapter two: Materials and methods	
2.1 Reagents	51
2.2 Culture media	51
2.3 Bacterial strains and plasmid DNA	52
2.4 Preparation of chromosomal DNA	58
2.5 PCR	59
2.6 Preparation of plasmid DNA	60
2.7 Restriction enzyme digestion of DNA and electrophoresis	60
2.8 Isolation and purification of DNA fragments	60
2.9 Ligation of DNA fragments	61
2.10 Preparation of *E. coli* competent cells	61

2.11 Transformation of *E. coli* 62

2.12 DNA sequencing 63

2.13 Preparation and transformation of *B. subtilis* competent cells 65

2.14 Protein expression in *B. subtilis* 66

2.15 Purification of PenPC, HybridF and HybridB by Celite 66

2.16 Expression and purification of GST fusion proteins 67

2.17 PreScission protease cleavage 69

2.18 Expression and purification of MBP fusion proteins 70

2.19 Protein gel electrophoresis (SDS/PAGE) 70

2.19 Determination of protein concentration 71

2.20 Enzyme kinetics 71

2.21 Thermal stability of β-lactamases 72

2.22 Circular dichroism 73

2.23 Site-directed mutagenesis 73

2.24 β-Lactamase inhibition assay 75

2.25 Softwares 75

Chapter three: Generation of hybrid β-lactamases

3.1 Introduction 77

3.2 Construction of recombinant plasmid containing two homologous β-lactamase genes 80

3.3 Generation of hybrid β-lactamase genes by *in vivo* intramolecular homologous recombination 81

3.4 Restriction analysis and DNA sequencing of hybrid β-lactamase genes 85

3.5 DNA sequence analysis of hybrid β-lactamase genes 87

3.6 Discussion 93

Chapter four: Expression of β-lactamases in *Bacillus subtilis*

4.1 Introduction 97

4.2 Construction of the pSG703 derivatives containing various β-lactamase genes 101

4.3 Transformation of the constructed pSG703 derivatives into *B. subtilis* 1A304(ϕ105 MU331) 105

4.4 Small scale expression of β-lactamases from *B. subtilis* 111

4.5 SDS/PAGE analysis and activity assay of expressed protein samples 111

4.6 Effect of the transcriptional terminator from the gene of *penPC* on the expression of PC1 from *B. subtilis* 115

4.7 Large scale expression of PenPC, HybridB, and HybridF β-lactamases 116

4.8 Purification of the β-lactamases expressed from *B. subtilis* 119

4.9 Discussion 123

Chapter five: Expression of β-lactamases in *Escherichia coli*

5.1 Introduction 128

5.2 Construction of the pGEX-6P-1 derivatives containing various β-lactamase genes 135

5.3 Expression of β-lactamases from *E. coli* 142

5.4 Solubility of expressed GST-β-lactamase fusion proteins 142

5.5 Expression of β-lactamases as fusion with MBP 148

5.6 Expression and purification of MBP-β-lactamase fusion proteins 153

5.7 Signal peptidase contaminants during fusion protein purification 158

5.8 Discussion 165

Chapter Six: Characterization of hybrid β-lactamases

6.1 Far-UV circular dichroism analysis of β-lactamases 169

6.2 Thermal denaturation of MBP-β-lactamases 173

6.3 Equilibrium unfolding 175

6.4 Thermo-inactivation studies 178

6.5 K_m measurement as a K_i with nitrocefin as the report substrate 182

6.6 Kinetic characterization of PenP, PenPC and their hybrid enzymes 186

6.7 Kinetic characterization of MBP fusion enzymes of PC1, PenPC and their hybrid enzymes 191

6.8 Discussion 209

Chapter Seven: Expression of β-lactamase inhibitory protein (BLIP) in *Bacillus subtilis*

7.1 Introduction 225

7.2 Subcloning the *blip* gene into the plasmids pSG1112 and pSG1113 232

7.3 Transformation of the recombinant plasmid into *B. subtilis* strain 1A304(ϕ105MU331) 235

7.4 Small scale expression of BLIP from *B. subtilis* 235

7.5 Optimizing conditions for BLIP expression 236

7.6 Large-scale expression and purification of BLIP 243

7.7 Discussion 247

Conclusion 249

References 257

Appendix

Appendix 1: Reagents 285

Appendix 2: ABL scheme 287

Abbreviations

aa	Amino acid(s)
Å	Angstrom (= 0.1 nm)
ABL	Class A β-lactamase numbering
6-APA	6-aminopenicillanic acid
Ax	Absorbance in x nm wavelength
B. cereus	*Bacillus cereus*
BHY	Brain heart infusion with yeast extract
bla	Gene conferring ampicillin resistance on *Escherichia coli*
B. licheniformis	*Bacillus licheniformis*
BLIP	β-Lactamases inhibitory protein
B. subtilis	*Bacillus subtilis*
cat	Chloramphenicol acetyl transferase gene
CD	Circular dichroism
Cm^R	Chloramphenicol resistance
Cm^S	Chloramphenicol sensitive
Da	Dalton
DD-peptidase	D-alanyl-D-alanine carboxypeptidase/transpeptidase
DEAE	Diethylaminoethyl
dH_2O	Distilled water
DMF	Dimethyl formamide

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Δ (Delta)	Change of
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetra acetic acid
Er^R	Erythromycin resistance
Er^S	Erythromycin sensitive
ermC	Gene conferring erythromycin resistance
ESBLs	Extended-spectrum β-lactamases
h	Hour(s)
hsps	Heat-shock proteins
MBP	Maltose binding protein
min	Minute(s)
OD	Optical density
IPTG	Isopropylthio-β-D-galatoside
IRT	Inhibitor-resistant TEM β-lactamase
kb	Kilobase pair(s)
lacZ	Gene encoding β-galatosidase
Ori	Origin of replication
PAGE	Polyacrylamide gel electrophoresis
PBPs	penicillin-binding proteins
PBS	Phosphate-buffered saline
pc1	*S. aureus* β-lactamase gene

PC1	*S. aureus* β-lactamase (protein)
penP	*B. licheniformis* β-lactamase gene
PenP	*B. licheniformis* β-lactamase (protein)
penPC	*B. cereus* β-lactamase gene
PenPC	*B. cereus* β-lactamase (protein)
RCF	Relative centrifugal force
rpm	Revolutions per minute
s	Second(s)
S. aureus	*Staphalococcus aureus*
S. clavuligerus	*Streptomyces clavuligerus*
SDS	Sodium dodecyl sulphate
SDS/PAGE	Polyacrylamide gel electrophoresis in the presence of SDS
S. R61	*Streptomyces* R16
T	Total concentration of acrylamide
Tris	Tris(hydroxymethyl) aminomethane
UV	Ultra violet light
VIS	Visible light

Chapter One

Introduction

Chapter 1 Introduction

1.1 β-Lactams and their target enzymes

The most widely used antibiotics are β-lactams such as penicillins and cephalosporins. The success of this family of drugs depends on their high clinical efficacy, broad-spectrum activity, and good safety profile. The β-lactam antibiotics are classified together as a result of their common core structure: the four-membered-lactam ring (Figure 1.1). The β-lactam antibiotics have structural similarities with the binding sites of bacterial substrates, which enable them to attach to and inactivate the transpeptidases involved in bacterial cell wall synthesis. Their broad spectrum of clinical effectiveness and tolerability mean that more than 50 years after their introduction, β-lactam antibiotics are still very widely used in the successful management of many common bacterial infections.

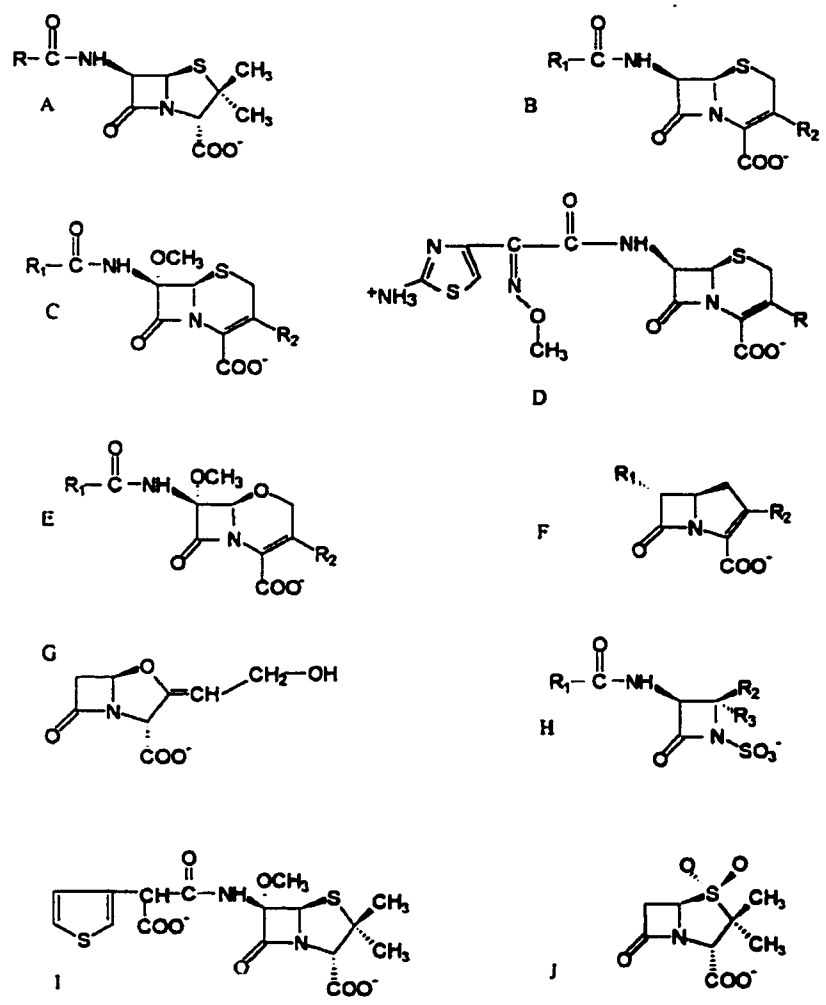


Figure 1.1 Structures of some β-lactam antibiotics (after Matagne *et al.*, 1998). (A) Penams (e.g. benzylpenicillin, ampicillin, amoxycillin), (B) cephems (cephalosporins), (C) cephamycins (e.g. cefoxitin), (D) cefotaxime (oximino cephalosporin; R = CH_2–O–CO–CH_3), (E) oxacephamycins (e.g. moxalactam), (F) carbapenems (e.g. imipenem), (G) clavulanate (oxapenam), (H) monobactams (e.g. aztreonam), (I) temocillin (6–methoxy penam) and (J) sulbactam (penam sulphone).

The history of the antibiotics is generally considered to begin with Alexander Fleming and his observation in 1928 that a strain of the mould *Penicillium* produced a diffusible antibacterial agent that he named penicillin (Fleming, 1929). Fleming carried out a number of studies on the antibacterial activity of penicillin *in vitro* using the filtrate from liquid cultures of *Penicillium*. He also showed that the culture filtrate appeared to be non-toxic when injected into mice and rabbits. However, he did not carry out any studies against experimental infections in animals, and thus failed to demonstrate an essential property of penicillin, namely its ability to overcome bacterial infection when administered systemically.

The first β-lactam antibiotics such as benzylpenicillin began making their way into clinical use in the 1940s and 50s. By killing the bacteria that cause many of humankind's worst infectious diseases, such as tuberculosis and pneumonia, they saved countless lives. They were hailed as miracle drugs, but not all miracles last forever. Bacteria evolve methods of evading their action with all antibiotics including β-lactams. Soon after β-lactams were introduced as chemotherapeutic weapons, their efficiency was challenged by the emergence of resistant pathogenic strains (Abraham and Chain, 1940; Kirby, 1944). In consequence, new molecules have been progressively introduced, with structures increasingly different from those of the original drugs; therefore, the β-lactam antibiotics form an ever-expanding family of

compounds. Table 1.1 lists the dates when β-lactam antibiotics were approved for use in the United States (Medeiros, 1997).

The β-lactam antibiotics inhibit a set of transpeptidase enzymes [also called penicillin-binding proteins (PBPs)] that are important for the construction of the peptidoglycan layer in the bacterial cell wall. This layer protects bacteria from lysis under osmotic pressure (Frere *et al.*, 1992; Ghuysen, 1991). The peptidoglycan is also an essential element in maintaining the shape and rigidity of the cell wall, in both Gram-positive and Gram-negative bacteria. This macromolecule is unique to the bacterial world, which explains the high specificity of antibiotics interfering with its biosynthesis.

Membrane DD-peptidases, the physiological targets of β-lactam compounds, are responsible for the synthesis and remodelling of the peptidoglycan (Frere *et al.*, 1992; Ghuysen, 1991; Nanninga, 1991). These lethal targets in bacteria are active-site serine enzymes which perform their catalytic cycle according to an acylation/deacylation mechanism, involving transient acyl-enzyme adducts (Frere and Joris, 1985; Jamin *et al.*, 1995) (Figure 1.2).

Table 1.1 Dates when β-lactam antibiotics were approved for use in the United States

Antibiotic era	Antibiotic	Approval date
1940-1960: the penicillin era	Penicillin	05/46
1960-1978: The era of broad- spectrum penicillins and early generation cephalosporins	Methicillin	10/60
	Oxacillin	01/62
	Ampicillin	12/63
	Nafcillin	01/64
	Cephalothin	07/64
	Carbenicillin	08/70
	Cefazolin	10/73
	Ticarcillin	11/76
	Cefamandole	09/78
1978-1995: The era of Cephamycins, Oxyiminocephalosporins, Monobactams, Carbapenems, And β-lactamase inhibitors (clavulanic acid and penicillanic acid sulfones)	Cefoxitin	10/78
	Cefotaxime	03/81
	Piperacillin	12/81
	Cefoperazone	11/82
	Ceftizoxime	09/83
	Cefuroxime	10/83
	Amocicillin/clavulanate	08/84
	Ceftriaxone	12/84
	Ticarcillin/clavulanate	04/85
	Ceftazidime	07/85
	Imipenem/cilastatin	11/85
	Cefotetan	12/85
	Ampicillin/sulbactam	12/86
	Aztreonam	12/86
	Piperacillin/tazobactam	10/93

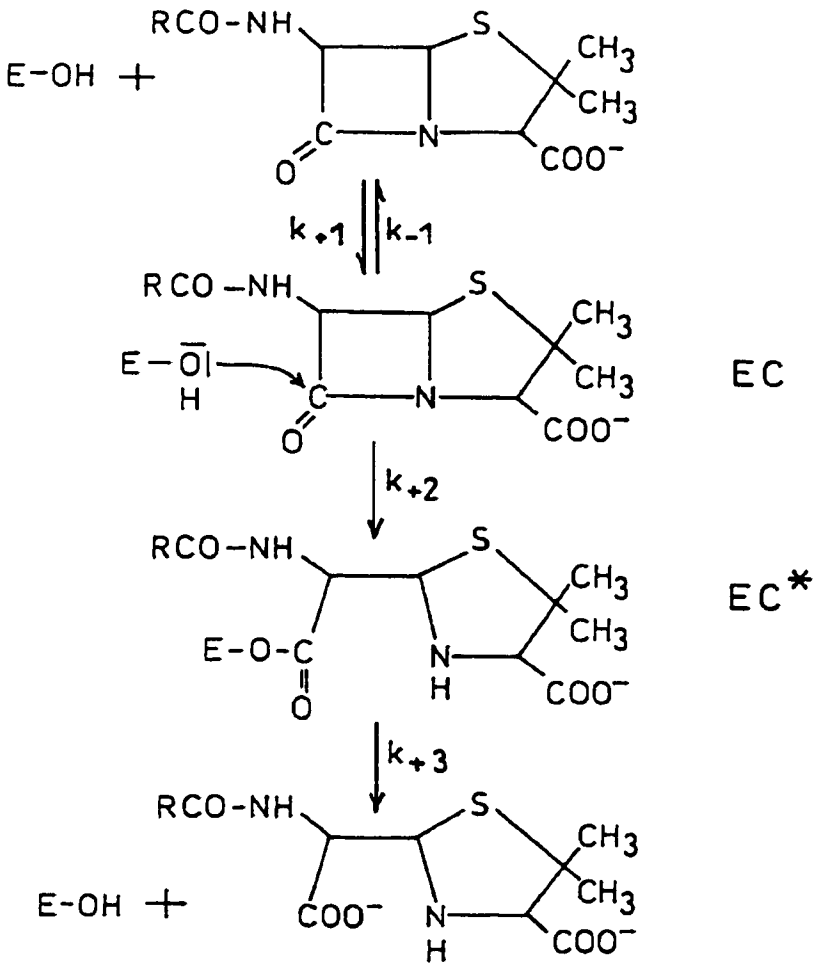


Figure 1.2 General catalytic pathway of active-site serine penicillin-recognizing enzymes (after Matagne *et al.*, 1998). In general, both acylation (k_2) and hydrolysis of the acyl-enzyme (k_3) are rapid, resulting in high turnover numbers [$k_{cat} = k_2 \times k_3/(k_2+k_3)$] and specificity constants [$k_{cat}/K_m = k_2/K'$ where $K' = (k_{-1}+k_2)/k_{+1}$]]. Note that the k_{cat}/K_m parameter (also sometimes referred to as the 'catalytic efficiency') corresponds to the apparent second-order rate constant for acyl-enzyme formation (k_2/K'), and thus is independent of the deacylation rate. Hence the genuine catalytic efficiency of the enzyme rests on high values of both k_{cat}/K_m and k_{cat}.

Interestingly, the β-lactam compounds appear to form a similar adduct when reacting with their physiological targets (the DD-peptidases) and with active-site serine β-lactamases. The difference between these two types of enzymes is purely quantitative. Indeed, with the former, the k_3 values are very low (hardly ever above 0.001 s^{-1}) and the acyl-enzymes are very stable ($t_{1/2}$ > 10 min), whereas with the latter very high k_3 values are observed (up to ~10000 s^{-1} for the interaction between the *B. licheniformis* and the *Streptomyces albus* G β-lactamases and benzylpenicillin at 30 °C). Also the rate of acylation (k_{cat}/K_m, see Figure 1.2) is generally larger with β-lactamases, sometimes close to the diffusion-limited values of 10^7–10^8 $M^{-1}\cdot s^{-1}$. These purely quantitative differences result in sharp qualitative differences, as β-lactamases inactivate penicillins, whereas penicillins inactivate PBPs (Matagne *et al.*, 1998).

β-Lactam resistance is becoming an increasing problem for clinicians worldwide, in both hospital and community settings. Resistance is mediated by four mechanisms: decreased access to the targets in the bacterial cell by the antibiotic; altered penicillin-binding proteins; upregulation of endogenous efflux mechanism; and destruction of the antibiotics by β-lactamases (Davies, 1994; Levy, 1994). The most frequent and most efficient mechanism of resistance to β-lactams is the production of β-lactamase enzymes (Sanders and Sanders, 1992; Sykes and Matthew, 1976), which

are now seen in a wide variety of clinically important bacteria. β-Lactamase production has been widely reported among the Enterobacteriaceae, *Haemophilus influenzae*, *Moraxella* spp., *Neisseria gonorrhoeae*, *Vibrio cholerae*, *Pseudomonas aeruginosa* (Garau, 1994) and anaerobes such as *Bacteroides fragilis*, *Prevotella* spp., *Fusobacterium* spp., and *Clostridium* spp. (Summanen *et al.*, 1993).

1.2. β -Lactamases

β-Lactamases (E.C. 3.5.2. 6, systematic name: β-lactam hydrolase) are a group of constitutive or inducible enzymes. They catalyze the hydrolysis of the β-lactam ring, splitting the amide bond and leaving a substituted β-amino acid. As a result, the antibiotics can no longer inhibit bacterial cell wall synthesis. The enzymes are of varying specificity, some acting more rapidly on penicillins (penicillinase), some acting more rapidly on cephalosporins (cephalosporinase). Cephalosporinase was formerly classified separately as E.C. 3.5.2.8.

Although all β-lactamases catalyze the same reaction, at the latest count, at least 340 β-lactamases, originating from clinical isolates, have been described with unique amino acid sequences or differentiated phenotypic behavior (Bush, 2001). They have been classified according to several schemes, based on, for example, amino acid sequence homology, and biochemical characteristics such as molecular weight or

substrate specificities. The location of genes encoding β-lactamases also varies; they may be an innate part of the chromosome, or are encoded on plasmids. Chromosomal β-lactamases are universal in a specific bacterial species, whereas the presence of those encoded by plasmids is variable, and they are transferable between bacterial species. Further genetic mobility may be provided by transposons, which can carry β-lactamase genes from plasmids to chromosomes. More rarely, chromosomal β-lactamase genes may escape onto plasmids. This mobility is important since it allows for the possibility of the spread of resistance genes through several bacterial communities.

Attempts to classify β-lactamases began in the late 1960s and the first scheme to achieve wide acceptance was proposed by Richmond and Sykes (1973). This scheme was based on whether an enzyme hydrolyzed penicillin more or less rapidly than cephaloridine and whether its activity was inhibited by cloxacillin and/or p-chloromercuibenzoate.

Based on structural comparisons of amino acid sequence relationships among the diverse series of β-lactamases, these enzymes have been categorized into several subgroups. Following an initial work by Ambler *et al.* (1980), these enzymes are grouped into four molecular classes: A, B, C, and D. Class A, C, D β-lactamases utilize an active-site serine residue and function by the three-step mechanism (shown

in scheme 1) similar to the DD-peptidases, involving the transient formation of an acyl-enzyme in which the hydroxyl group of the essential serine residue is esterified by the carbonyl group of the antibiotic moiety (Frere *et al.*, 1991; Waley, 1992; Fisher *et al.*, 1980).

$$E + C \underset{k_{-1}}{\overset{k_{+1}}{\longleftrightarrow}} EC \xrightarrow{k_2} EC^* \xrightarrow{k_3} E + P$$

Scheme 1

where E is the enzyme, C the antibiotic, EC a non-covalent Heri-Michaclis complex, EC* a covalent acyl-enzyme and P the inactive degradation product of the antibiotic. Most clinically important β-lactamases belong to classes A and C. Class A β-lactamases prefer penicillins as substrates, whereas class C enzymes turn over cephalosporins better. On the other hand, class D β-lactamases hydrolyze oxacillin-type β-lactams efficiently. In particular, Class A includes the chromosomal β-lactamases of *Klebsiella spp.*, *Citrobacter diversus*, *Proteus vulgaris* and most *Bacteroides spp.*, as well as virtually all the common plasmid-encoded β-lactamases. Class C comprises the chromosomal AmpC (Richmond & Sykes Class I) cephalosporinases. Class D comprises the OXA enzymes, which are widely scattered in Enterobacteriaceae as plasmid-encoded types (Livermore, 1998).

Class B β-lactamases are metalloenzymes. Although *in vitro* experiments have

shown that the Co^{2+} and Cd^{2+} derivatives are enzymatically active (Davies and Abraham, 1974a; Davies and Abraham, 1974b), it seems that the naturally occurring cation is always Zn^{2+}, requiring zinc for activity. The detailed mechanism of metallo-β-lactamases has no been established, but the overall catalytic function is similar to that of serine-based enzymes. There are a number of uncertainties as to whether the mechanism involves a general base catalysis, a Zn atom behaving simply as an eletrophile, a second Zn atom involvement in the mechanism, or a zinc atom acting as a provider of Zn-bound hydroxide ion (Ambler, 1980). Whatever the mechanism, it is likely that Zn (II) stabilizes the tetrahedral intermediate presumed to be formed during the catalysis process. Class B enzymes can rapidly hydrolyze a broad range of substrates, including carbapenems, which resist hydrolysis by most of the other classes of enzymes. They are resistant to β-lactamase inhibitors. Class B enzymes were originally confined to a few isolated strains of *Bactrtoides fragilis* and *Bacillus cereus* as chromosomal enzymes, but have been identified in Japen later on plasmids carried by *Bactrtoides fragilis*, *Klebsiella pneumoniae*, *Serratia marcescens* and *Pseudomonas aeruginosa*. Such strains appear to be confined to localized area, but they have the potential for widespread dissemination.

Recently, the cysteine-rich protein A from *Helicobacter pylori* (HcpA) was found to be a β-lactamase and was designated as class E β-lactamases (Mittl *et al.*,

2000). HcpA slowly hydrolyzes 6-aminopenicillinic acid (6-APA) and 7-aminocephalosporanic acid (ACA) derivatives. The turnover for 6-APA derivatives is 2-3 times greater than for ACA derivatives. The enzyme is efficiently inhibited by cloxacillin and oxacillin but not by ACA derivatives or metal chelators. However, the designation of class E β-lactamase is still not widely accepted.

With the discovery of more β-lactamases and the availability of additional sequence data and biochemical information, an alternative classification scheme for β-lactamases based on functional characteristics has been proposed by Bush and coworkers (Bush *et al.*, 1995), who tried to correlate molecular structure with catalytic profiles and sensitivity to inactivator. This divides the enzymes into four groups according to substrate and inhibitor susceptibilities. Group 1 consists of cephalosporinases, which are not inhibited by clavulanic acid. Group 2 consists of penicillinases, including broader-spectrum β-lactamases that are generally inhibited by mechanism-based inhibitors. Subgroups of enzymes, namely, 2a, 2b, 2c, 2d, 2e and 2f, were defined based on the rates of hydrolysis of carbenicillin, cloxacillin, extended-spectrum β-lactams ceftazidime, cefotaxime, or aztreonam and of inhibition profile by clavulanate, respectively. Enzymes that are inhibited by the metal-chelating agent ethylenediame tetra-acetic acid (EDTA) are classified as group 3. Group 4 consists of β-lactamases that are only poorly inhibited by the β-lactamase inhibitor

clavulanic acid. It is summarized in Table 1.2, together with an updated summary of the numbers of enzymes and their major attributes. As seen in Table 1.2, from 1995 through 2000, 5 of the 11 (sub)groups of β-lactamases increased in number by at least 50%: groups 1, 2be, 2br, 2d, and 3. Of these, at least 3 different groups of enzymes are increasing in prevalence worldwide: the plasmid-encoded functional group 1 cephalosporinases, the group 3 metallo – β-lactamases, and the group 2be extended-spectrum β-lactamases (ESBLs), which represent the largest group of β-lactamases described. The other 2 budding groups are somewhat less problematic in North America, in part because of the confinement of the group 2br inhibitor-resistant TEM β-lactamases to Western Europe and the restriction of the newer, less efficient group 2d OXA-related enzymes primarily to Eastern Europe (Bush, 2001). The heterogeneity of group 2 in the classification emphasizes the amazing diversity of the catalytic properties of class A β-lactamases (Matagne *et al.*, 1990).

However, for simplicity, the consensus-numbering scheme (ABL scheme) proposed by Ambler *et al.* (1991) will be used throughout this thesis to facilitate comparisons between different class A β-lactamases.

Table 1.2 Functional and molecular characteristics of the major groups of β-lactamases

Functional group	Major subgroups	Molecular class	Attributes of β-lactamases in functional group	Estimated no of enzymes[a]	
				1995	2000
1		C	Often chromosomal enzymes in gram-negative bacteria but may be plasmid-encoded. Confer resistance to all classes of β-lactams, except carbapenems. Not inhibited by clavulanic acid.	32	51
2		A, D	Most enzymes responsive to inhibition by clavulanic acid (unless otherwise noted)	136	256
	2a	A	Staphylococcal and enterococcal penicillinases included. Confer high resistance to penicillins.	20	23
	2b	A	Broad-spectrum β-lactamases, including TEM-1 and SHV-1, primarily from gram-negative bacteria.	16	16
	2be	A	Extended-spectrum -lactamases conferring resistance to oxyimino-cephalosporins and monobactams.	36	119
	2br	A	Inhibitor-resistant TEM (IRT) β-lactamases; one inhibitor-resistant SHV-derived enzyme.	9	24
	2c	A	Carbenicillin-hydrolyzing enzymes.	15	19
	2d	D	Cloxacillin-(oxacillin)–hydrolyzing enzymes; modestly inhibited by clavulanic acid	18	31
	2e	A	Cephalosporinases inhibited by clavulanic acid.	19	20
	2f	A	Carbapenem-hydrolyzing enzymes with active site serine, inhibited by clavulanic acid.	3	4
3	3a, 3b, 3c	B	Metallo-β-lactamases conferring resistance to carbapenems and all β-lactam classes except monobactams. No inhibited by clavulanic acid.	13	24
4		?[b]	Miscellaneous unsequenced enzymes that do not fit into other groups	7	9

Note: The table is adapted from (Bush, 2001); a, Identified from clinical isolates. b, Unknown.

1.3. Fighting the β-lactamases

Two strategies have been used to overcome β-lactamase-mediated resistance to β-lactam antibiotics: modification of the antibiotic's structure so that it is no longer a substrate for the enzyme, and inhibition of the enzyme using a compound that is structurally related to the β-lactam substrate. The use of β-lactamase inhibitors has proved the more successful in recent years, because the rapid evolution of pathogens has resulted in the eventual emergence of β-lactamases that are capable of hydrolysis of the modified β-lactams that have been developed (Moosdeen, 1996).

The ability of certain natural and semi-synthetic β-lactam agents to inhibit selected β-lactamases has been known for a long time. A wide search for more potent compounds resulted in the discovery of carbapenems and clavulanic acid from natural products, and the subsequent synthesis of the sulphones, sulbactam and tazobactam (Muratani *et al.*, 1993). The β-lactamase inhibitors in therapeutic use have poor antimicrobial activity and act synergistically in combination with β-lactamase-labile penicillins. None is effective against metallo-β-lactamases. The penicillins, cephalosporins and monobactams are primarily competitive inhibitors, or, more specifically competitive substrates. Their action is often reversible leaving the enzymes intact, because they simply act as poor substrates that bound tightly to the β-lactamase and are hydrolyzed slowly. The most effective inhibitors that have been

developed commercially are irreversible, the enzyme and inhibitor interacting competitively initially and then progressively forming a complex in which both enzyme and inhibitor are inactivated: progressive or 'suicide' inhibitors. Inactivation usually occurs after a fixed amount of inhibitors has been hydrolyzed like a normal substrate. Clavulanic acid, sulbactam, and tazobactam are all of this form, although the precise nature, rate and degree of inactivation differ considerably among the various agents and enzyme (Muratani *et al.*, 1993). The reaction mechanism is quite complicated and, although the key intermediates are the same, the reaction probably follows a slightly different course in the various types of class A enzymes. For clavulanic acid, the reaction with *S. aureus* PC1 enzyme is nearly stoichiometric. The reaction with other class A enzymes requires higher molar stoichiometric to achieve complete inhibition. TEM-2 β-lactamase has been reported to require 115 moles/mole enzyme for complete inhibition. Brown *et al.* (1996) showed that the reaction leading to irreversible inhibition involves cross-linking to Ser 130 in the active site.

There are three β-lactamase inhibitors in clinical use: clavulanic acid, sulbactam, and tazobactam (Figure 1.3). Five β-lactam/β-lactamase inhibitor combinations are commercially available for the treatment of common infections, although some are only available as parenteral preparations and others used for community-acquired infections because of their oral bioavailability (Table 1.3). These combination

therapies are being successfully used for the treatment of infectious due to β-lactamases producing organisms. However, these inhibitors are becoming less effective for certain infectious. During the past two decades, considerable efforts has been expended to identify better inhibitors by exploiting the newly acquired knowledge of structure and mechanism of action of these enzymes and their inhibitors (Sandanayaka and Prashad, 2002).

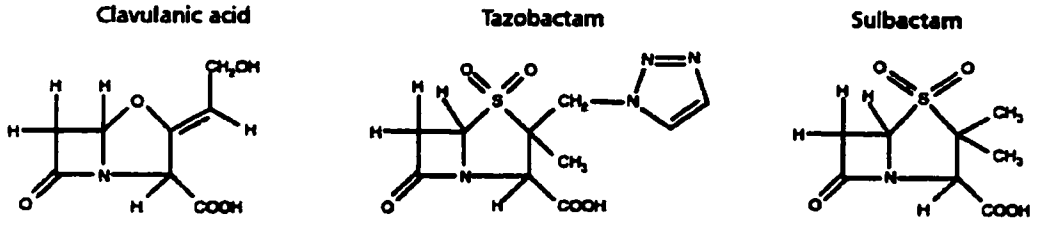


Figure 1.3 Structure of β-lactamase inhibitors

Table 1.3 β-Lactams/β-lactamase inhibitors for clinical use

β-Lactam	β-Lactamase inhibitor	Administration route
Ampicillin	Sulbactam	Parenteral and oral
Cefoperazone	Sulbactam	Parenteral only (not available in the USA)
Piperacillin	Tazobactam	Parenteral only
Ticarcillin	Clavulanic acid	Parenteral only
Amoxicillin	Clavulanic acid	Parenteral and oral (only oral form available in the USA)

1.4 Conserved elements among DD-peptidases and β-lactamases

The similarity between β-lactamases and DD-peptidases is not restricted to 'mechanistic' properties. Indeed, X-ray diffraction studies have highlighted striking structural analogies between the *Streptomyces* R61 (*S.* R61) DD-peptidase and several class A and class C β-lactamases (Figure 1.4) (Jamin *et al.*, 1995; Kelly *et al.*, 1986; Lobkovsky *et al.*, 1993; Oefner *et al.*, 1990; Samraoui *et al.*, 1986). There are striking similarities in the organization of the secondary structure elements, despite very low degrees of sequence similarity. These enzymes are all medium-sized monomeric proteins (M_r values of about 29000 and 39000 for class A and class C β-lactamases respectively, and 37500 for the *S.* R61 DD-peptidase), which are made up of two structural domains (an all-α and an α/β domain) with the active site situated in a groove between the two domains (Figure 1.4). Compared with the class A β-lactamases, the class C enzymes and the *S.* R61 PBP have additional loops and secondary structures on the surface of the all-α domain (α-20 helix).

Several conserved elements have been identified in the vicinity of the active-site serine residue of DD-peptidases and β-lactamases, which appear to be directly or indirectly involved in the substrate recognition and catalytic processes (Table 1.4) (Matagne *et al.*, 1999). Careful comparisons of the primary and tertiary structures have allowed identification of the same structural and functional elements in all

active-site serine penicillin-recognizing enzymes (Ghuysen, 1991, Joris *et al.*, 1991; Joris *et al*, 1988; Sanschagrin *et al.*, 1995).

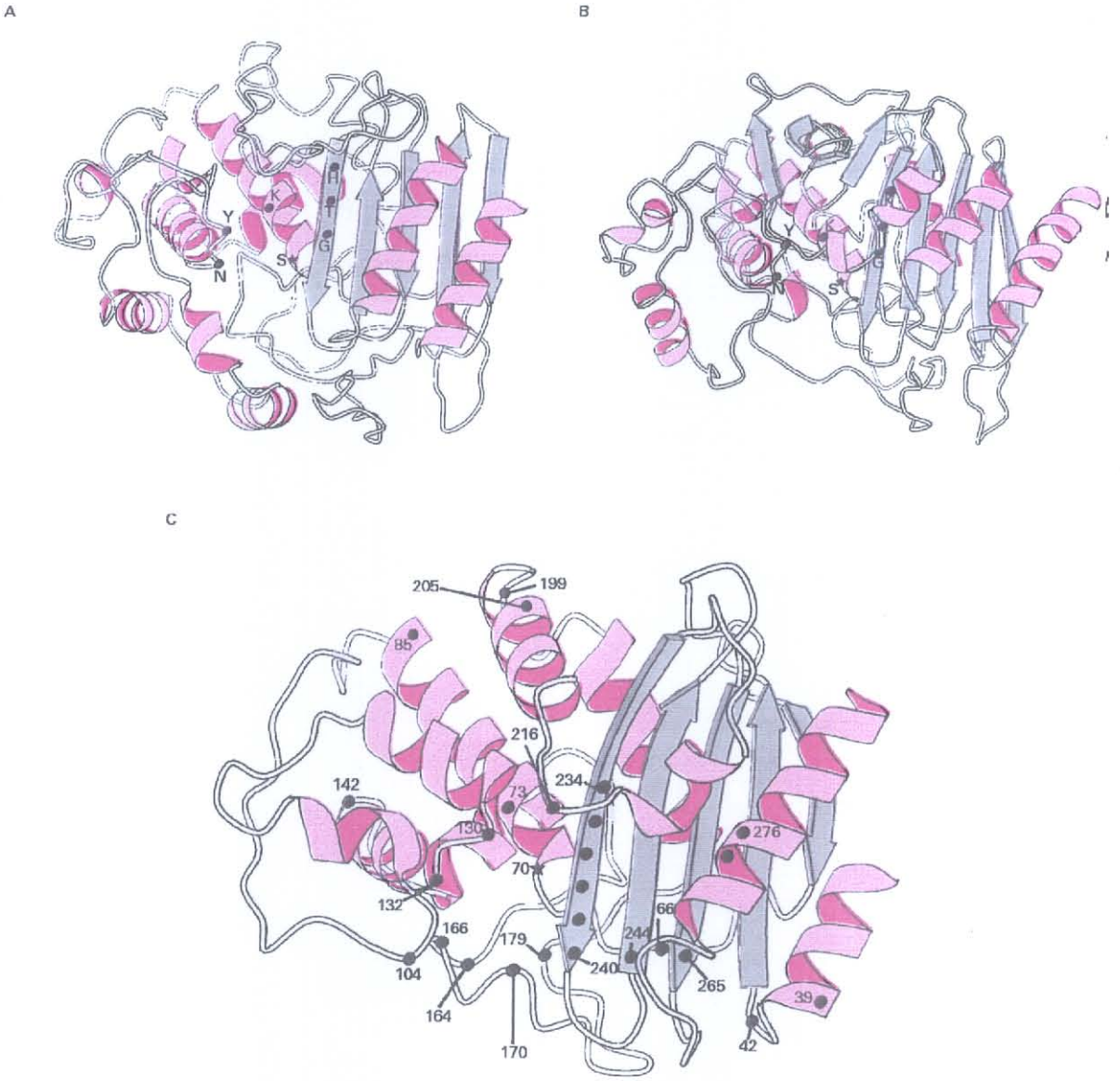


Figure1.4 Comparison of tertiary structures of the *S.* R61 DD-peptidase (A), the *E. cloacae* class C β-lactamase (B) and the class A TEM β-lactamase (C). The α-carbon of the active-site serine is marked by a star and those of the residues discussed in the text by a dot (after Matagne *et al.* ,1998).

Table 1.4 The three equivalent structural and functional elements of penicillin-recognizing enzymes. The active-site serine is indicated by *.

	Element 1	Element 2	Element 3
Class A	S70*-Xaa-Xaa-Lys	Ser130-Asp-Asn	Lys234-Thr-Gly
		Ser130-Asp-Ser	Lys234-Ser-Gly
		Ser130-Asp-Gly	Arg234-Thr-Gly
			Arg234-Ser-Gly
Class C	Ser64*-Xaa-Ser-Lys	Tyr150-Ala-Asn	Lys314-Thr-Gly
		Tyr150-Ser-Asn	
Class D	Ser70*-Xaa-Xaa-Lys	Tyr144-Gly-Asn	Lys214-Thr-Gly
*S.*R61 DD-peptidase	Ser60*-Val-Thr-Lys	Tyr159-Ser-Asn	His298-Thr-Gly
Other known PBPs	Ser60*-Xaa-Xaa-Lys	Ser159-Xaa-Asn	Lys298-Thr-Gly
		Ser159-Xaa-Cys	Lys298-Ser-Gly
		Tyr159-Gly-Asn	

The first element contains the active serine (Ser 70) and, one helix-turn downstream, a lysine (Lys 73) residue whose side-chain also points into the active site (Ser-Xaa-Xaa-Lys sequence). The proximity of the serine and lysine side-chains, which are hydrogen-bonded, suggested the likely involvement of the lysine side-chain amino group in the catalytic process (Herzberg and Moult, 1987; Knox and Moews, 1991; Strynadka *et al.*, 1992). Lys 73 is conserved active-site residues in the class A β-lactamases, as well as other members of the serine penicillin–binding enzyme family; its role in catalysis remain controversial and uncertain (Chen *et al.*, 1996; Lietz *et al.*, 2000). It is likely that both Glu 166 and Lys 73 are important to each other in

terms of maintaining the optimum electrostatic environment for fully efficient catalytic activity to occur.

The second element is situated on a short loop in the all α-domain, where it forms one side of the catalytic cavity. It consists of Tyr-Xaa-Asn (β-lactamases of classes C and D, some PBPs) or Ser-Xaa-Asn (β-lactamases of class A, most PBPs) sequences. In class A β–lactamases, the Ser^{130}-Asp^{131}-Asn^{132} motif (also called the SDN loop) is nearly invariant. From the study of the SDN loop in *Streptomyces albus* G β-lactamase by site-directed mutagenesis (Jacob *et al.*, 1990b), all three residues are indeed important, either for the β-lactamase structure or good functioning, and their respective roles are quite distinct. Ser 130 seems to help maintain the structure of the active-site cavity, Asp 131 seems to be a key residue in protein structural stability, and Asn 132 seems to act in the catalytic process. The structural and functional roles of Ser 130 also have been studies via Ala, Asp, and Gly mutant (Matagne and Frere, 1995), these studies highlighted the participation of Ser 130 in the complex hydrogen bond network in the active site and its potential role as a proton shuttle from the hydroxyl group of Ser 70 to the leaving N atom.

The third element, on a piece of the β-sheet, forms the opposite wall of the catalytic cavity. It is generally a Lys-Thr-Gly sequence, but Lys is replaced by His or Arg in a few exceptional cases and Thr by Ser in several class A β-lactamases. A

positive side-chain, followed by one bearing a hydroxyl group, appears to be universally conserved. Lys 234 is an important site in class A β-lactamase, it may be involved in the network of hydrogen bonds composited of Ser 70, Lys 73, Glu 66 and Ser 130 (Herzberg, 1991). By calculation, the pKa of Lys234 is raised in the tetrahedral intermediate, suggesting a probable role of this residue in the stabilization of the tetrahedral intermediate.

The fourth element, containing a negatively charged residue, has been tentatively identified in all enzymes, but it seems to play a catalytic role only in class A β-lactamases. It is situated on a 16–19 residues loop [residues 161-179 in TEM-1 (Jelsch *et al.* 1993); residues 163-178 in PC1 (Herzberg and Moult, 1987)], and usually referred to as the Ω-loop (Figure 1.5). In most case, this loop contains the Glu166-Xaa-Glu-Leu-Asn170 sequence where the two residues Glu 166 and Asn 170 seem essential in positioning the conserved water molecule W1 very close to the active site serine (Lamotte-Brasseur *et al.*, 1991). TEM-1 β-lactamase substrate specificity can be significantly altered by mutation of amino acid 161 thought 170 (Palzkill *et al.*, 1994). The wild-type *Staphylococcus aureus* PC1 β-lactamase hydrolyzes penicillin compounds better than cephalosporins. In contrast, the deletion of the Ω-loop led to a variant enzyme that acts only on cephalosporins, including third generation compounds (Banerjee *et al.*, 1998). It seems that the Ω-loop may plays

importance roles in the activity, substrate specificity, and structure of class A β-lactamases.

These 'structural and functional conserved elements', described in Table 1.4, are also found in the sequences of penicillin–recognizing enzymes of unknown three-dimensional structures, including the class D β-lactamases and the large number of PBPs whose primary structures have been deduced from the corresponding gene sequences (Joris *et al.*, 1988; Joris *et al.*, 1991; Sanschagrin *et al.*, 1998). However, it is still not clear whether these conserved elements play identical roles in the catalytic mechanism of the various groups of enzymes.

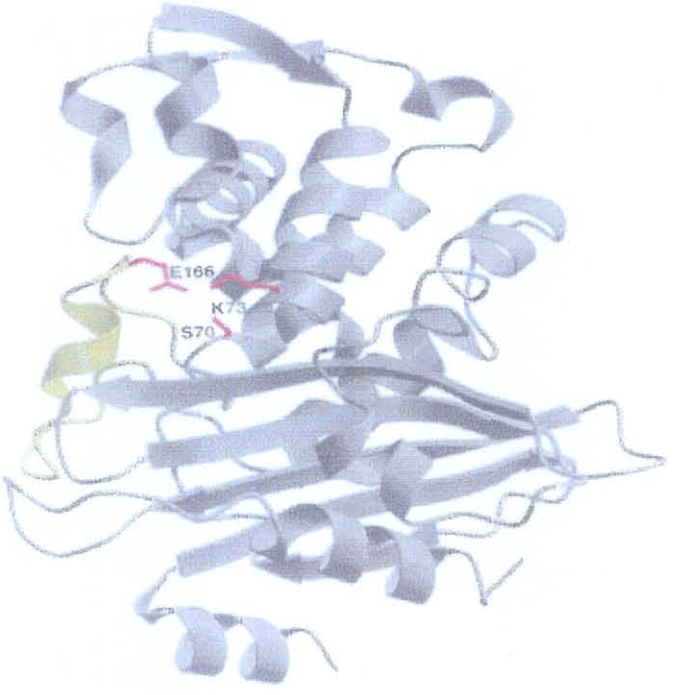


Figure 1.5 Overall fold of β-lactamase, highlighting the Ω-loop (gold) and three residues, Ser 70, Lys 73, and Glu 166 (red) (after Banerjee *et al.*, 1998).

1.5 Class A β-lactamases and their catalytic mechanism

Of all these enzymes, class A β-lactamases are the most commonly encountered by clinicians and hence, have been extensively studied. A very high number of enzymes have been reported and more than 45 sequences determined (not considering the numerous variants in the TEM and SHV families) (Matagne *et al.*, 1998). The three-dimensional structures of six class A enzymes are known, most of them at high resolution [*B. cereus* I (Samraoui *et al.*, 1986); *S. albus* G (Lamotte-Brasseur *et al.*, 1991; Dideberg *et al.*, 1987); *Staphylococcus aureus* PC1 (Herzberg and Moult, 1987; Herzberg, 1991); *B. licheniformis* 749/C (Knox and Moews, 1991; Moews *et al.*, 1990); TEM-1 (Jelsch *et al.*, 1992); NMC-A (Swaren *et al.*, 1998)]. Also, the function of many residues has been probed by site-directed mutagenesis (Matagne and Frere, 1995). The catalytic properties and primary structures of class A differ considerably, making them a highly diverse class (Waley, 1992).

These medium-sized proteins (Mr about 29,000) show a wide distribution of pI values, ranging from ~3.5 to ~10 (Waley, 1992). Of their 260-280 residues, it appears that only nine residues are strictly conserved (Matagne *et al.*, 1999). Four of them (Ser 70, Lys 73, Ser 130, Glu 166) are essential residues for catalysis, whereas five other residues (Gly 45, Pro 107, Asp 131, Ala 134 and Gly 236) are conserved most probably for structural reasons. Additionally, residues at positions 132 (Asn in most

sequences), 234 (Lys or Arg) and 235 (Ser or Thr) have also been shown to be important for the enzyme activity (Matagne *et al.*, 1999).

High resolution X-ray crystallography combined by detailed chemical studies has served to elucidate the essential features in the catalytic mechanism of these enzymes. The current consensus with respect to the active site residues directly contributing to catalysis includes Ser 70, Lys 73, Ser 130, and Glu 166; Lys 234 and Arg 244 are also believed to act as electrostatic catalysts. It is also now generally agreed that the Glu 166 carboxylate is the general-base catalyst of the deacylation step where an occluded water molecule, observed directly adjacent to the carboxylate in crystal structures (Jelsch *et al.*, 1993; Waley, 1992), is believed to be the nucleophile. There is much less agreement concerning the mechanism of the acylation step where various general acid/base roles have been attributed to Lys 73, Ser 130, and Glu 166. The investigation of a wide range of mutants of these residues (Matagne and Frere, 1995) has not yet led to consensus.

The most controversial situation prevails for class A enzymes, in which two distinct residues have been proposed as potential general bases. The first hypothesis (Damblon *et al.*, 1996; Lamotte-Brasseur *et al.*, 1991; Lamotte-Brasseur *et al.*, 1992) assumes a symmetrical mechanism, where both acylation and deacylation involve Glu 166 as a general base via a conserved water molecule. The second hypothesis

(Strynadka *et al.*, 1992) assumes an non-symmetrical mechanism, with two different general bases, Lys 73 and Glu 166, participating in acylation and deacylation, respectively. In this mechanism, the enzyme active site would provide a favorable local environment, namely a very positive electric field that strongly reduces the pKa value of the alkylammonium group of Lys 73 (by 5-6 pH units), enabling the lysine to remain unprotonated at neutral pH.

A recent comparison of two x-ray structures, one of the *Staphylococcus aureus* PC1 β-lactamase modified by a phosphonate (Chen *et al.*, 1993), and another of the TEM-1 β-lactamase modified by the same phosphonate (Maveyraud *et al.*, 1998), indicated a pathway for the formation of the acyl-enzyme intermediate. The phosphonate mimicked the transition state for acylation process. However, there are intriguing differences between the two structures. In the *S. aureus* PC1 β-lactamase, the side chains of Ser 70 and Lys 73 interact closely, giving the appearance of the function of Lys 73 for abstracting the proton from Ser 70. On the other hand, the structure of TEM-1 enzyme shows strong interaction between Ser 130 and the phosphonate oxygen corresponding to the leaving group, indicating that the complex mimics the collapse of the tetrahedral species *en route* to the formation of the acyl-enzyme intermediate. The process would take place by the transfer of a proton from Ser 130 to the departing amine in the β-lactam substrate. Then a proton would be

transferred to Ser 130 from the now-protonated Lys 73 (Maveyraud *et al.*, 1998). These analyses collectively argue for the existence of Lys 73 in its unprotonated form.

Kinetic studies (Gibson *et al.*, 1990; Guillaume *et al.*, 1997) indicated that both acylation and deacylation rates of Glu 166 mutants were decreased and that Glu 166 may play more important role than Lys 73 in acylation step. Moreover, the first hypothesis is further strengthened by kinetic and modeling studies (Matagne *et al.*, 1993; Matagne *et al.*, 1993; Raquet *et al.*, 1994). Thus it seems that the present evidence substantiates the view that Glu 166 is the genuine general base catalyst in both formation and hydrolysis of the acyl-enzyme intermediate formed with class A β-lactamases (Matagne *et al.*, 1999).

The known three-dimensional structures of the class A β-lactamases are superimposible, which is not surprising given their extensive amino acid sequence homology. Nevertheless, a systematic study of the catalytic properties of class A β-lactamases with a number of β-lactam substrates has revealed a wide range of variation (Matagne *et al.*, 1990) despite their extensive tertiary structure similarities (Jelsch *et al.*, 1993). For example, the K_m values of the *E. coli* TEM-2 (Matagne *et al.*, 1990) and *Bacillus cereus* 569/H β-lactamases for S-type penicillins (such as penicillin G, ampicillin) are generally higher relative to those of the *Staphylococcus aureus* PC1 enzyme (Matagne *et al.*, 1990) and the converse situation exists with

A-type penicillins (such as oxacillin, methicillin). These observations are highly significant given that type A penicillins are thought to elicit a conformational change in the enzyme unfavorable to catalysis (Citri and Zyk, 1982). The conformational properties of β-lactamases are strongly influenced by the structure of the side-chains of the substrates. Briefly, two types of derivatives have been recognized. S-type induces a compact conformation that is favorable to the catalytic reaction. Conversely, the open conformation induced by all A-type derivatives is unfavorable to catalysis (Citri *et al.*, 1976). The high variability of the kinetic parameters of class A β-lactamases for a given β-lactam is also clear illustrated by Table 1.5 (Matagne *et al.*, 1999). Table1.5 also indicates that with their best substrates, the interactions are characterized by very high values of both k_{cat}/K_m (close to the diffusion limit, i.e. 10^8 $M^{-1}s^{-1}$), and k_{cat} (up to 7000 s^{-1}).

Table 1.5 Comparison of the kinetic parameters $k_{cat}(s^{-1})$ and $k_{cat}/K_m(M^{-1}s^{-1})$ for the hydrolysis of some characteristic β-lactams by various class A β-lactamases

	Group		PG	AMPI	CARB	OXA	CR	CT	CTX	CAZ	AZT	IMI
B .licheniformis	2a	K_{cat}	2200	1500	400	10	630	48	6.7	15	ND	ND
		K_{cat}/K_m	29000	11000	8100	1200	5000	2500	33	9	ND	ND
S. albus G	2a	K_{cat}	2800	3900	>1000	270	200	260	>1	<0.001	>0.5	0.004
		K_{cat}/K_m	2800	6100	100	630	620	370	1	<0.001	0.4	0.32
S. aureus PC1	2a	K_{cat}	130	240	190	>7	0.074	0.006	ND	ND	ND	ND
		K_{cat}/K_m	40000	20000	70	3	100	8	ND	ND	ND	ND
TEM-1	2b	K_{cat}	1600	1050	150	60	1500	170	9	0.3	1	0.04
		K_{cat}/K_m	84000	33000	9000	20000	2200	650	1.5	0.07	0.7	1.5
S. fonticola	2be	K_{cat}	770	ND	100	105	2530	7150	920	30	38	no activity
		K_{cat}/K_m	33500	ND	2500	2500	14500	40900	11500	60	930	no activity
TEM-7	2be	K_{cat}	40	18	ND	9.5	26	20	1.5	9	4	ND
		K_{cat}/K_m	13000	1100	ND	9500	300	250	15	9	3	ND
A. R39	2d	K_{cat}	370	1900	220	940	440	200	280	>13	20	<0.0001
		K_{cat}/K_m	7500	16000	870	5900	11600	3700	400	13	76	9
P. vulgaris	2e	K_{cat}	3.8	4.7	ND	0.8	85	37	78	1.9	3.3	0.004
		K_{cat}/K_m	640	450	ND	710	1100	1700	470	10	160	1000
IMI-1	2f	K_{cat}	36	190	ND	ND	2000	120	3.4	0.0068	51	89
		K_{cat}/K_m	560	240	ND	ND	1900	920	18	0.0024	550	520

PG, penicillin G; AMPI, ampicillin; CARB, carbenicillin; OXA. oxacillin; CR, cephaloridine; CT, cephalothin; CTX, cefotaxime; Caz, ceftazisdime; AZT, aztreonam; IMI, imipenem.

In addition to the differences in substrate specificity profiles, the class A β-lactamases also vary in their susceptibility to inactivation by mechanism-based inhibitors. Thus, *S. aureus* PC1 β-lactamase has been found to be extremely sensitive to clavulanic acid (IC_{50} 0.06 μg /ml, Cartwright and Coulsen, 1979) whereas the *B. cereus* 569/H β-lactamase I exhibits considerable resistance (IC_{50} 17 μg /ml) to undergo inactivation by this inhibitor (Durkin *et al.*, 1978). Interestingly, the situation with the two enzymes is exactly the converse with 6-bromopenicillanic acid (Pratt and Loosemore, 1978). Complete inactivation of the *B. cereus* enzyme is achieved with a 10-fold molar excess of this β-lactam whereas the *S. aureus* enzyme is only partially inhibited. Likewise, 6-(trifluoromethane sulfonyl)amido-penicillanic acid sulfone has been shown to be a potent mechanism-based inhibitor of the *B. cereus 569/H*, *B. licheniformis* 749/C and *E. coli* R6K β-lactamases (Hilhorst *et al.*, 1984; Clarks *et al.*, 1983; Mezes *et al.*, 1982), whereas the *S. aureus* PC1 is less susceptible to this compound.

A number of mechanistic properties of class A β-lactamases are beginning to be elucidated by both biochemical studies and the structural information. Two broad types of class A β-lactamases with defined phenotypic properties are emerging as a result of recent clinical selection pressures. One is the inhibitor-resistant phenotype, which was first observed in the TEM family, for which the term inhibitor-resistant

TEM (IRT) was coined. This type of phenotype has now been seen in the SHV family as well (Lin *et al.*, 1998). The second phenotype is that of the so-called extended-spectrum β-lactamases (ESBLs). As the name suggests, these variants have broadened their substrate profiles to include such β-lactams as expended-spectrum cephalosporins and carbapenems. These extended-spectrum β-lactamases are exemplified by the Imi-1, Per-1, Sme-1, Toho-1 and NMC-A β-lactamases (Naas *et al.*, 1994; Nordmann *et al.*, 1993; Rasmussen *et al.*, 1996; Swaren *et al.*, 1998). Both these phenotypes are the main causes for serious concern in the clinic.

More recent studies have shown that variation in the amino acids that line the active-site pocket of β-lactamases imparts altered activities. Naturally occurring substitutions of one to three amino acids in this region of the TEM-1 enzyme (Jacoby and medeiros, 1991) or those generated *in vitro* by both random-replacement (Palzkill and Botstein, 1992) and site-directed mutagenesis (reviewed by Matagne and Frere, 1995) enhance reactivity toward the extended-spectrum cephalosporins. And also, the resistance of IRTs (inhibitor-resistant TEMs) to inhibitors has been accounted for by a few naturally occurring amino acid replacements (Canica *et al.*, 1997; Farzaneh *et al.*, 1996; Blazquez *et al.* 1993). Changes involving a single amino acid residue comprising the active-site pocket thus appear to dramatically influence the activity of β-lactamases toward some substrates and inhibitors, and this precision could account

for the wide range of specificity noted among the class A enzymes (Matagne *et al.*, 1990).

To summarize, the introduction of β-lactam antibiotics in clinical trials more than 50 years ago clearly represents one of the major breakthroughs in modern chemotherapy. Till now, β-lactams still account for approximately 50% of global antibiotic consumption. The production of one or several β-lactamases by pathogenic bacteria represents the most widespread resistance mechanism to antibiotics. Despite numerous kinetic, structural and site-directed mutagenesis studies, we have not completely succeeded in explaining the diversity of the specificity profiles of β-lactamases and their surprising catalytic power. The solutions to these problems represent the cornerstones on which better antibiotics and inhibitors of β-lactamase can be designed, hopefully on a rational basis.

1.6 Hybrid enzymes

While engineering enzymes is used to improve their properties, it has also become an essential tool of research for basic protein biochemistry. *In vitro* DNA synthesis and recombinant DNA technology make it possible to design and produce any kind of polypeptide. So far, the majority of protein engineering relies on the modification of pre-existing proteins. For this reason, recombination of pre-existing

elements provides a very powerful tool to study the structure-function relationships of proteins and to generate proteins with new properties.

The simplest approach to altering the structure of pre-existing protein is site-directed mutagenesis. Site-directed mutagenesis, also called 'rational mutagenesis', perhaps the most commonly employed method, makes it possible to generate any desired protein sequence at will. However, given the large number of possible derivatives [for a 20-amino-acid region there are 380 possible single mutants and $>10^5$ possible double mutant(s)], it is completely impractical to alter protein function by directed mutation in the absence of detailed structural information. The advantage of site-directed mutagenesis is that a site can be precisely probed by specific substitutions. The results allow one to conclude whether a specific site can tolerate a specific change and to determine if changes alter the properties of a particular variant. However, it should be noted that interpretation of the results might be difficult. Loss of enzyme activity by certain substitution does not indicate, by itself, that the replaced amino acid is directly involved in catalysis (Skandalis *et al.*, 1997). Moreover, a series of examples showing that mutation of essential residues generally does not annihilate activity and sometimes even leaves most of the catalytic power of the enzyme intact (Plapp, 1995). Enzymes deprived of important catalytic groups perform catalysis through mechanisms that can be significantly modified with respect

to the wild-type mechanism. Such altered mechanisms rely on the presence in the active sites of multiple catalytic devices and, sometimes, on the surrogate action of groups that do not participate in the wild-type reaction, blurring the distinction between `essential' and `nonessential' residues. Furthermore, the efficiency of the alternative mechanisms can depend heavily on the specific enzyme and on the type of amino acid substitution (Peracchi, 2001). In the case of class A β-lactamases, replacing Ser 70 with Ala in β-lactamase from *Bacillus licheniformis* yielded a severely impaired mutant that, nevertheless, retained a k_{cat} ~10^6-fold higher than background (Hokenson *et al.*, 2000). Analogously, the S70A mutant of the enzyme from *Streptomyces albus* could still accelerate β-lactam hydrolysis by nearly a million fold, even in the presence of β-iodopenicillinate, a covalent inhibitor of β-lactamase that selectively modifies Ser 70 in the wild-type enzyme (Jacob *et al.*, 1991a). The *S. albus* S70A mutant also showed a specificity towards different substrates that was markedly altered with respect to the wild-type enzyme (Jacob *et al.*, 1991a). These results suggested the existence of an alternative catalytic mechanism in the S70A mutants, involving the direct hydrolysis of the β-lactam amide bond (Hokenson *et al.*, 2000; Jacob *et al.*, 1991a).

Moreover, site-directed mutagenesis, as well as other conventional approaches, has significant limitations with respect to the frequency and diversity of variant

proteins. Growing evidences suggest that many protein functions cannot be ascribed to single or a few amino acids but they rather depend on regions located far away from the active and regulatory sites, and therefore hard to be predicted *a priori*.

Less stochastic approach to protein engineering is to make use of properties in existing enzymes and, guided by the detailed sequence and structural knowledge currently available, construct chimeric or hybrid enzymes. The term 'hybrid enzyme' is a rather nebulous term that warrants further definition. For the purposes of this thesis, a hybrid enzyme is considered to be composed of elements of more than one enzyme. The construction of hybrid enzymes (Figure 1.6) parallels the strategies that nature uses to evolve enzymes. It is generally thought that enzymes have evolved to fit a specific niche in biology through such processes as gene duplication, domain recruitment and fixation of multiple point mutations. Similarly, hybrid-enzyme approaches seek to recruit established functions and properties from existing enzymes and incorporate them into the engineered enzyme. These techniques have been shown to be useful in the alteration of nonenzymatic as well as enzymatic properties and also as tools for understanding structure–function relationships. In addition, the creation of hybrid enzymes can expand the potential uses of natural enzymes. Enzymes, or fragments of enzymes, could potentially serve as building blocks for proteins capable of catalyzing reactions not observed in nature.

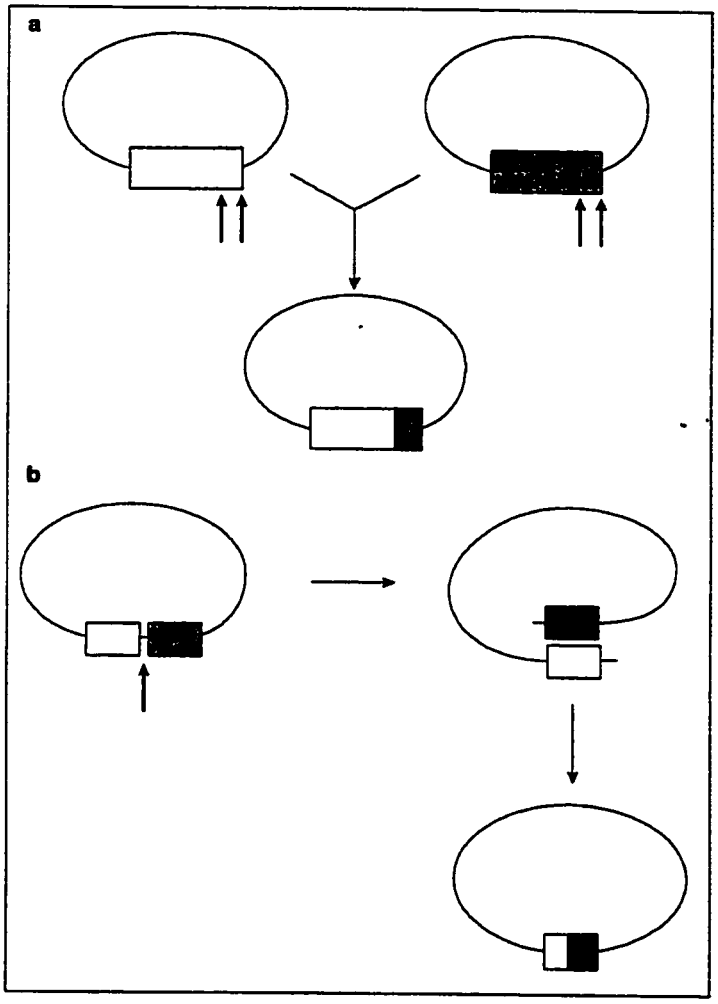


Figure 1.6 Hybrid-gene formation by using restriction enzymes or homologous recombination. (a) Two common restriction sites, indicated by arrows, are used for swapping DNA fragments. (b) Two genes whose sequences are similar (normally >70%) are cloned in tandem in a vector. This recombinant plasmid is linearized by restriction enzyme(s) as indicated by the arrow, and the resultant plasmid is used to transform *E. coli* (or other organism). The homologous recombination between genes forms a hybrid gene.

Early demonstrations of the functionality of interspecies hybrids (Schneider *et al.*, 1981; Mas *et al.*, 1986; Rey *et al.*, 1986) have led to many studies on hybrids between highly homologous enzymes in order to confer some enzymatic properties, such as substrates specificity and thermal stability (Olsen *et al.*, 1991; Zulli *et al.*, 1990), of one enzyme on the other. These hybrids are created by the exchange of residues or structures between homologous regions of related enzymes. Such exchanges have generally resulted in hybrid enzymes with properties intermediate between those of the two parent enzymes. For example, hybrids of *Agrobacterium tumefaciens* β-glucosidase (optimum activity at pH 7.2–7.4 and 60°C) and *Cellvibrio gilvus* β-glucosidase (optimum activity at pH 6.2–6.4 and 35°C) resulted in hybrids that were optimally active at pH 6.6–7.0 and 45–50°C, and possessed K_m values for various saccharides that were intermediate between those of the parent enzymes (Singh *et al.*, 1995; Singh and Hayashi, 1995).

Often, the exchange of homologous regions between related enzymes results in hybrid enzymes with diminished activity. Not too surprisingly, the lower the similarity, the more likely that the hybrid will have diminished or no activity. However, random mutagenesis can be used to restore the enzyme's activity, presumably by restoring proper interactions for folding, stability and obtaining the correct structural formation. For example, RTEM-1 β-lactamase and a β-lactamase from *Proteus vulgaris* have

37% similarity; of a series of 18 hybrids between the two β-lactamases, most were inactive, with a few hybrids with partial or trace levels of activity (Hosseini-Mazinani *et al.* 1996). However, random mutagenesis of some of the hybrids with partial or trace activity dramatically improved their activity, even though the residues mutated did not interact with the substrate.

Furthermore, hybrid enzymes have often been used to determine the differences between related enzymes, identifying those residues or structures that impart a specific property that one enzyme has but another, homologous, enzyme does not. For example, hybrids between two highly homologous proteinases from *Lactococcus lactis* were used to determine which residues were responsible for their cleavage specificity and rate towards α_{s1}- and β-casein (Vos *et al.* 1991). The hybrids were also used to identify an additional unique domain involved in substrate binding that was absent from related subtilisins. Highly homologous *Staphylococcus hyicus* lipase (SHL) and *Staphylococcus aureus* lipase (SAL) show remarkable differences in their biochemical characteristics. Testing of the enzymatic activity of the hybrids toward p-nitrophenyl esters showed that chain length selectivity is defined by elements within the region of residues 180-253. Moreover, residues along the stretch 275-358 contribute to the binding of acyl chains. Interestingly, several chimeras were even more active than the parent enzymes on long-chain p-nitrophenyl esters (van Kampen

et al., 1998). Hybrid enzymes have also been used to investigate the relative merits of structural and sequence alignments between related enzymes (Baker *et al.*, 1997; Caramori *et al.*, 1991; Kimura *et al.*, 1997; Vuilleumier and Fersht, 1994).

In summary, studies of hybrid enzymes provide a broader understanding of how an enzyme's structure relates to its function and what changes can be tolerated within a particular framework. It is one of the most powerful tools for determining structure-function relationships of enzymes and the creation of enzymes with novel activities.

1.7 The objective of this study

Understanding of the catalytic mechanism of β-lactamases would be an important step in the design of improved β–lactam antibiotics or inhibitors of β–lactamases. As mentioned above, even though site-directed mutagenesis has been widely applied to the study of β–lactamases (reviewed by Matagne and Ferere, 1995), it is still difficult to establish correlation between the primary structures and the highly variable substrate profiles. It is interesting that the minor sequence changes can bring about major changes in the stability, specificity, catalytic activity and antigenic properties of β–lactamases. The analysis of a family of enzymes provides more information than a combination of separate studies of each member. A comprehensive

study allows us to make predictions about some family members based on the properties of others. Comparisons of known enzyme structures can give us an "enzyme's eye view" of evolution: their similarities show us what is important to an evolving enzyme, whereas their differences give us an appreciation of the flexibility of an active site (Hasson et al, 1998). As class A β–lactamases show highly homologous structures and exhibit an amazing spectrum of specificity profiles, it would be very informative to study structure-function relationships of β–lactamase by the use of hybrid enzyme approach.

Of the well-characterized class A β–lactamases, *Bacillus cereus* 569/H β–lactamse I (PenPC), *Bacillus licheniformis* 749/C β–lactamase (PenP) and *Staphalococcus aureus* PC1 β–lactamase (PC1) were well studied. Although *B. cereus* is not usually regarded as a pathogen, the molecular properties of the β–lactamases it produces have been subjected to detailed study. One reason for the attention given to these β–lactamases is that they are easily obtained in pure form in the relatively large amounts needed for investigation by simple techniques. Many wild-type isolates of *B. licheniformis* possess an inducible β–lactamase, and the studies of this system are proved to be of considerable fundamental interest. PenP is the most homologous to PenPC among all β–lactamases (Ambler, 1980), both are chromosomally-encoded proteins secreted to the growth medium as multiple processed forms (Izui *et al.*, 1980;

Lampen *et al.*, 1980; Thatcher, 1975a; Thatcher, 1975b). The study of the β–lactamases of *S. aureus* has been sustained largely because of the problem of resistance of this species to penicillin G, and PC1 has also been used as a model for studying the folding of globular proteins. PC1 contains neither cysteine nor tryptophan and it is somewhat unusual in that more than 60% of the residues are polar. The remarkable feature of the amino acid sequence of PC1 is the extensive homology shown to PenPC and PenP. The most likely explanation is that there was a common ancestral gene for these three enzymes (Dyke, 1979). The alignments of the three mature β–lactamases are shown in Figure 1.7, both the identity (53%) and similarity (74%) between PenPC and PenP are higher than the identity (34%) and similarity (57%) between PenPC and PC1. The three-dimensional structures of all of these three enzymes (Figure 1.8) are also known, and the function of many residues has been studied by site-directed mutagenesis. Further, the properties of these three enzymes (such as substrate profiles and responses to β–lactamase inhibitors) are very different, which make the study of hybrid constructs more interesting and informative. Therefore, the three class A β-lactamases PenPC, PenP and PC1 were chosen for hybrid studies in this thesis. Table 1.6 and Table 1.7 summarize some published kinetic parameters and properties of the three enzymes.

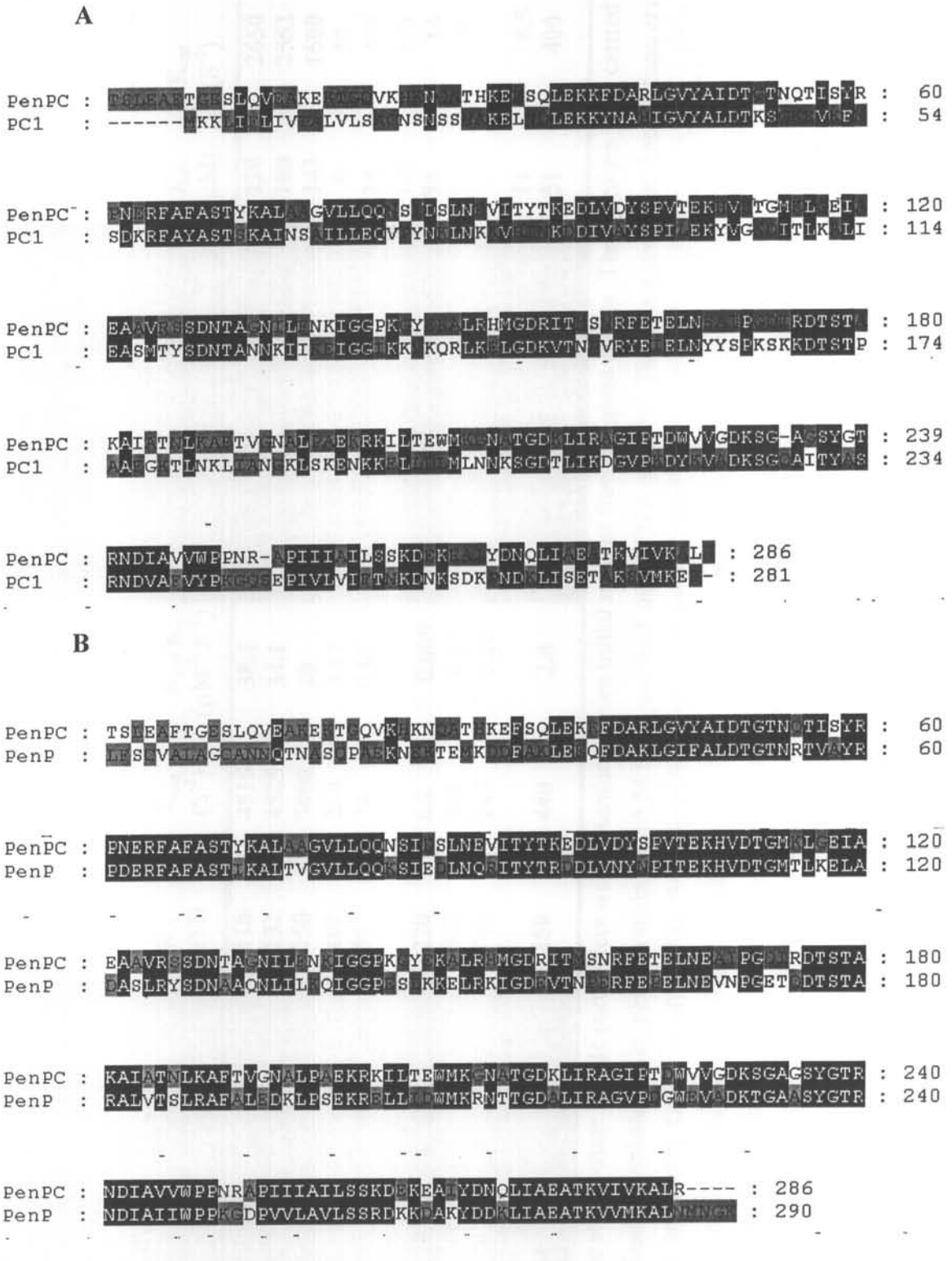


Figure 1.7 Alignment of the mature protein primary structures of PenPC and PenP (A), or PenPC and PC1 (B). Conserved amino acids are indicated by black box. The homologous protein sequences are found from GeneBank at NCBI. The alignments of the amino acid sequence were performed with Clustal W.

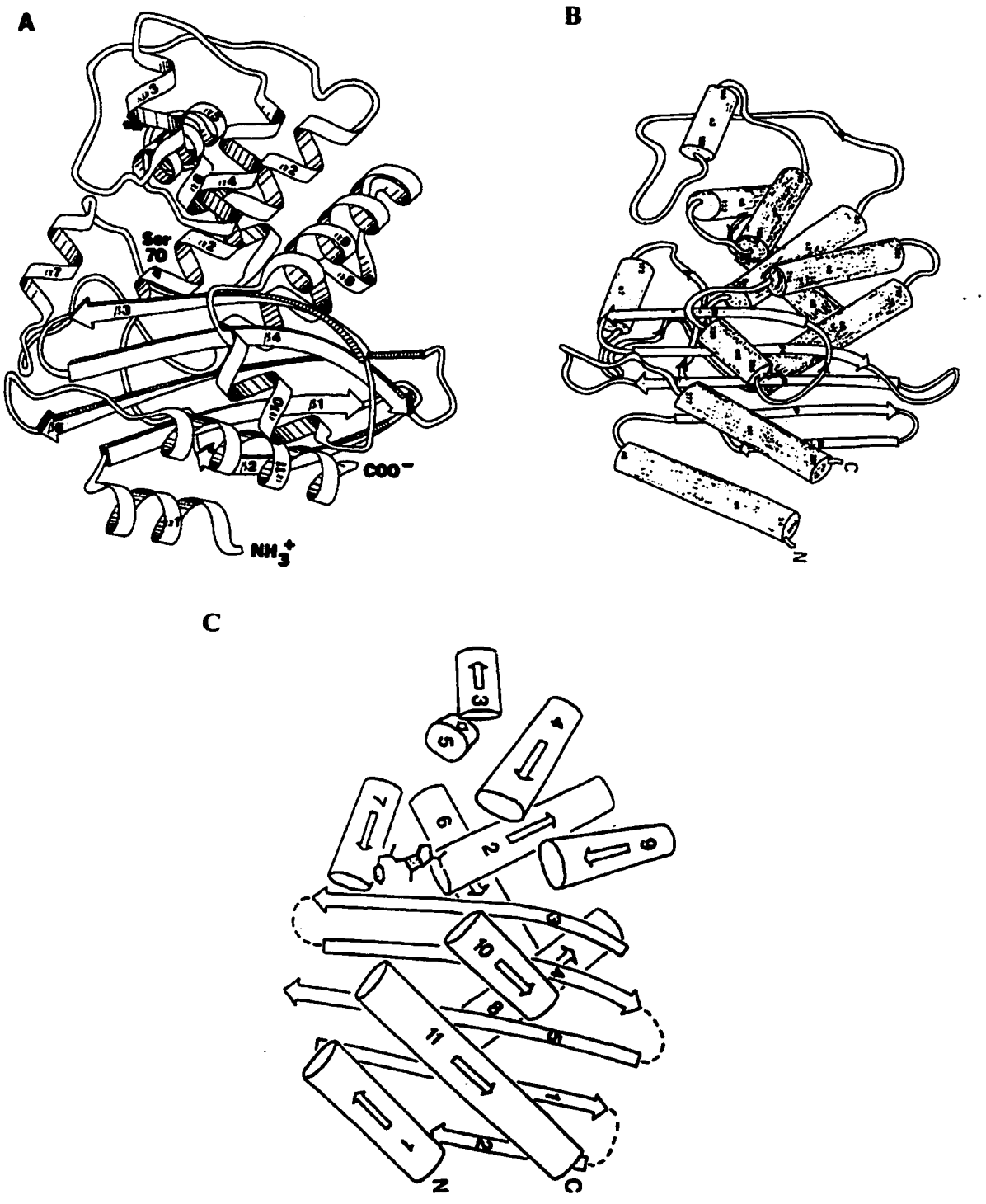


Figure 1.8 Structure of the three β-lactamases PC1 (A) (after Herzberg and Moult, 1987), PenPC (B) (after Samraoui *et al.*, 1986), and PenP (C) (after Knox and Moews, 1991).

Table 1.6 Published kinetic parameters of the three class A β-lactamases: PenPC, PC1 and PenP

Substrates	PenPC[a]			PC1[b]			PenP[c]		
	K_m (μM)	K_{cat} (S^{-1})	K_{cat}/K_m ($\mu M^{-1}.S^{-1}$)	K_m (μM)	K_{cat} (S^{-1})	K_{cat}/K_m ($\mu M^{-1}.S^{-1}$)	K_m (μM)	K_{cat} (S^{-1})	K_{cat}/K_m ($\mu M^{-1}.S^{-1}$)
Penicillin G	**118**	**4515**	**38.1**	**5**	**400**	**80**	**120**	**2650**	**21.5**
Penicillin V	**132**	**4525**	**34.1**	**7**	**440**	**60**	**100**	**2552**	**24.8**
Ampicillin	**250**	**5000**	**20**	**35**	**550**	**16**	**143**	**1500**	**11**
6-Aminopenicillanic acid	**1600**	**260**	**0.17**	**70**	**40**	**0. 6**	**9**	**57**	**6**
Nitrocefin	**55**	**34**	**0.62**	**7**	**14.0**	**2**	**38**	**470**	**13**
Cephaloridine				**<1**	**0.008**		**135**	**630**	**5**
Cephalosporin C	**220**	**0.2**	**0.009**	**100**	**5**	**50**	**98**	**14**	**0.15**
Oxacillin	**826**	**261**	**0.32**	**1000**	**8.2**	**0.008**	**8**	**10**	**1.2**
Methicillin	**740**	**145**	**0.20**	**>5000**	**ND**				
Cloxaccillin				**10000**	**8**	**0.00.8**	**11**	**8.5**	**0.775**
Carbenicillin	**150**	**440**	**2.8**				**51**	**400**	**8.1**

(a) The steady-state kinetic parameters were determined from initial rates or from progress curves. The reactions were carried out in 0.1 M sodium phosphate buffer, pH 7, containing 0.5 M NaCl and 0.01 mM EDTA at 20 ^{0}C. The data were taken from Gibson *et al.* (1990), Guo *et al.* (1999), Leung *et al.* (1994); (b) all reactions were performed at 30°C in 50 mM sodium phaophate buffer, pH7.0. The data were taken from Guo *et al.* (1999) and Matagne *et al.* (1990); (c) all reactions were performed at 30°C in 50 mM sodium phosphate buffer, pH 7.0.The data were from Escobar *et al.* (1994), Matagne *et al.* (1990).

Table 1.7 Some kinetic properties of PenPC, PC1 and PenP

PenPC	The enzyme has a broad pH optimum between 6.0 and 7.0 and displays a characteristic substrate profile similar to that of many other gram-positive beta-lactamases. Penicillin V and ampicillin are hydrolyzed at rates similar to that of benzylpenicillin, whereas the enzyme degrades methicillin, oxacillin, cloxacillin, and cephalosporins at low rates. Many penicillins which are poor substrates for the enzyme also act as competitive inhibitors.
PC1	The enzyme is most active against the naturally occurring penicillins (penicillin G and penicillin V) because it combines a high V_{max} against these substrates with a high affinity (low K_m). Two classes of beta-lactam antibiotics have shown themselves to be effective against PC1-producing cultures. They are the semisynthetic penicillins (methicillin and cloxacillin), and some of the cephalosporins (notably cephaloridine and cephalothin). The effectiveness of both groups of compounds depends crucially on their interaction with PC1 since both induce the enzyme and have to act in the presence of large amounts of it. They solve the problem in distinct ways. The new penicillins have an extremely low affinity for the enzyme so that although they are susceptible to hydrolysis in laboratory tests at high substrate concentrations, this sensitivity is unavailing at therapeutic levels. The cephalosporins, on the other hand, are more conventional in their resistance: they show a much reduced V_{max} for the enzyme when compared with penicillins.
PenP	With penicillin G as substrate, the enzyme has a broad pH profile. Slight differences in pH profile are observed when methicillin is hydrolyzed. The different charge variants of the enzymes were observed and these isoenzymes were found to have identical substrate profiles.

In our previous studies, five hybrids (Hybrids A-E, Figure 1.9) were successfully constructed from the *penPC* and *penP* genes by *in vivo* homologous recombination in *B. subtilis* 168 (Leung, 1994). By the comparison of the properties of these purified hybrid enzymes and their parents, the HybridA was found to be more similar to PenP in terms of its enzyme kinetics (with penicillin G, penicillin V and cephaloridine as substrates), thermostability and pH stability than the other hybrids. The properties of the Hybrids B-E are more similar to those of PenPC than those of HybridA (Cheung, 2000). Therefore, some important residues to differentiate PenPC from PenP may lie in between the crossover sites of HybridA and HybridB. More hybrids would be helpful to pinpoint some important determinants of PenPC and PenP.

The genes of *penPC* and *penP* share 61% identity. The recombinant machinery of *B. subtilis* 168 was utilized to produce the hybrids. However, in this study, *E. coli*, which is reported to have a similar machinery to carry out *in vivo* homologous recombination to *B. subtilis* and yeast, was chosen to generate hybrids because it can be more conveniently handled than *B. subtilis*. *E. coli* RR1 was successfully used to generate hybrid enzymes crossing the human pyridoxal kinase and the procine pyridoxal kinase in our laboratory, and the efficiency of homologous recombination for these two highly identical kinases was proved to be very high, almost 100% (Kwok, 2000).

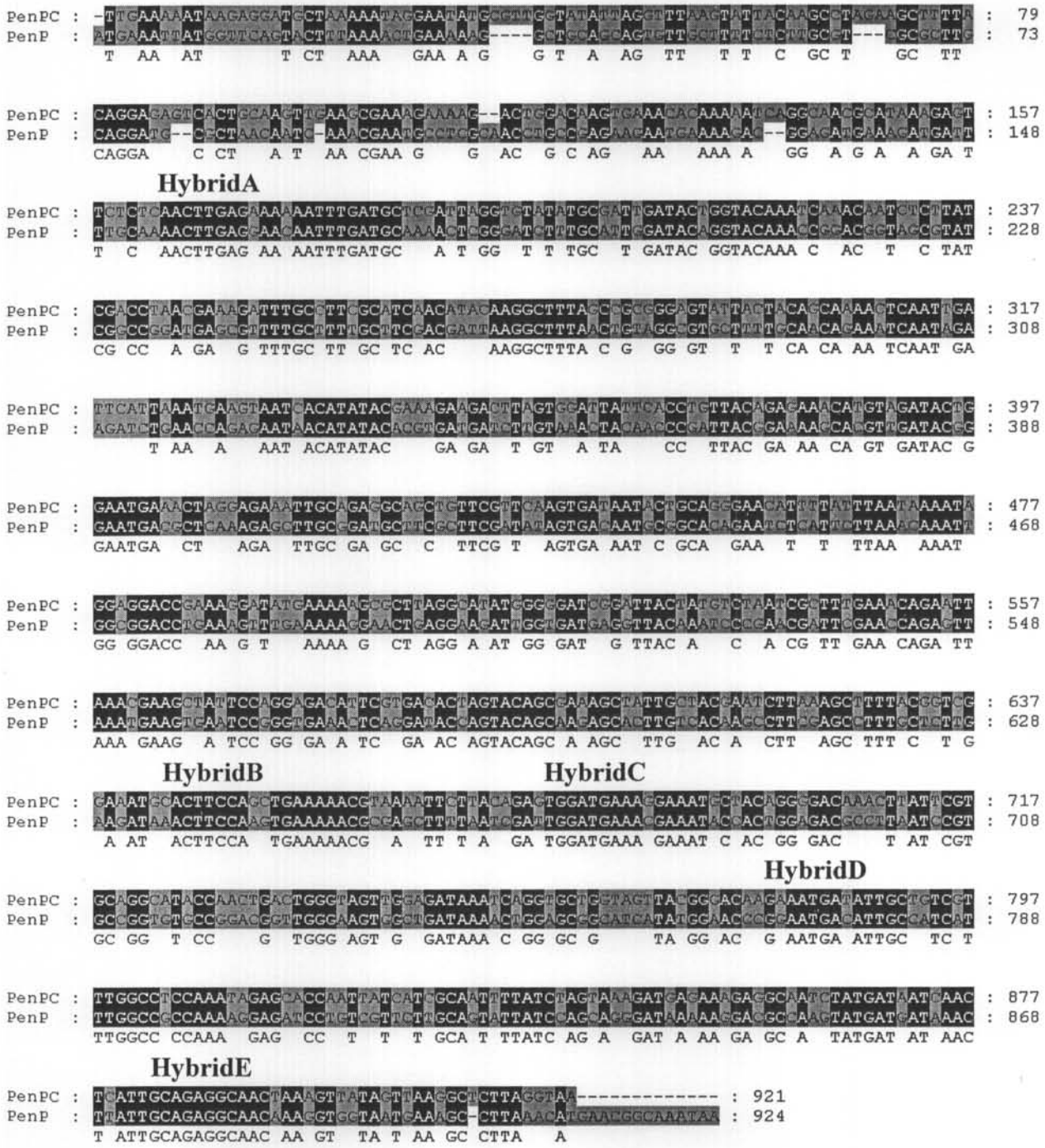


Figure 1.9 Crossover regions of the five hybrid genes obtained from *penPC* and *penP*, which were generated by *in vivo* intramolecular homologous recombination in *B. subtilis* 168. The two genes (*penPC* and *penP*) share 61% nucleotide sequence identity. The alignments of the amino acid sequence were performed with Clustal W.

In addition to the creation of new hybrids from *penPC* and *penP*, the main aim of the present study is to generate hybrids from *penPC* and *pc1*. Considering that the two enzymes show relatively low similarity and identity, it is not surprising to see aforementioned differences in substrate specificity profiles and their susceptibility to inactivation by mechanism-based inhibitors between PenPC and PC1.

In addition, the thermal stability of different β-lactamases also varies to some extents (Table1-8) (Arnold and Viswanatha, 1983; Arriaga *et al.*, 1992; Vanhove *et al.*, 1995a; Vanhove *et al.*, 1995b; Rahil and Pratt, 1994). Since homologous enzymes share the same catalytic mechanism, a high sequence homology, and a rather similar three-dimensional structure, the comparison of homologous enzymes with different thermostabilities offer a unique opportunity to elucidate strategies for the thermal adaptation. Thermostability in different thermozymes seems not to be achieved by a general universal strategy but by a combination of individual strategies, such as an increased number of hydrogen bonds and salt bridges, an optimized packing of the hydrophobic core, shortened surface loops, increased number of prolines, and an increase in buried hydrophobic residues (Vielle *et al.*, 1996; Wallon *et al.*, 1997; Korndorfer *et al*, 1995; Fritt *et al.*, 2001). Five class A β-lactamases, the TEM-1 β-lactamase and the enzymes produced by *Staphylococcus aureus* PC1, *Streptomyces albus*, *Bacillus lichenisformis* 749/C and *Actinomadura* R39, had been compared by

Vanhove *et al.* (1995). The analysis revealed that higher stability appeared to correlate with increased numbers of intramolecular hydrogen bonds and of salt bridges. By contrast, the global hydrophobilicity of the protein seemed to play a relatively minor role. A strong unfavorable balance between charged residues and the presence of a cis-peptide bond preceding a non-proline residue might also contribute to the particularly low stability of two of the enzymes. A more precise picture of protein regions containing thermostability determinants may be drawn by the construction and characterization of hybrid β-lactamases in this study.

Table1.8 Comparison of the enthalpy changes of some different β-lactamases

β-lactamase	pH	T_{trs}	ΔH	Approach	Ref
PC1	7	41.8 ± 0.3	418 ± 25	heat, v.H.	Vanhove et al., 1995
PC1	7.5	41.6 ± 1.0	464 ± 63	heat, v.H	Rahil and Pratt, 1994
TEM-1	7	50.1 ± 0.2	469 ± 29	heat, v.H	Vanhove et al., 1995
PenPC	7	51.03	464 ± 25	DSC	Arriaga et al., 1992
PenPC	7.15	57.5 ± 0.1	646	DSC	Arnold and Viswanatha, 1983
PenP	7	63.2 ± 0.6	686 ±38	heat, v.H	Vanhove et al., 1995

T_{trs}, transition temperature; ΔH (J/mol), the enthalpy change; heat, v. H, heat-induced protein undolding; DSC, differential scanning calorimetry.

For PC1, an interesting feature of its structure is the presence of a non-proline *cis* peptide bond between Glu 166 and Ile 167 (Herzberg, 1991). Non-proline *cis* peptide bonds are rare in proteins of known structure. This is not surprising because there is an approximately 2.8 kcal/mol energy difference between the *cis* and *trans*

conformations of a non-proline peptide bond as revealed by NMR experiments (Drakenberg *et al.*, 1972), and the *cis* conformation may be further destabilized by longer range interactions, resulting in an expected frequency of occurrence of about 0.1% in the denatured state (Ramachandran & Mitra, 1976). The importance of the *cis* peptide bond for the *S. aureus* PC1 β-lactamase function is obvious in light of the crucial role of the carboxylate group of Glu166. The *cis* peptide bond is located on an Ω-loop encompassing residues 163-178, a structural unit which is not well packed against the rest of the molecule, with several intervening internal water molecules, which could contributed to a larger conformational flexibility of the Ω loop (Herzberg & Moult, 1987; Herzberg, 1991). The study of hybrid β-lactamases in this thesis may be very helpful to decipher the precise function of the Ω loop.

Nevertheless, PC1 has been proved to be a useful model for folding studies, not least because of its large size and the absence of disulphide bonds, and of its high solubility and stability, and also of the high degree of reversibility of denaturation. So, the studies on hybrid enzymes generated from PenPC and PC1 may also be of great help for the identification of protein-folding pathways.

In short, not only may the results of the study shed some lights on the elucidation of structure-function relationships of β-lactamases and their folding pathways, but also may supply important information for the design of new β-lactams and inhibitors.

Chapter Two

Materials and Methods

2.1 Reagents

General chemicals used were of analytical grade, and were obtained from BDH Chemicals Ltd., Difco, Pharmacia Ltd. and Sigma Chemical Company.

Antibiotics used for the selection of bacteria containing plasmids or phage DNA were ampicillin trihydrate, chloramphenicol, erythromycin hydrochloride, and were from Sigma Chemical Company. Lithium clvunate was generously supplied by Prof. Yoshikazu Ishii. Antibiotic stocks were sterilized by filtration, if necessary (through an Agrodisc disposable filter, pore size 0.22 μm), and added to medium after autoclaving to the final concentration shown in Table 2.1. All stock solutions were kept at -20°C.

Table 2.1 Preparation of antibiotics

Antibiotics	Stock	Final concentration (ug/ml)	
		B. subtilis	*E. coli*
Ampicillin	100 mg/ml in distilled water	100	100
Chloramphenicol	34 mg/ml in 100% ethanol	5	10
Erythromycin	20 mg/ml in 50% ethanol	20	100

2.2 Culture media

All media were made up in dH_2O (distilled water) to a volume of 1 liter, unless stated otherwise, and were sterilized by autoclaving at 15 lb.p.s.i.,121°C for 20 to 25 min. *E. coli* strains were routinely grown in LB broth (Luria-Bertani Broth,

consisted of 10 g trptone, 5 g Yeast-extract and 5 g NaCl per 1 liter), the 2 x TY broth was used for the growth of *E. coli* for the preparation of competent cells, transformation and protein expression, 2 x TY broth is made by the addition of 16 g trptone, 10 g yeast-extract and 5 g NaCl into one liter dH_2O.

The rich medium BHY, which consisted of 37 g Brain Heart Infusion Broth and 5 g Yeast-extract per liter, was used for the growth of *B. subtilis* strains for protein expression.

Nutrient agar plates were used for growth of bacteria, antibiotics were added when the sterilized agar had cooled to about 50°C. However, when erythromycin was in use, nutrient agar was replaced by LB agar.

2.3 Bacterial strains and plasmid DNA

The geneotypes of *B. subtilis* strains and ϕ105DI:lt bacteriophage derivatives used in this study are detailed in Table 2.2. The bacteriophage derivatives were lysogens of 1A304 strains. The convention used in the text to describe lysogens is strain (prophage). The genotypes of *E. coli* strains are shown in Table 2.3. The relevant characteristics of the plasmids are shown in Table 2.4. The construction of plasmids will be described in the various chapters in detail. The convention in the text to describe strains constructed by transformation with plasmid is strain (plasmid).

Bacteria were maintained on nutrient agar plates, with antibiotics if required, and were stored at room temperature (*B. subtilis*) or at 4°C (*E. coli*). The plates were

subcultured every 3 to 4 weeks by streaking a single colony onto a fresh plate using a sterile wire loop or wooden toothpick.

E. coli or *B. subtilis* frozen stocks were prepared for long-term storage as the follows: a late log phase culture (700 µl) was added to sterile 50% glycerol (300 µl) in a sterile eppendorf tube and was stored at -20°C or -80°C.

Table 2.2 Bacterial strains and bacteriophages of *B. subtilis*

Strain or Phages	Relevant characteristics*	Source/construction**
B. subtilis strains		
168	trpC2	Lab stock
1A304	*trp*C2*met*B52 *xin*-1 SPβ(S)	Lab stock
Phages		
ϕ105MU331	ind-125 cts-52 Ω(lacZcat)2	Thornewell(1992)
ϕ105HB1	ind-125 cts-52 Ω(lacZΔClaIpenPCcat)1	pSGpenPC DNA→ 1A304(ϕ105MU331)
ϕ105HB2	ind-125 cts-52 Ω(lacZΔClaIPC1cat)2	pSGPC1 DNA→ 1A304(ϕ105MU331)
ϕ105HB3	ind-125 cts-52 Ω(lacZΔClaIhybridFcat)3	pSGhybridF DNA→ 1A304(ϕ105MU331)
ϕ105HB4	ind-125 cts-52 Ω(lacZΔClaIhybrid1cat)4	pSGhybrid2 DNA→ 1A304(ϕ105MU331)
ϕ105HB5	ind-125 cts-52 Ω(lacZΔClaIhybrid2cat)5	pSGhybrid2 DNA→ 1A304(ϕ105MU331)
ϕ105HB6	ind-125 cts-52 Ω(lacZΔClaIhybrid3cat)6	pSGhybrid3 DNA→ 1A304(ϕ105MU331)
ϕ105HB7	ind-125 cts-52 Ω(lacZΔClaIhybrid4cat)7	pSGhybrid4 DNA→ 1A304(ϕ105MU331)

ϕ105HB8	ind-125 cts-52 Ω(lacZΔClaIhybrid5cat)8	pSGhybrid5 DNA→ 1A304(ϕ105MU331)
ϕ105HB9	ind-125 cts-52 Ω(lacZΔClaIhybrid6cat)9	pSGhybrid6 DNA→ 1A304(ϕ105MU331)
ϕ105HB10	ind-125 cts-52 Ω(lacZΔClaIhybrid7cat)10	pSGhybrid7 DNA→ 1A304(ϕ105MU331)
ϕ105HB11	ind-125 cts-52 Ω(lacZΔClaIhybrid8cat)11	pSGhybrid8 DNA→ 1A304(ϕ105MU331)
ϕ105HB12	ind-125 cts-52 Ω(lacZΔClaIhybrid9cat)12	pSGhybrid9 DNA→ 1A304(ϕ105MU331)
ϕ105HB13	ind-125 cts-52 Ω(lacZΔClaIhybrid10cat)13	pSGhybrid10 DNA→ 1A304(ϕ105MU331)
ϕ105HB14	ind-125 cts-52 Ω(lacZΔClaIhybrid11cat)14	pSGhybrid11 DNA→ 1A304(ϕ105MU331)

* : Ω indicates an insertion into the prophage of the genes shown in parenthesis; the allele number following the parenthesis is derived from the isolation number of the prophage carrying the original insertion. xxxΔXxx represents the 5' portion of the gene xxx obtained when the DNA was digested with the restriction endonuclease Xxx.

** : → indicates transformation of DNA into the lysogen shown, the transformants were selected for chloramphenicol resistance.

Table 2.3 Bacterial strains of *E. coli* used in this study

E. coli strain	Genotype	Source/reference
XL1-blue	*recA1 endA1 gyr*A96 *thi-1* *hsd*R17 *sup*E44 *rel*A1 *lac* [F' *proAB lacI*^{q}*ZΔM15 Tn10* (Tet^r)][c]	Stratagene
DH5α	U169 *recA1 endA1* λ^-*gyrA96 thi-1* *hsd*R17(rk$^-$ mk$^+$) *deo*R *sup*E44 *rel*A1 F- ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-argF)	Gibco BRL
HB101	*supE44 ara14 galK2 lacY1* Δ*(gpt-proA)*62 *rpsL20* (Str^r) *xyl-5 mtl-1 recA13* Δ(*mcrC-mrr*) HsdS$^-$(r$^-$m$^-$)	Stratagene
RR1	HB101 RecA$^+$	Stratagene
BL21	*E. coli* B F$^-$ *dcm ompT hsd*(rB$^-$ mB$^-$) *gal*	Stratagene
BL21(DE3)	BL21 λ(DE3)	Stratagene

Table 2-4 Plasmids

Plasmid	relevant characteristics	Source/reference
PGK13[a]	5.0 kb, *catermC* with pWVO1*ori*$^+$	Kok et al. (1984)
pYCL18	8.2 kb, *cat ermC penPCpenP*	pGK13
pHBL1	8.2 kb, *cat ermC penPCpc1*	pGK13
pHBL2	5.2 kb, *ermC hybrid12*	pGK13
pHBL3	5.2 kb, *ermC hybrid1*	pGK13
pHBL4	5.2 kb, *ermC hybrid2*	pGK13
pHBL5	5.2 kb, *ermC hybrid3*	pGK13
pHBL6	5.2 kb, *ermC hybrid4*	pGK13
pHBL7	5.2 kb, *ermC hybrid5*	pGK13
pHBL8	5.2 kb, *ermC hybrid6*	pGK13
pHBL9	5.2 kb, *ermC hybrid7*	pGK13
pHBL10	5.2 kb, *ermC hybrid8*	pGK13
pHBL11	5.2 kb, *ermC hybrid9*	pGK13
pHBL12	5.2 kb, *ermC hybrid10*	pGK13

pHBL13	5.2 kb, *ermC hybrid11*	pGK13
pHBL12	5.2 kb, *ermC hybridF*	pGK13
pSG703[b]	5.3 kb, *lacZΔEcoRV cat bla*	Thornewell (1992)
pSGpenPC	6.5 kb, *lacZΔSmaI penPCcat bla*	pSG703
pSGPC1	6.5 kb, *lacZΔSmaI PC1cat bla*	pSG703
pSGhybridF	6.5 kb, *lacZΔSmaI hybridFcat bla*	pSG703
pSGhybrid1	6.5 kb, *lacZΔSmaI hybrid1cat bla*	pSG703
pSGhybrid2	6.5 kb, *lacZΔSmaI hybrid2cat bla*	pSG703
pSGhybrid3	6.5 kb, *lacZΔSmaI hybrid3cat bla*	pSG703
pSGhybrid4	6.5 kb, *lacZΔSmaI hybrid4cat bla*	pSG703
pSGhybrid5	6.5 kb, *lacZΔSmaI hybrid5cat bla*	pSG703
pSGhybrid6	6.5 kb, *lacZΔSmaI hybrid6cat bla*	pSG703
pSGhybrid7	6.5 kb, *lacZΔSmaI hybrid7cat bla*	pSG703
pSGhybrid8	6.5 kb, *lacZΔSmaI hybrid8cat bla*	pSG703
pSGhybrid9	6.5 kb, *lacZΔSmaI hybrid9cat bla*	pSG703
pSGhybrid10	6.5 kb, *lacZΔSmaI hybrid10cat bla*	pSG703
pSGhybrid11	6.5 kb, *lacZΔSmaI hybrid11cat bla*	pSG703
pGEX-6P-1[b]	4.9 kb, *lacI^q bla* with pBR322 *ori*	Phamacia Biotech
pGEXpenPC	6.1 kb, *lacI^q bla penPC*	pGEX-6P-1
pGEXpc1	6.0 kb, *lacI^q bla pc1*	pGEX-6P-1
pGEXhybrid1	6.1 kb, *lacI^q bla hybird1*	pGEX-6P-1
pGEXhybrid2	6.1 kb, *lacI^q bla hybrid2*	pGEX-6P-1
pGEXhybrid3	6.1 kb, *lacI^q bla hybrid3*	pGEX-6P-1
pGEXhybrid4	6.1 kb, *lacI^q bla hybrid4*	pGEX-6P-1
pGEXhybrid5	6.1 kb, *lacI^q bla hybrid5*	pGEX-6P-1
pGEXhybrid6	6.1 kb, *lacI^q bla hybrid6*	pGEX-6P-1
pGEXhybrid7	6.1 kb, *lacI^q bla hybrid7*	pGEX-6P-1
pGEXhybrid8	6.1 kb, *lacI^q bla hybrid8*	pGEX-6P-1
pGEXhybrid9	6.1 kb, *lacI^q bla hybrid9*	pGEX-6P-1
pGEXhybrid10	6.1 kb, *lacI^q bla hybrid10*	pGEX-6P-1
pGEXhybrid11	6.1 kb, *lacI^q bla hybrid11*	pGEX-6P-1
pMAL-c2[b]	6.6 kb, *lacI^q bla*	New England Biolabs

pMALpenPC	7.8 kb, *lacI^q bla penPC*	pMAL-c2
pMALpc1	7.8 kb, *lacI^q bla pc1*	pMAL-c2
pMALhybrid1	7.8 kb, *lacI^q bla hybrid1*	pMAL-c2
pMALhybrid2	7.8 kb, *lacI^q bla hybrid2*	pMAL-c2
pMALhybrid3	7.8 kb, *lacI^q bla hybrid3*	pMAL-c2
pMALhybrid4	7.8 kb, *lacI^q bla hybrid4*	pMAL-c2
pMALhybrid5	7.8 kb, *lacI^q bla hybrid5*	pMAL-c2
pMALhybrid6	7.8 kb, *lacI^q bla hybrid6*	pMAL-c2
pMALhybrid7	7.8 kb, *lacI^q bla hybrid7*	pMAL-c2
pMALhybrid8	7.8 kb, *lacI^q bla hybrid8*	pMAL-c2
pMALhybrid9	7.8 kb, *lacI^q bla hybrid9*	pMAL-c2
pMALhybrid10	7.8 kb, *lacI^q bla hybrid10*	pMAL-c2
pMALhybrid11	7.8 kb, *lacI^q bla hybrid11*	pMAL-c2
pSG1112	3.5 kb, ϕ105ΔEcoRI/MunI *cat bla*	Lab stock
pSG1113	3.5 kb, ϕ105ΔEcoRI/MunI *cat bla*	Lab stock
pSG1112blipF	4.2 kb, ϕ105ΔEcoRI/MunI *cat bla blipF*	pSG1112
pSG1112blipM	4.2 kb, ϕ105ΔEcoRI/MunI *cat bla blipM*	pSG1112
pSG1113blipF	4.2 kb, ϕ105ΔEcoRI/MunI *cat bla blipF*	pSG1113
pSG1113blipM	4.2 kb, ϕ105ΔEcoRI/MunI *cat bla blipM*	pSG1113

(a): Plasmids based on the pWVO1 origin of replication can replicate in both *E. coli* and *B. subtilis*.

(b): All the plasmids based on the ColE1-type origin (pSG703) or the pBR322 origin (pGEX-6P-1, pMAL-c2, pSG1112, pSG1113) can only replicate in *E. coli*.

2.4 Preparation of chromosomal DNA

The freeze-thaw method was used for the preparation of *Staphylococcus aureus* PC1 (*Staphylococcus aureus* PC1 strain was generously supplied by Prof. Richard Virden) chromosomal DNA. Single colony of *S. aureus* PC1 was transferred from agar plate to a universal bottle containing 5 ml LB medium, and incubated at

37°C overnight. A 1 ml overnight culture was transferred into an eppendorf tube and centrifuged at 12,000 rpm for 1 min. After the supernatant was removed, and added to the cell pellet was resuspended in 400 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The suspension was freezed at -80°C for 20 min, and then was thawed at 50°C for 1 min. The above procedure of freeze and thaw was repeated 6 times/cycles. The cell culture was vortexed 15 se per two cycles. After 6 cycles, cell suspension was heated to 85°C for 5 min, then centrifuged at 12,000 rpm for 15 s. And the supernatant (containing the bacterial chromosomal DNA) was collected and stored at -20°C. A 10 µl of the supernatant was used for agarose gel electrophoresis in order to estimate the concentration of the chromosomal DNA.

Wizard® Genomic DNA Purification Kit (Promega) was used for the preparation of chromosomal DNA from *Stretomyces clavuligerus* (ATCC 27064). Single colony of *S. clavuligeus* was transferred from a nutrient agar plate to a universal bottle containing 5 ml LB medium or #1877 broth (5 g Tryptone and 3 g Yeast extract in 1 liter distilled water), and incubated at 30°C for several days. 1ml of culture was transferred into an eppendorf tube and centrifuged at 12,000 rpm for 1 min. Chromosomal DNA was prepared following the steps described in the manual (Technical Manual No. 050, Promega).

2.5 PCR

All PCR reactions in this study were carried out using the Expand™ High Fidelity PCR System (Boehringer Mannheim). Expand™ High Fidelity PCR System

composed of a unique enzyme mix containing thermostable Taq DNA and Pwo DNA polymerases. This powerful polymerase mixture is designed to give PCR products with high yield, high fidelity and high specificity. For each reaction, 50 μl reaction solution was set up in a sterile microfuge tube according to the manufacturer's instruction as follows: 0.1 - 0.75 μg template DNA, 300 nM dNTPs, 200 μM of each primer, 5 μl 10 x Expand HF buffer with 15 mM $MgCl_2$, 2.6 units of Expand™ High Fidelity PCR System enzyme mix and sterile re-distilled water. After the reaction solution was denatured at 94°C for 5 min, thirty-five cycles of amplification were performed with the following temperatures: 0.5 min at 94°C, 0.5 min at 50°C, 1 min at 72°C. Finally, the ragged ends were filled at 72 °C for 7 min. Throughout this thesis, this setting of cycles was used for the PCR reactions with a few exceptions, which were mentioned in the various chapters.

2.6 Preparation of plasmid DNA

Colonies from plate were inoculated into 5 ml LB medium with an appropriate amount of antibiotic in a universal bottle. When shaken at 300 rpm at 37°C overnight, the culture became saturated. The cells were harvested from the culture and used to isolate plasmid DNA by the Wizard® Plus SV Minipreps DNA Purification System Kit (Promega).

2.7 Restriction enzyme digestion of DNA and electrophoresis

Restriction digestions of DNAs by appropriate restriction enzymes were performed according to the manufacturer's instruction. The digested DNA fragments of the DNA were analyzed by electrophoresis on 0.8% (wt/v) horizontal slab agarose gels containing 0.008% ethidium bromide, with 1 X TBE as the running buffer. And then the DNAs were visualized by UV light using an UV transilluminator, Gel Doc 1000 (BIO-RAD), or Lumi-Imager™ (Boehringer). The 1kb DNA Ladder (Promega) was used as the marker to estimate the sizes of DNA fragments.

2.8 Isolation and purification of DNA fragments

After gel electrophoresis, the agarose gel containing the desired DNA fragment was excised quickly with a scalpel under long-wavelength UV light. The DNA fragment was purified from the excised agarose by using the QIAEX II Gel Extraction Kit (Qiagen) or the Agarose Gel DNA Extraction Kit (Roche) following the manufacturer's instruction.

2.9 Ligation of DNA fragments

For ligation, after the digestions of vector and insert DNA, the restriction enzymes were inactivated at 65°C for 20 min. The vector and insert DNA fragments were recovered and purified from agarose gels and appropriate amount of vector and insert (normally at a molar ratio of 1:3) were used for ligation. Before the addition of T4 DNA ligase, the ligation mixture was heated to 42°C for 5 min and then kept on

ice for 3 min. After the addition of ligase and well mixture, the ligation mixture was incubated overnight at 10°C and then was used for transformation.

For rapid ligation using Quick Ligation™ Kit (New England Biolabs), vector and insert were combined and adjusted to 10 μl with distilled water, and 10 μl of 2 X Ligation Buffer was added. After addition of 1 μl of the Quick T4 DNA ligase, the mixture was centrifuged and incubated at room temperature (25°C) for 5 min. After chilling on ice, the ligation mixture was used for transformation.

2.10 Preparation of *E. coli* competent cells

Strains of *E. coli* XL1-Blue, *E. coli* RR1, *E. coli* DH5α, *E. coli* BL21, and *E. coli* BL21 (DE3) were used in this study. The competent cells of these strains were prepared by the $CaCl_2$ method, which is described as follows.

Overnight culture of *E. coli* was inoculated into 2 x TY medium by 1: 100 dilution, and the fresh culture was incubated at 37°C with shaking at 300 rpm for 2-3 h until A_{600} reached 0.3 - 0.4. After cooling down on ice, the cells were pelleted by centrifugation at 3,000 rpm, 4°C for 15 min. And the cells were resuspended in ice-cold sterile 100 mM $CaCl_2$ solution (using 1/2 of the original culture volume) by pipetting gently and pelleted again by centrifugation. The procedure was repeated once and the cells were stored at 4°C overnight. Finally, the competent cells were pelleted again and resuspended in ice-cold sterile 100 mM $CaCl_2$ (containing 15% glycerol) and aliquoted into sterile eppendorf tubes before freezed rapidly in liquid nitrogen. They were stored at -80°C for future use.

For the preparation of competent cells for electroporation, overnight culture of *E. coli* was inoculated into LB broth by 1: 100 dilution, and the fresh culture was incubated at 37°C with shaking at 300 rpm for 4-5 h until A_{600} reached 0.6 - 0.7. After cooling down on ice for 10 min, the cells were pelleted by centrifugation at 3,000 rpm, 4°C for 15 min. The cells were resuspended in cold sterile Millli-Q water by pipetting gently and pelleted again by centrifugation. The procedure was repeated twice and the cells were resuspended in cold sterile 10 % glycerol and aliquoted into sterile eppendorf tubes (50 μl of cells /tube) before freezed rapidly in liquid nitrogen. They were stored at -80°C for future use.

2.11 Transformation of *E. coli*

The ligation product or plasmid DNA was added into 50 μl thaw competent cells, and mixed thoroughly. The mixture was incubated on ice for 25 min and heat shocked at 42°C for 2 min. An appropriate amount of transformed cells were plated on agar plate containing suitable nutrients and antibiotics and incubated at 37°C overnight.

For electroporation, less than 1 μl of ligation mixture (before transformation, low DNA concentration ligation mixture was precipitated by ethanol using tRNA as carrier and resuspended in sterile nuclease free water) was added to 50 μl of competent cells. These were transferred to the bottom of a pre-chilled electroporation cuvette making that there was no air bubbles trapped. After electroporated at 2.5 kV, 1 ml of 2 X TY (or SOC) was added to the cells before incubating at 37 °C for 30-60

min. A 10 μl or 50 μl portion of cells were spread on the plate containing the appropriate antibiotic. Normally, the transformation efficiency by electroporation is greater than 10^8 colonies/μg supercoiled plasmid DNA.

2.12 DNA sequencing

DNA sequencing in this study was carried out by using the ABI PRISM BigDye™ Terminator Cycle Sequencing Kit (PE Applied Biosystem). ABI PRISM™ 310 Genetic Analyzer (an automated instrument) was used for analyzing fluorescently labeled DNA fragments by capillary electrophoresis. Cycle sequencing is a simple method in which successive rounds of denaturation, annealing, and extension in a thermal cycle result in linear amplification of extension product. AmpliTaq® DNA polymerase, FS is the sequencing enzyme used in the kit. It is a mutant form of *Thermus aquaticus* (Taq) DNA polymerase and contains a point mutation in the active site, replacing phenylalanine with tyrosine at residue 667 (F667Y). This mutation results in less discrimination against dideoxynucleotides, and leads to a much more even peak intensity pattern (Tabor and Richardson, 1995). With dye terminator labeling, each of the four dideoxy terminators (ddNTPs) is tagged with a different fluorescent dye. The growing chain is simultaneously terminated and labeled with the dye that corresponds to that base. The recently developed BigDye terminators are 2-3 times brighter than the rhodamine dye terminators when incorporated into cycle sequencing products. Also, the BigDye terminators have narrower emission spectra than the rhodamine dye terminators,

giving less spectral overlap and therefore less noise. The procedures of DNA sequencing were made as recommended by the kit's manufacturer, which are described in brief as follows. A 8 μl portion of the master mix (from the kit), 0.5 -1 μg of plasmid DNA, 3.2 - 4 pmol of primer and sterile water (which made the total volume up to 20 μl) were mixed into a 0.2-ml PCR tube. We had tried using 5 μl total volume (2 μl of master mix, 2 μl of plasmid containing 0.5 -1ug DNA, 1 μl of 4 pmol/μl primer) to perform sequencing reaction, and obtained almost the same results by increasing the injection time to 60 s. Twenty-five cycles of amplification were carried out in a DNA thermal cycler (GenAmp PCR System 2400 or 9600, Perkin-Elmer) with the recommended condition (96°C 10 s, 50°C 50 s, 60°C 4 min). To remove unincorporated dye, the entire contents of each extension reaction were mixed thoroughly with 2 μl of 3 M sodium acetate (pH 4.8) and 50 μl of 95% ethanol in a 0.5-ml microcentrifuge tube. After incubation at room temperature for 15 min, the extension products were precipitated by centrifugation at 14,000 rpm for 20 min. The supernatant was carefully aspirated with a pipette tip as completely as possible, and the pellet was washed with 250 μl of 70 % ethanol. After the pellet was dried at 90°C for 1 min, 15 – 20 μl template suppression reagent (TSR) was added. Heat denaturation of the products was performed by boiling for 3 min and then ice-chilled. The samples were loaded onto the instrument for automated electrophoresis. The results were analyzed with the ABI PRISM® DNA Sequencing Analysis Software.

2.13 Preparation and transformation of *B. subtilis* competent cells

The method of transformation of *B. subtilis* is based on that of Anagnostopoulos and Spizizen (Anagnostopoulos and Spizizen, 1961).

The recipient strain 168 or 1A304(ϕ105MU331) was streaked on nutrient agar containing 5 μg/ml erythromycin and incubated overnight at 37°C. Cells from the plate were heavily inoculated into a 150ml Erlenmeyer flask containing 5 ml of freshly prepared PTM (pre-transformation medium) so that A_{600} of the culture was within 0.7-0.9. The culture was then incubated at 37°C with shaking at 300 rpm for several h (2.5-3.5 h). When A_{600} of the culture reached 3-3.3, the cells were in stationary phase and were most competent, and this competent state of the cells may only last for about 30 min. The competent cells could also be stored at -80°C by the addition of glycerol to a final concentration of 15%, but the competency may be lower. For transformation, 100 μl of competent cells were transferred to 1 ml pre-warmed TM (transformation medium) in a universal bottle, and an appropriate amount of DNA (1-2 μg) was added into the mixture. The suspension was incubated at 37°C with shaking at 300 rpm for 90 min. Certain amount of the culture was plated on nutrient agar containing an appropriate antibiotic. For the plates containing erythromycin or chloramphenicol, the colonies were usually visible after 2 days' incubation at 37°C.

2.14 Protein expression in *B. subtilis*

After the required gene was subcloned into pSG703, the recombinant plasmid was transformed into *B. subtilis* 1A304(ϕ105MU331). The bacteria which were resistant to chloramphenicol were used for protein expression. The bacteria were inoculated from an agar plate into a 150 ml flask containing 5 ml BHY with 5 μg/ml chloramphenicol. For comparison, cells without the gene of interest were also inoculated into a 150 ml flask containing 5 ml BHY with 5 μg/ml erythromycin. All the cultures were incubated at 37°C with shaking at 300 rpm overnight (for about 11 h). A 1 ml portion of the overnight culture was transferred into other 150 ml flask containing 15 ml BHY without antibiotics added. The flask was then incubated at 37°C with shaking of 300 rpm. A 0.1 ml portion of the culture was removed every h to measure the absorbance at 600 nm. When A_{600} reached 3.1- 4.0, the culture was heat-shocked at 50°C for 5 min with vigorous shaking. The culture was then re-incubated at 37°C with shaking (300 rpm). Sample was taken every h for SDS-PAGE and activity assay to detect the protein expression level.

2.15 Purification of PenPC, HybridF and HybridB by Celite

The purification procedure using Celite is modified from Davies and Abraham (Davies and Abraham, 1974) and similar to that reported by Leung (Leung, 1994) and Cheung (Cheung, 2000).

Dry Celite 545 (40 g) was pre-washed and stirred with 500 ml of dH_2O in a beaker. After completely sedimentation, the small and fine particles were discarded with dH_2O. This step was repeated for 7 times. Culture supernatant with expressed

β-lactamase was poured into the pre-washed Celite and was stirred continually on ice for 20 min. Stirring was then stopped and Celite was allowed to settle down for about 30 min. After the supernatant containing unbound proteins was removed, Celite with bound β-lactamase was then washed with 500 ml of dH_2O for 3-5 times. Finally, enzyme was repeatedly eluted by adding 80 ml elution buffer (100 mM Tris-HCl, 2 M NaCl, 0.1 M tri-sodium citrate, pH 7.0) for 5 times.

All the eluted fractions containig were pooled together and concentrated in a 200-ml ultrafiltrition cell (Amicon Inc., Model 8200) at 4°C under a pressure of 4 bar. YM-1 membrane (Diafco Inc.) was used for retaining β-lactamase during ultrafiltration. Finally, the buffer of concentrated β-lactamase was buffer-exchanged into 20 mM ammonium bicarbonate, and the enzymes were aliquoted and freeze-fried for long-term storage.

2.16 Expression and purification of GST fusion proteins

A single colony of *E. coli* cells containing a recombinant pGEX plasmid were inoculated into 2-100 mL of 2 x TY medium and the culture was incubated at 37°C overnight with vigorous shaking. The overnight culture was diluted 1:100 into fresh pre-warmed 2 x TY medium, and distributed into appropriate flasks and to grow at 37°C with shaking until A_{600} reached 0.5-2 (to ensure adequate aeration, flasks were filled to only 20-25% capacity). IPTG (100mM) was added to a final concentration of 0.1 mM and the culture was continually incubated for an additional 2-6 h. Then, the culture was transferred to appropriate centrifuge bottles or tubes and

centrifuged at 7,700g for 10 min at 4°C to sediment the cells. The supernatant was discarded and the cell pellet was drained and stored at -20°C or -80°C for protein extraction and purification.

For purification, the harvested cells were resuspended in appropriate amount of PBS (20 ml PBS for 400 ml culture). After the addition of DTT (final 5-10 mM), EDTA (final 2 mM) and PMSF (final 1 mM), the suspended cells were sonicated on ice for about 3 min, and 20% triton X-100 was added to a final concentration of 1-2%. After mixed gently for 30 min in a cold room to aid the solubilization of the fusion protein, the suspension was centrifuged at 12,000g for 10 min at 4°C. The supernatant was collected and subjected to protein purification. At the mean time, certain amount of Glutathione Sepharose 4B (1 ml matrix per 8 mg fusion protein) was packed into a column and equilibrated with PBS (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3). After sonication, the extract was clarified by filtration through a 0.45 μm filter before applied to the column. After all sonicate had flowed through the column, the matrix was washed by the addition of 30 bed volumes of PBS. The column was drained and the fusion protein was eluted by the addition of Glutathione Elution Buffer (10mM reduced glutathione in 50 mM Tris-HCl, pH 8.0). Following the elution steps, a significant amount of fusion protein might still remain bound to the matrix. Conditions used for elution might vary among fusion proteins. All eluates were analyzed by SDS-PAGE to estimate the yield and purity, and the fractions containing the required enzyme were pooled together for protease cleavage or other further studies.

2.17 PreScission protease cleavage

In most cases, the fusion partner of interest retains functional activity such that functional test can be performed using the intact fusion with GST. If removal of the GST affinity tail is necessary, fusion proteins containing a PreScission protease recognition site, Leu-Phe-Gln/Gly-Pro (for pGEX-6P vectors), may be cleaved in solution. For cleavage, protease was added to pooled eluates (10 units of protease for per mg fusion protein), and the solution was mixed gently and incubated at 4°C for 2-16 h. During these procedures, samples should be removed at various time and analyzed by SDS-PAGE to estimate the extent of digestion. The amount of protease, temperature and length of incubation required for complete digestion of a given GST fusion protein varies. Optimal conditions for each fusion should be determined in pilot experiments. Once digestion was complete, GST and PreScission protease [which is a fusion protein of GST and human rhinovirus (HRV) type 14 3C protease, Walker *et al.*, 1994] can be removed by first removing gluthathione by extensive dialysis (e.g. 2000 volumes) against PBS followed by column purification on Glutathione Sepharose 4B. The purified protein of interest would be found in the flow-through.

2.18 Expression and purification of MBP fusion protein

For expression, bacteria transformed with recombinant plasmids were grown in 200 ml 2 x TY medium with 0.1 mg/ml ampicillin and 0.2% glucose to an A_{600} of

0.6-0.8 at 37°C. Induction was performed with 0.2 mM isopropyl β-D-thiogalactoside (IPTG) at 37°C. Bacteria were harvested 3 hours latter after induction by centrifugation at 6,000 g for 20 min and stored at –80°C. For purification, 100 ml MBP column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4) was added to the cell pellet obtained from 400 ml of culture. After the addition of DTT (final 2-5 mM) and PMSF (final 1 mM), the suspended cells were lysed with a probe sonicator for about 5 min. The lysate was centrifuged at 18,000 rpm for 1 h and was subjected to amylose affinity column. The supernatants containing the MBP fusion proteins were loaded onto a 16 mm x 60 cm column packed with amylose affinity gel (New England Biolabs) and washed with 2 column volume of the binding buffer. Elution was performed with a maltose gradient (0-10 mM). Fractions of 6 ml were collected. All the steps were conducted at 4 °C.

2.19 Protein gel electrophoresis (SDS/PAGE)

SDS/PAGE is based on the method of Laemmli (Laemmli, 1970). Proteins were separated by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) in a Mini-PROTEIN II dual slab cell (Bio-Rad). Each polyacrylamide gel was a discontinuous composite gel containing a short stacking gel of low % T (3-4%) layered on top of a long resolving gel of higher % T and was subjected to electrophoresis in Laemmli running buffer at 200 V for about one h. The low-range protein marker from Bio-Rad was run with the samples at the same time and was used to estimate the molecular weight of protein bands. When the

electrophoresis was finished, the gel was stained in Coomassie Bule R-250 staining solution (25% methanol, 10% acetic acid and 0.06% Coomassie Blue R-250) for about 0.5 h with agitation and then was destained in destaining solution (10% acetic acid and 10% methanol) until the background of the gel was clear. After soaked in water for several h, the gel was dried by the use of the gel drying films for record.

2.19 Determination of protein concentration

The yield of fusion protein can be estimated by measuring the absorbance at 280 nm. The GST tag can be approximated by 1 $A_{280} \approx 0.5$ mg/ml (This is based on the extinction coefficient of the GST monomer using a Bradford assay). Concentrations of the β-lactamase can be determined by using the absorption coefficient 1.95 x 10^4 $M^{-1}cm^{-1}$ at 276.5 nm (Carrey and Pain, 1978). However, the BIO-RAD Protein assay, based on the method of Bradfold, was widely used to determine the concentrations of protein in this study according to the manufacturer's instruction.

2.20 Enzyme kinetics

Kinetic measurements were made in a Perkin-Elmer Lambda Bio20 (or Lambda35) UV/visible spectrometer. All assays were performed at 25°C in 0.05 M sodium phosphate buffer at pH 7.0. Initial velocities of substrate hydrolysis were monitored by loss of UV absorption for penicillin G ($\Delta\varepsilon_{232} = 755$ $M^{-1}cm^{-1}$), penicillin V ($\Delta\varepsilon_{232} = 940$ $M^{-1}cm^{-1}$), ampicillin ($\Delta\varepsilon_{235} = 820$ $M^{-1}cm^{-1}$), oxacillin ($\Delta\varepsilon_{260} = 1{,}010$

$M^{-1}cm^{-1}$), methicillin ($\Delta\varepsilon_{235}$ = 960 $M^{-1}cm^{-1}$), 6-APA ($\Delta\varepsilon_{240}$ = 397 $M^{-1}cm^{-1}$), cephaloridine ($\Delta\varepsilon_{260}$ = 10,700 $M^{-1}cm^{-1}$), cefuroxime ($\Delta\varepsilon_{260}$ = 7,600 $M^{-1}cm^{-1}$) and by the increase in absorbance at 500 nm for nitrocefin ($\Delta\varepsilon_{500}$ = 15,900 $M^{-1}cm^{-1}$). The wavelength for measuring the enzyme activity for specific substrate is the wavelength at which the difference in absorbance between substrate and product is maximum. The kinetic parameters were determined from measurements of initial rates (from the first 5% of the reaction) by fitting the Michaelis-Menten equation with the non-linear regression program of EnzymeKinetics version 1.1. Enzyme Kinetics version 1.1, which was written by Don Gilbert, is a Hypercard stack for Macintosh computers. When the value of K_m was too high or below 10 μM, it was measured as a K_i with nitrocefin as the reporter substrate (De Meester *et al.*, 1987). Dilutions of the enzymes below a concentration of 0.1 mg/ml were performed with buffer solutions containing 0.1 mg/ml bovine serum albumin. Nitrocefin is hard to dissolve in assay buffer, nitrocefin stock solution was prepared in DMF by the ratio of 0.0015 g powder in 200 μl of DMF. The stock solution can be kept at –20°C for one month. Before use, 210 μl of stock solution was added slowly (drop by drop) into 5.79 ml of assay buffer. The final concentration of nitrocefin for use was calculated from the measurement of the value of A_{386} (ε = 19.2 $mM^{-1}cm^{-1}$). The nitrocefin solution can only be used for one day and has to be freshly prepared from the stock solution.

2.21 Thermal stability of β-lactamases

The enzymes were incubated at various temperatures in assay buffer containing 0.1 mg/ml bovine serum albumin. Samples were withdrawn after various periods of time and the residual activity was determined with penicillin G, cephaloridine or nitrocefin as substrate at 25 °C.

2.22 Circular dichroism

Circular dichroism (CD) spectra were measured with a Jasco 810 spectropolarimeter using a water-jacketed cylindrical cell with a path length of 1.0 mm. Temperature control was provided by a Neslab RTE-211 circulating water bath interfaced with a MTP-6 temperature programmer. The protein samples were dissolved in 10 mM potassium phosphate (pH 7.0) and their concentrations ranged from 0.25 mg/ml to 0.5 mg/ml. Thermal denaturation of β-lactamases was monitored by measuring the far-UV molar ellipticity at 222 nm. Data were collected as a function of temperature with a scan rate of 1°C/min over the range of 20-90 °C.

2.23 Site-directed mutagenesis

Site-directed mutagenesis was carried out by the use of QuickChange™ Site-directed Mutagensis Kit (Stratagene). This method allows the rapid introduction of point mutation into sequences of interest, using a pair of complementary mutagenesis primers to amplify the entire plasmid in a single PCR reaction. Destruction of the parental template plasmid by DpnI digestion, followed by transformation into *E. coli* cells, allows introduction of the desired mutation with high efficiency (70-90%), in

as little as 24 h. Two mutagenesis primers, which contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid, were designed to make sure that the melting temperature (T_m) was greater than or equal to 78 °C according to the following formula: $T_m = 81.5 + 0.41$ (%GC) – 675/N - % mismatch (where N is the primer length in base pairs), even if this makes the primers 60 bp long. The reaction was prepared by mixing the following ingredients: 5 μl of 10X reaction buffer, 125 ng of the two primers, 10-100 ng of template DNA, 1 μl of dNTP mix, and sterile ddH_2O to a final volume of 50 μl. After the mixture was preheated at 95°C for 5 min, 1 μl of *PfuTurbo* DNA polymerase (2.5 U/μl) was added to start the PCR reaction using the following setting: 95°C for 30 s, 52°C for 1 min, 68 °C for several min (2 min/kb of plasmid length). The number of cycles depends on the type of mutation desired: 12 for point mutation, 16 for single amino acid changes, and 18 for multiple amino acid deletions or insertions. During thermal cycling, *Pfu* DNA polymerase extended and incorporated the mutagenic primers and resulted in nicked circular double stranded DNA. After 10 μl of the amplified product was checked by electrophoresis on agarose gel, 1 μl of DpnI (10 U/μl) was added to certain amount of amplification product and incubated at 37 °C for 1 h to digest the parental supercoiled dsDNA. Then digested DNA was transformed into *E. coli* XL1-Blue competent cells. Transformants were screened from LB plate with 100 μg/ml of ampicillin.

When longer primers are attempted, the mutagenesis efficiency is drastically decreased, largely because of the more favorable primer dimer formation (100%

complementary), compared with the primer-template annealing. A simple modification, two-stage PCR mutagenesis protocol, was used to address the problem (Wang and Malcolm, 2002).

Additionally, primers with a very low %GC and long stretches of A's and T's will most likely lead to secondary structure. The possible formation of primer secondary structure may be limited by performing a two-step PCR (as opposed to a traditional three-step PCR) as follows: 95°C for 30 s, 68°C for a few min, which is suggested by Dr. James Hatteroth (Company's technical support scientist, personal communication)

2.24 β-Lactamase inhibition assays

β-Lactamase inhibition assays were conducted as described by Doran *et al.* (1990), using Penase concentrate (Bacto™, BD Biosciences) as the source of β-lactamase and nitrocefin (or penicillin G) as the substrate. One unit of BLIP was defined as the amount of material that would give 50% inhibition of 2×10^4 U of Penase used in the spectrophotometric β-lactamase inhibition assay. For the assay, certain amount of Penase concentrate was incubated with BLIP sample at 25°C for 5 min, and then the residual activity of Penase was determined with Penicillin G or nitrocefin as substrate.

2.25 Softwares

Whiles the normal processing of experimental data was carried out by Microsoft Excel, Graph Pad PRISM® (VERSION 3.0) was used for data fitting and the calculation of the standard deviation. For kinetic study, the slope of the straight line in the progress curve was obtained by the KinLab program (supplied by Perkin Elmer) and kinetic parameters such as K_m and k_{cat} were calculated by computer program EnzymeKinetics (Version 1.1, Trinity Software). The normal analysis of DNA and protein was performed using computer program Gene Runner (Version 3.05), which was free downloaded from the website (www.generunner.com). The alignment of DNAs or proteins was made by the use of computer programs Cluster W (Multiple sequence alignment) and GeneDoc. Cluster W was developed by Kyoto University Bioinformatics Center and was free downloaded from the website (www.clustalw.genome.ad.jp). GeneDoc is also free software and was downloaded from the website (www.psc.edu/biomed/genedoc).

Chapter Three

Generation of hybrid β-lactamases

3.1 Introduction

Site-directed mutagenesis has become one of the most commonly used methods in molecular biology. The site-directed mutagenesis of one or several amino acid side chains in an enzyme guided by X-ray crystallographic data and followed up by a detailed mechanistic data analysis can yield unique insight into structure-function relationships of protein. It is apparent, however, that an enormous amount of structure-function information is essentially inaccessible through this high-definition methodology (Armstong, 1990). The major limitation of site-directed mutagenesis is that it can only generate one specific mutation at a time which means that important amino acid can only be discovered slowly. What is more, simple mutations generally are not expected to drastically alter an enzyme's substrate recognition pattern, as many amino acid residues which often not close to one another in the primary structure may affect the binding pocket of the substrate in the enzyme. In contrast, the accelerated exploration is possible by hybrid enzyme approach, which involves replacing chunks of protein primary sequence by formation of hybrid enzymes between two evolutionarily related proteins and results in multiple mutations to a section of the structure thought to be important to a particular function. The avowed objective of this experimental exercise is to assess whether a particular structural module can confer a defined functional characteristic of the donor to the newly constructed chimeric or

hybrid enzyme. The replacement of defined structural motifs or regions of primary structure with related sequences is as reasonable as the site-directed mutagenesis of a single amino acid side chain but the changes are more complicated.

A library of chimeric genes encoding hybrid enzymes can be generated by recombination between two partially homologous genes either *in vitro* or *in vivo*. It was proved that the hybrids generated by *in vitro* homologous recombination might contain deletions or rearranged sequences, whereas the *in vivo* process is both more efficient and simpler to carry out (Weber and Weissmann, 1983).

Two methods of *in vivo* homologous recombination can be used to generate libraries of chimeric genes encoding hybrid enzymes. Hybrid genes have been efficiently assembled in *E. coli* (Weber and Weissmann, 1983; Rey *et al.*, 1986; Abastado *et al.*, 1987), *B. subtilis* (Conrad *et al.*, 1995) and *Saccharomyces cerevisiae* (Pompon and Nicolas, 1989) by intramolecular recombination within homologous regions of the two parent genes (Figure 3.1a). Chimeric genes also have been constructed both in *E. coli* (Schneider *et al.*, 1981) and in yeast (Orr-Weaver and Szostak, 1983; Pompon and Nicolas, 1989) by intermolecular recombination (Figure 3.1b). Both of the two methods offer the potential to generate libraries of chimeric genes that can be used for further studies to map functional residues or regions of the polypeptides.

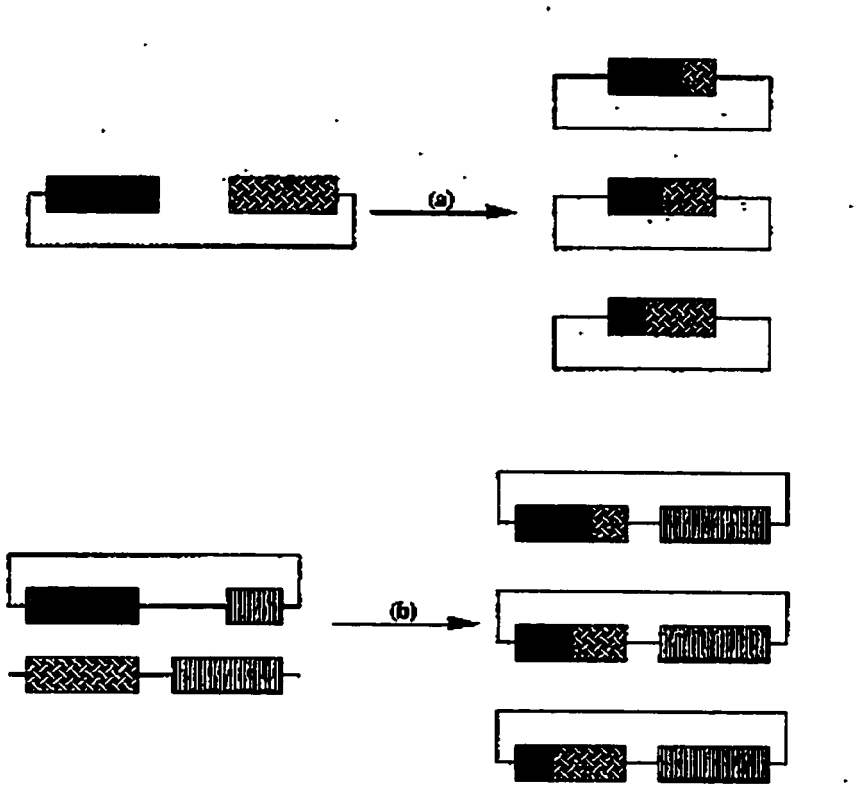


Figure 3.1 Construction of hybrid genes by homologous recombination.Libraries of hybrid genes were constructed by (a) *in vivo* intramolecular recombination between partially homologous genes located at the same linear DNA and (b) intermolecular recombination, which requires two crossover events, one between the two partially homologous genes and the other one between the selective marker (e.g., the chloramphenicol resistance gene) sequences.

The *penPC* gene and the *penP* gene share 61% sequence identity. In our previous studies (Leung, 1994), five hybrids (Hybrids A-E, Figure 1.9) were

successfully constructed between the two genes by *in vivo* homologous recombination in *B. subtilis* 168, and all these hybrids showed enzymatic function. Surprisingly, for unknown reasons, no hybrid β-lactamase could be created by intermolecular homologous recombination. Hybrid genes were successfully obtained by intermolecular recombination in *E. coli* between two *trpA* genes of *E. coli* and *Salmonella typhimurium* (Schneider *et al.*, 1981), which share 75% identity. The lower identity may account for the lack of crossing over between *penPC* and *penP*.

In the present study, hybrids from crossing between *penPC* and *pc1* (the two genes share 53% identity), as well as hybrids from crossing between *penPC* and *penP*, were generated by *in vivo* intramolecular recombination in *E. coli* RR1 and have been used for the study of the structure-function relationships of β-lactamase.

3.2 Construction of a recombinant plasmid containing two partially homologous β-lactamase genes

While pYCL18, which was constructed by the insertion of the *penPC* gene and *penP* gene into plasmid pGK13 (Leung, 1994), was directly used for the generation of hybrid β-lactamases in *E. coli* RR1, a new recombinant plasmid (pHBL1) containing *penPC* and *pc1* was constructed from pYCL18 for the generation of hybrid β-lactamase genes from *penPC* and *pc1*.

The chromosomal DNA of *Staphylococcus aureus* PC1was prepared by the

freeze and thaw method. Two primers, LHB1 (5'-cataa**AGATCT**attgacaccgatatta caattg-3') and LHB2 (5'-cggac**ATCGAT**gtcaacgataatacaaaatataatac-3'), were designed to amplify the *pc1* gene from the upstream of the start codon to the downstream of the transcriptional terminator by PCR. The PCR product and the pYCL18 plasmid DNA were digested by *Bgl*II and *Cla*I, and then the two digested fragments were purified and ligated together. The correct recombinant plasmids were screened by restriction enzymes digestions (Figure 3.2) and further confirmed by DNA sequencing.

3.3 Generation of hybrid β-lactamases by *in vivo* intramolecular homologous recombination

Both plasmids pYCL18 and pHBL1 carry two β-lactamase genes (*penPC* and *penP* for pYCL18, *penPC* and *pc1* for PHBL1) with the *cat* gene in between to facilitate characterization. They also contain the *ermC* gene, which confer the resistance for erythromycin (Figure 3.2). The initial plasmids confer Er^RCm^R. There are several restriction enzyme sites at the region between or in the two β-lactamse genes, which may be used to linearize the plasmids for intramolecular recombination. To generate hybrid genes by intramolecular homologous recombination, linearized plasmids were transformed into *E. coli* RR1 competent cells, with selection for Er^R.

Er^R transformants were examined for the absence of the *cat* gene, which is lost upon homologous recombination between *penPC* and *penP* or *pc1*. Recombination would arise via a "snail" intermediate following a crossover event at any site with sufficient homology. All hybrid genes generated would have their N-terminal coding moiety derived from *penPC* and their C-terminal coding moiety derived from *penP* or *pc1* (Figure 3.3).

Different enzyme digestions were used to linearize pYCL18 and pHBL1 before transformation was performed. The locations of the restriction enzymes for pYCL 18 and pHBL1 were shown in Figure 3.2. The transformation efficiency of the competent *E. coli* RR1 was about 10^7colonies/ug pUC18 or 10^6cononies/ug pGK13. Plasmids from Er^RCm^S transformants were prepared and analyzed for restriction enzyme digestions and finally confirmed by DNA sequencing for correct hybrids.

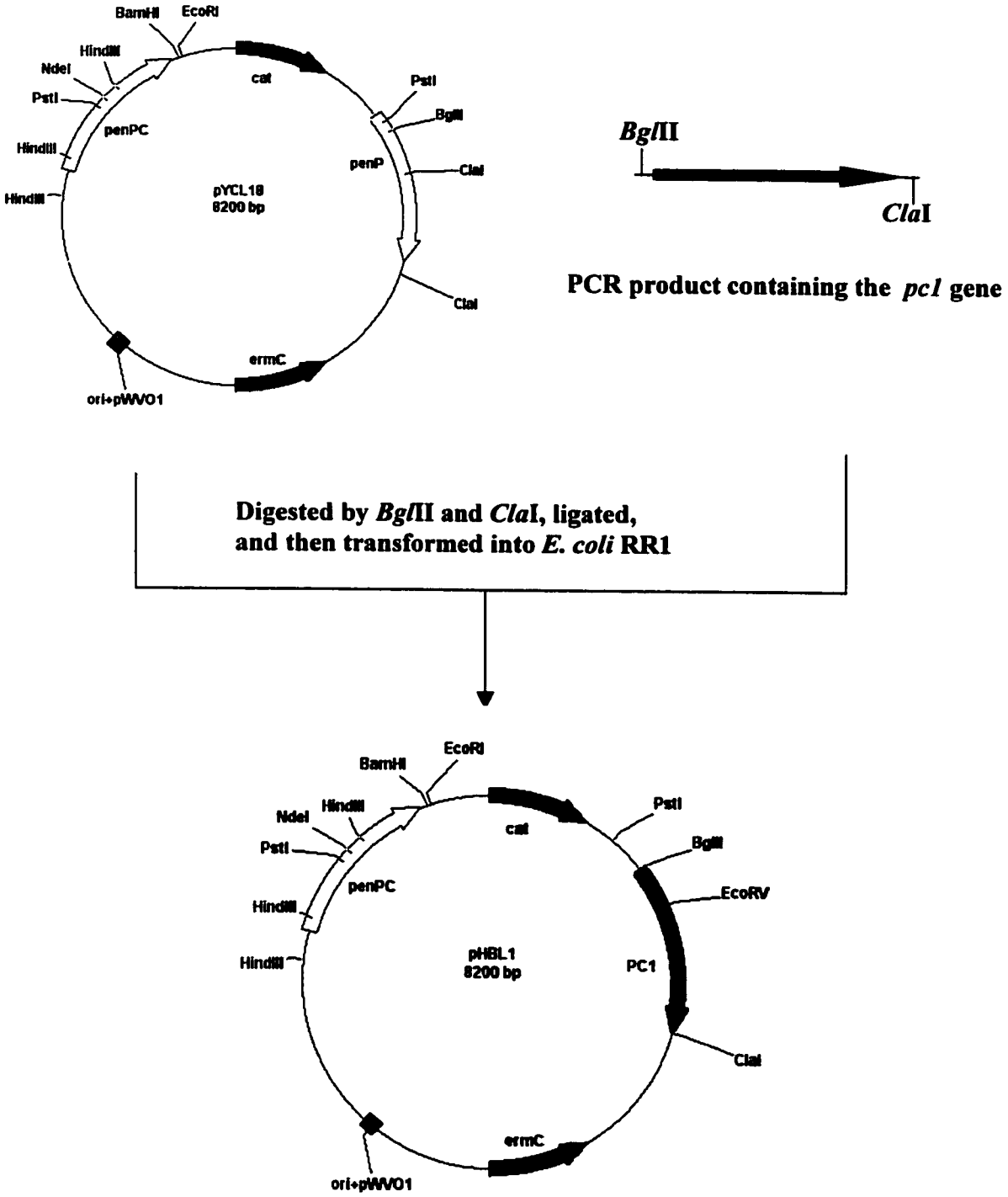


Figure 3.2 The construction of plasmid pHBL1. Plasmid pYCL18 and PCR product containing *pc1* gene were digested by *Bgl*II and *Cla*I. Digested fragments were purified and ligated together. The ligation product was transformed into *E. coli* RR1. The correct recombinant plasmids were screened by restriction enzyme digestions and DNA sequencing.

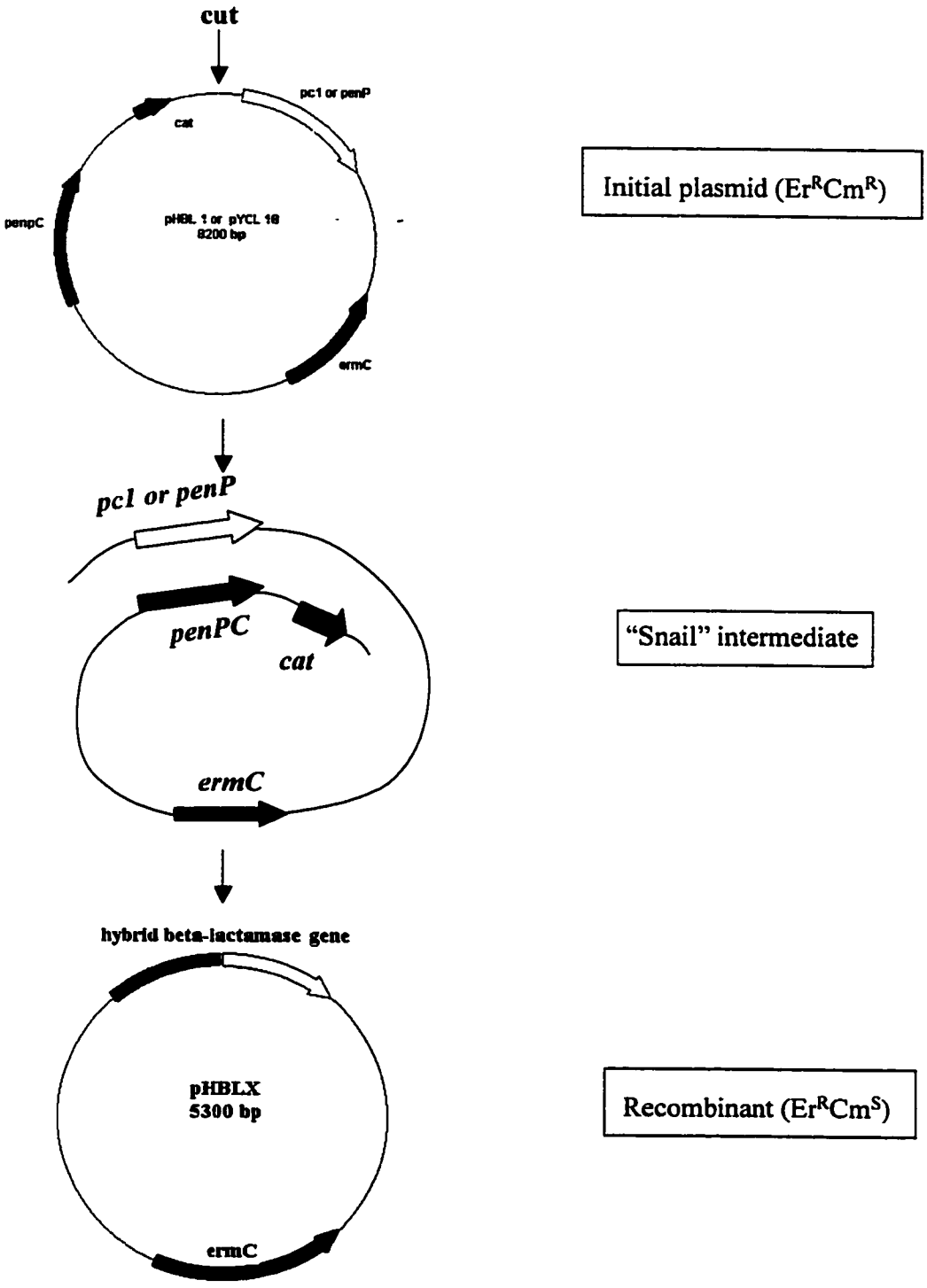


Figure 3.3 Intramolecular homologous recombination in *E. coli* RR1 for the generation of hybrid β-lactamase genes. The initial plasmid confers Er^RCm^R. To generate hybrid genes, linearized plasmids were transformed into *E. coli* RR1 (recombination positive) cells, with selection for Er^R. Er^R transformants were examined for the absence of the *cat* gene, which was lost upon homologous recombination between *penPC* and *pc1* or *penP*. Recombination would arise via a "snail" intermediate following a crossover event at any site with sufficient homology. All hybrid genes generated would have their N-terminal coding moiety derived from *penPC* and their C-terminal coding moiety derived from *pc1* or *penP*.

3.4 Restriction analysis and DNA sequencing of hybrid β-lactamase genes

Because the *penPC* gene and *pc1* gene only share 53% identity, transformation of linear pHBL1 mainly resulted in self-ligation of the linear DNA, the efficiency of homologous recombination was less than 30%. Therefore, a large number of colonies had to be screened in order to obtain the hybrid genes. *Cla*I was used to linearize the plasmids for the estimation of the size of the plasmid DNA. The predicted size of real hybrid plasmids is 5.3 kb.

Considered that there are three *Hind*III sites in pHBL1, one at the upstream of the *penPC* gene and two inside the *penPC* gene (Figure 3.2). The crossover site can be roughly decided by *Hind*III digestion. One band was seen if the recombination happened between the first and the second sites of *Hind*III after enzyme digestion, two bands were seen if the recombination happened between the second and the third sites, while three bands were seen if the recombination happened at the site downstream the third *Hind*III site. Figure 3.4 shows the electrophoresis results of pHBL1 and hybrid plasmids by *Cla*I and *Hind*III after restriction enzyme digestions.

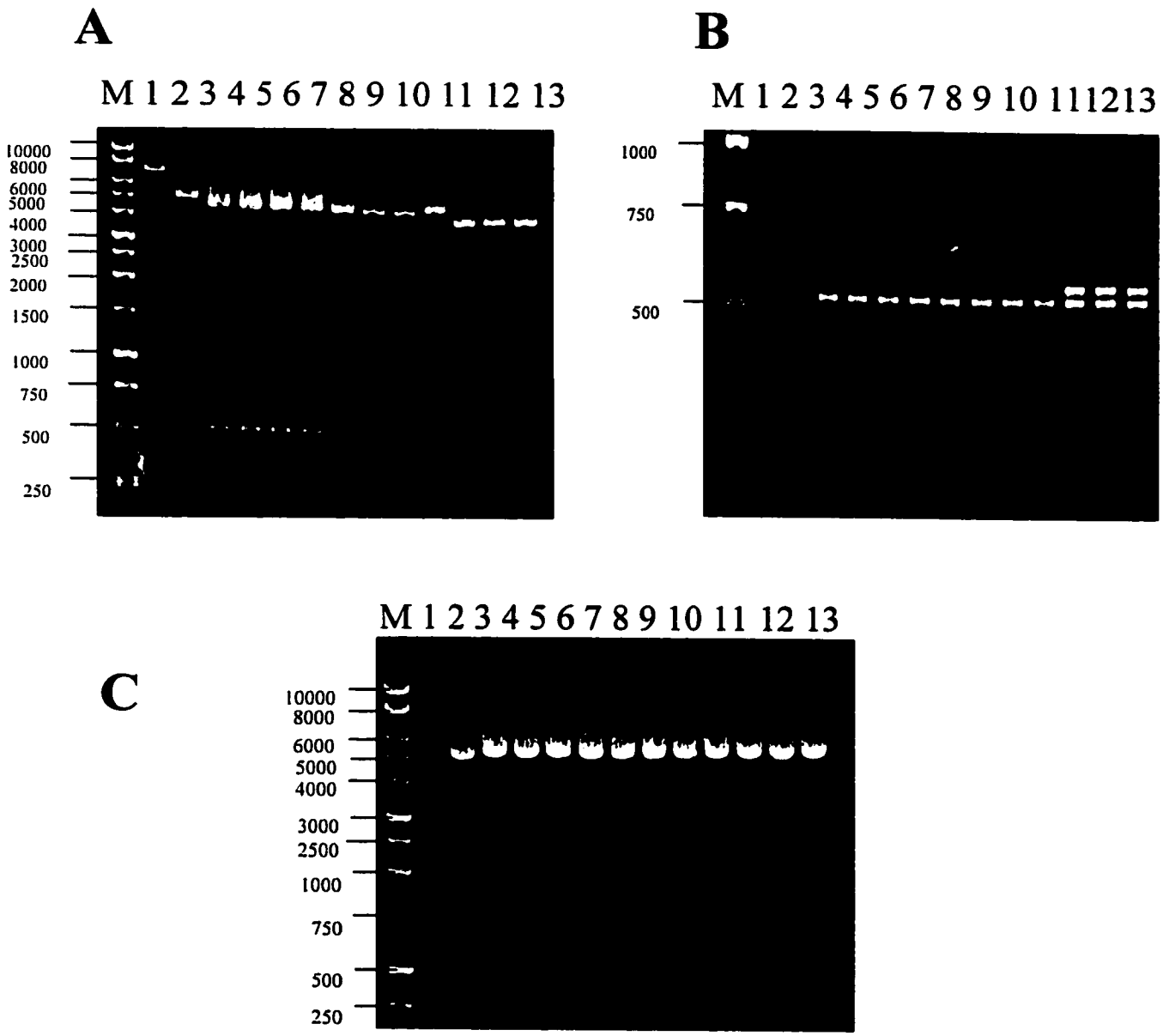


Figure 3.4 Gel electrophoresis of *Hind*III digested plasmids using 0.8% agarose gel (A) or using 1.6% agarose gel (B) and *Cla*I digested plasmids in 0.8% agarose gel (C). 1 kb DNA ladder (Promega) was loaded as marker (M) to estimate the size of DNA fragments. Lanes 1 to 13 represented the samples from plasmids pHBL1, pHBL2, pHBL3, pHBL4, pHBL5, pHBL6, pHBL7, pHBL8, pHBL9, pHBL10, pHBL11, pHBL12 and pHBL13 respectively No sample was loaded onto the Lane 2 in (C).

3.5 DNA sequence analysis of hybrid β-lactamase genes

To determine the precise crossover regions of the hybrid β-lactamase genes, the potential plasmids, which had been analyzed by restriction enzyme digestions, were futher confirmed by DNA sequencing, using primers LHB2, LHB3 (5'-gattgtctattatgtgtacg-3', for sequencing 5' of *penPC*), and LHB4 (5'-gagaaacatg tagatactgg-3', for sequencing the middle part of hybrid plasmids) for hybirds derived from pHBL1, or using primers LHB3, LHB4, LHB9 (5'-gcctg**GTCGAC**aatgata tgagcttgatca-3', for sequencing 3' of the *penP* gene and amplification of *hybridF* gene) for hybrids derived from pYCL18. The results were summarized in Table 3.1. The DNA sequences of crossover regions of different β-lactamase hybrid genes were also listed in Table 3.2, the length of identical region for *in vivo* homologous recombination varied from 4 to 14 nucleotides. The AT content of the region was found quite high, range from 40% to 100%.

Table 3.1 Hybrid genes obtained from intramolecular homologous recombination

Linear DNA used for transformation	Hybrid genes obtained
*Bgl*II digested pYCL18	*hybridA hybridB hybridC*
	hybridD hybridE hybridF
*Pst*I digested pYCL18	*hybridA*
*Pst*I and *Bgl*II digested pHBL1	*hybrid12 hybrid1 hybrid2 hybrid3*
	hybrid4 hybrid5 hybrid6 hybrid7
*Bgl*II digested pHBL1	*hybrid1 hybrid4*
*Bam*HI digested pHBL1	*hybrid1 hybrid3*
*EcoR*I and *EcoR*V digested pHBL1	*hybrid7 hybrid8 hybrid9 hybrid11*
*EcoR*I and *Bgl*II digested pHBL1	*hybrid10*

Table 3.2 DNA sequences of crossover regions of different β-lactamase hybrid genes

Hybrid gene	Origin	Crossover sequence	Length of sequence	AT% of sequence
hybrid A	*penPC* x *penP*	AACTTGAG	8	50
hybrid B	*penPC* x *penP*	ACTTCCA	7	57.1
hybrid C	*penPC* x *penP*	TGGATGAAA	9	66.7
hybrid D	*penPC* x *penP*	AATGA	5	60
hybrid E	*penPC* x *penP*	ATTGCAGAGGCAAC	14	50
hybrid F	*penPC* x *penP*	AAAT	4	100
*hybrid*1	*penPC* x *pcI*	AAAAAAT	7	100
*hybrid*2	*penPC* x *pcI*	ATGCTC	6	50
hybrid 3	*penPC* x *pcI*	GGTGT	5	40
hybrid 4	*penPC* x *pcI*	GATACT	6	66.7
hybrid 5	*penPC* x *pcI*	AGATTTGCCT	10	60
hybrid 6	*penPC* x *pcI*	ATGTAG	6	66.7
hybrid 7	*penPC* x *pcI*	AGTGATAATAC	11	72.7
hybrid 8	*penPC* x *pcI*	AGAATTAAA	9	88.9
hybrid 9	*penPC* x *pcI*	AAAAC	5	80
hybrid 10	*penPC* x *pcI*	AAAATTCTTAC	11	81.8
hybrid 11	*penPC* x *pcI*	TTGCT	5	60
*hybrid*12	*penPC* x *pcI*	TTGAAAAA	8	87.5

From *penPC* and *penP*, in addition to hybridA-E, which had been generated in *B. subtilis*, a new hybrid (hybridF) was produced by homologous recombination in *E. coli* RR1 (Figure 3.5). From *penPC* and *pc1*, twelve hybrids were generated, the crossover regions of these hybrids are showed in Figure 3.6. The crossover site of *hybrid12* is located at the begining code of *pc1*, while it encodes the whole PC1 β-lactamase including the signal peptide, the upstream of the start codon was from *penPC* gene. Therefore, it may be used for the study of whether the promoter and ribosome binding site of the *penPC* gene is functional or not in PC1 protein expression. The coding frame of the regions where crossover events happen for the generation of hybrid9 and hybrid10 are different in *penPC* from that in *pc1*, therefore, recombination generated a new stop codon "TGA" downstream of the crossover sites in hybrid gene hybrid9 or hybrid10.

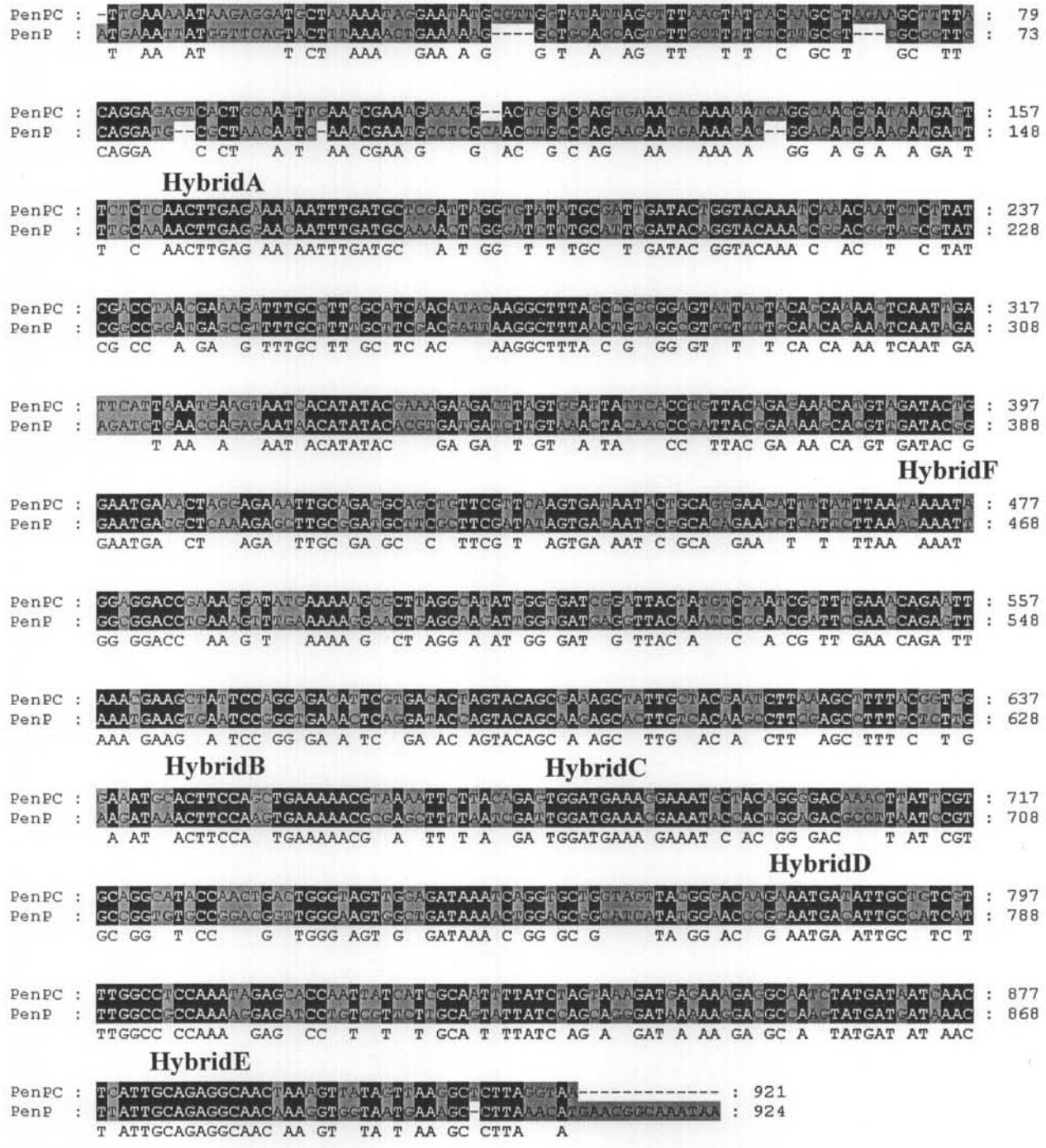


Figure 3.5 The crossover region locations of the six hybrid genes obtained from the *penPC* and *penP*, which were generated by *in vivo* intramolecular homologous recombination in *E. coli* RR1. The two genes (*penPC* and *penP*) share 61% nucleotide sequence identity. Conserved nucleotides are indicated by black box. The DNA sequences are found in Genebank at NCBI. The alignment of the DNA sequence was performed with Clustal W.

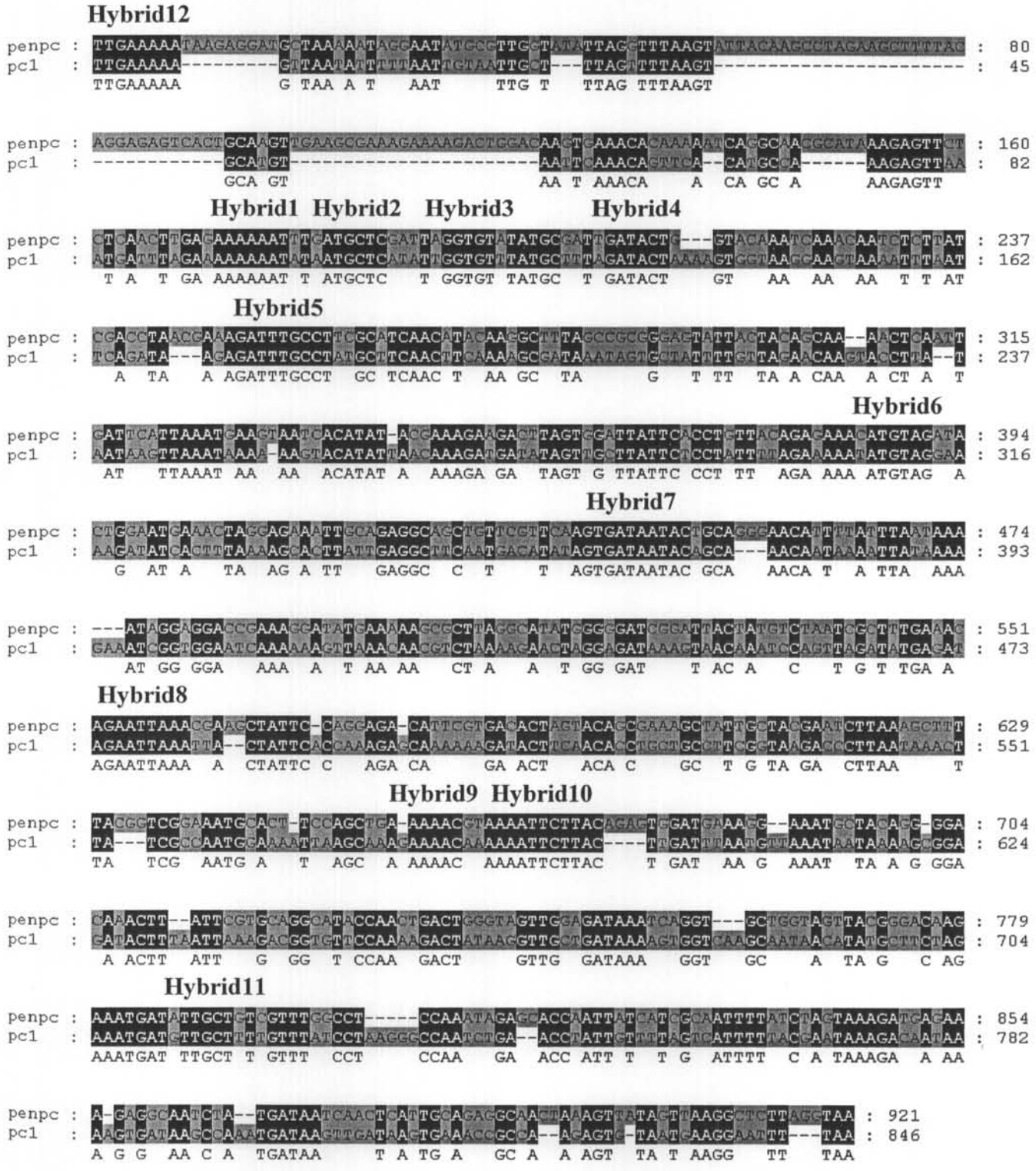


Figure 3.6 Locations of the twelve different crossover regions between the *penPC* and *pc1* genes as determined by DNA sequencing. The two genes share 53% nucleotide sequence identity. Conserved nucleotides are indicated by black box. The DNA sequences are found in Genebank at NCBI. The alignment of the DNA sequence was performed with Clustal W. The hybrid genes were generated by *in vivo* homologous recombination in *E. coli* RR1. Recombination results in a new stop codon "TGA" downstream of the crossover sites in *hybrid9* or *hybrid10*. Other hybrid genes do not have any changes in coding frame downstream of the crossover sites.

3.6 Discussion

From this study, we found that most of the plasmids (>90%) prepared from the colonies transformed with single cut pHBL1 were identical to pHBL1, which were probably due to plasmid molecules that escaped cutting by the restriction enzyme or were end-to-end ligated after the transformation. However, even when two restriction enzymes (e.g. *EcoR*I and *Bgl*II) were used to linearize pHBL1, about 70% plasmids were still found to be self-ligated. It is amazing that noncompatible DNA ends can be ligated together *in vivo.* However, single cut pKSY2 DNA (containing two pyridoxal kinase genes with more than 80% identity) resulted in the yield of almost 100% recombinants in transformants and single cut pYCL18 resulted in the yield of more than 90% recombinants. Therefore, it was proposed that both self-ligation and homologous recombination would compete with each other when linear DNAs were transformed into the cells of *E. coli* RR1. The more sequence identity between the two concerned genes, the higher frequency of homologous recombination, and lower frequency of end-to-end ligation. Considering the notion that the frequency of intermolecluar recombination is a function of the sequence similarity between the genes concerned (Leung, 1994), the same situation may also exist for intramolecular recombination. Therefore, it is concluded that the frequency of homologous recombination is a function of the sequence identity between the genes concerned.

Because two crossover events are needed for intermolecular recombination, it is reasonable that the two genes for intermolecular recombination require more sequence identity than the two genes for intramolecular recombination. This may be the reason why no hybrid β-lactamase genes were created by intermolecular recombination even with the stimulation of double-strand breaks inside the homologous region of the *penPC* gene while hybrid genes were successfully obtained by intramolecular recombination (Leung, 1994). We also propose that 53% sequence identity may be marginal for performing intramolecular homologous recombination between the genes of interest.

Abstado *et al.* (1987) suggested that double-strand breaks inside a region of homology stimulate recombination. Such stimulation of recombination was very obvious in this study when the linear pHBL1, cut by *Pst*I and *Bgl*II, was used to generate hybrid β-lactamase genes. There are two *Pst*I sites in pHBL1. One is upstream of the *pc1* gene, the other is inside the *penPC* gene. The region containing *Pst*I cutting site is highly homologous with *pc1*. Consequently, eight of twelve hybrid genes between *penPC* and *pc1* were generated by the transformation with this linear pHBL1. Our results strongly support this hypothesis, which should be an important guideline when experiments are designed to generate hybrid genes.

Abstado *et al.* (1987) also suggested that recombination was most frequent

near the homologous end, and the frequency decrease with the distance. In our studies, when linear pHBL1 digested by *Pst*I and *Bgl*II was used to transform *E. coli* RR1, about half of the recombinants were proved to be hybrid7. As the crossover site of hybrid7 is very near to the *Pst*I site of *penPC*, our observation is fully consistent with the first part of the suggestion. However, the gradient of recombination frequencies related to the location of the break was not observed in our study. However, when we tried to generate more hybrid β-lactamase genes between *penPC* and *penP*, linear pYCL18 digested by *Pst*I was transformed into competent cells. Surprisingly, all recombinants were found to be *hybridA*, the crossover site (5'-AACTTGAG-3') of *hybridA* is very far from the PstI site. More strikingly, even the neighbor homologous region of *hybridA* (5'-AATTTGATGC-3') is more homologous, no crossover event happened there at all. One possible explanation is that the crossover site of *hybridA* is a particular hot spot of recombination, with sequence different from the octameric Chi (χ) seqnence (5'-GCTGGTGG-3'). The Chi sequence is a recombination hot spot in *E. coli*. Recent studies (Eggleston and West, 1997) suggest a singular mechanism by which χ regulates not only the nuclease activity of RecBCD enzyme, but also the ability of RecBCD to promote loading of the stand exchange protein, RecA, onto χ-containing DNA. The mechanism by which the sequence (5'-AATTTGATGC-3') may also function as a recombination hot spot is still unclear. From our results, the

distribution of crossover sites over the homologous regions of two genes is almost systematical but nonrandom. This leads us to believe that crossover loci are not strictly a function of local DNA homology, but may reflect the mechanism of recombination in this system, which is under investigation.

In summary, even though the molecular details of the recombination processes are still far from being completely deciphered, homologous recombination is proved to be a very powerful approach to generate hybrid genes between two partially homologous genes. For protein engineering, it is very useful to construct enzymes with altered properties and to identify the sequences that may correlate with changed properties, such as thermostability and substrate specificity.

Chapter Four

Expression of β-lactamases in *Bacillus subtilis*

4.1 Introduction

A broad range of prokaryotic and eukaryotic cells have been used through genetic engineering for the overproduction of proteins, which are widely used for research and industry. Among them, *Bacillus subtilis* may be an optimal host for many applications. *Bacillus subtilis* is a very attractive host for the production of heterologous secretary proteins (Wong, 1995).

It is capable of secreting functional extracellular proteins directly to the culture medium. Product recovery is thereby greatly simplified compared with high-level intracellular expression, which may lead to the formation of inclusion bodies containing insoluble and inactive proteins. An important feature of *B. subtilis* is its apathogenicity and GRAS (Generally Recognized As Safe) status. It lacks the cellular components or metabolic products toxic to humans or animals, which facilitates the production of proteins of medical interest. In particular, as gram-positive organism, *B. subtilis* does not produce endotoxins (lipopolysaccharide), which are ubiquitous in all gram-negative bacteria, including *E. coli*, and are difficult to remove from many proteins in the process of purification. *B. subtilis* has been characterized extensively both physiologically and genetically, second only to *E. coli*, a great deal of vital information concerning its transcription and translation mechanisms, genetic manipulation and large-scale fermentation has now been acquired. Furthermore, *B. subtilis* has no significant bias in codon usage, it has been successfully applied to produce various industrial enzymes in large quantities, up to 1 g/L of α-amylase of *B. amyloliquefaciens* was secreted into the growth medium when it was expressed in *B. subtilis* (Palva *et al.*, 1992). Even so, however, using *B. subtilis* as a production host has also met some obstacles, the most prominent ones being the proteolysis degradation of many proteins by host proteases and the poor

compatibility of many nonbacillar proteins for secretion, especially those of eukaryotic origin. Production of high-value human proteins for pharmaceutical applications remains a major challenge. Most medical applications require intact proteins with both authentic primary sequences and properly folded three-dimensional structures.

Temperate phages have been widely used as tools for genetic manipulation and analysis of *B. subtilis*, the vast majority of cloning experiments were based on the use of prophage vectors, such as ϕ105 and SPβ (Errington, 1993). Previously, the most important application for phage vector in *B. subtilis* has been in gene cloning. However, it has recently become possible to use bacteriophage vectors for the overexpression of genes encoding heterologous or genetically manipulation proteins. Although a number of plasmid expression vectors are available, the prophage-based vectors have the advantages of increased stability because of their chromosomal locations and convenient regulation provided by the phage immunity system. Moreover, phage induction, which can be controlled by temperature shift in appropriate mutants, results not only in the expression from strong phage promoters but also in an increased copy number through phage DNA replication.

Expression vectors have been developed from two phages, ϕ105 and a defective phage, PBSX, which is present in most of the genetic strain of *B. subtilis* (Seaman *et al.*, 1964). One of the systems has been derived by manipulation of the PBSX prophage of *B. subtilis* 168 (Errington, 1993). Campbell-type integration introduces a plasmid carrying the gene to be expressed into the PBSX prophage and downstream from a strong prophage promoter (O'Kane *et al.*, 1986). The prophage carries a mutation, *xhi-1479*, that allows thermoinduction of prophage development, and the plasmid insertion blocks lysis of the host cell because the gene needed lie

downstream from the same promoter (Wood *et al.*, 1990). The construction is relatively stable and is maintained in single copy during the growth phase. Transcription and DNA amplification are activated by thermoinduction.

Similar systems have been developed from phage ϕ105. One vector utilizing the phage promoter that directs transcription through the ϕ105J106 cloning site, and mutations preventing host cell lysis and allowing thermoinduction, gave a relatively modest yield (about 20 mg/L) of a secreted heterologous protein (Gibson and Errington, 1992). Vectors giving substantially greater yield were also developed, mainly by the identification and use of a strong ϕ105 promoter. ϕ105MU209 was isolated as a colony exhibiting extremely strong expression of β-galctosidase among transformants generated by random insertion of a *lacZ* reporter gene into a temperature-inducible ϕ105 prophage (Errington, 1986). The structure of the insertion in ϕ105MU209 is complex and has not yet been fully resolved. However, it is clear that the insertion has not only conferred strong inducible expression of *lacZ* following temperature induction but has also somehow blocked host cell lysis. Despite the fact that the structure of the insertion is not fully known, it has been possible to use homologous recombination to replace part of the coding *lacZ* with heterologous genes, which are then overexpressioned following thermoinduction. This system has been used to produce 6000 to 900 Miller units of β-galctosidase (βGal) (approximately 5 to 10 % of total cellular protein) and wild type and mutant versions of the secreted β-lactamase I of *B. cereus* (Errington, 1993).

A novel expression vector based on the *B. subtilis* phage ϕ105 was then developed to permit the high-level synthesis and secretion of β-lactamase I (PenPC) from *B. cereus* (Thornewell *et al.*, 1993). The insertion of a promoterless *lacZ* gene into the phage genome by shotgun permitted the identification of a clone producing

large amounts of βGal. The insertion also blocked lysis of the host cell. Although the insertion in the original prophage was complex, plasmid vectors and prophage derivatives have been developed to facilitate the replacement of *lacZ* with other genes for expression. The new prophage contains two additional mutations: an *ind* mutation, which greatly enhances the normally poor transformability of ϕ105 lysogens, and a *cts* mutation, which allows thermoinduction of phage development and protein production. Induction of a derivative prophage containing the *penPC* gene resulted in the production of up to 500 mg of secreted PenPC per liter of culture supernatant. The ϕ105MU331 has several advantages as an expression vector. It is stable in the absence of selective pressure because the prophage is covalently inserted, in a single copy, into the host chromosome (Rutberg *et al.*, 1969). The lysogenic state also involves strong repression of phage transcription, minimizing expression of potentially toxic genes during the growth phase. On prophage induction, a strong phage promoter is activated and expression is further enhanced by phage DNA replication giving a rapid increase of copy number. Although induction of ϕ105 prophage normally results in lysis of the host cell, the ω(*lacZ cat*)3 insertion, by which the vector differs from ϕ105, has the additional property of blocking host cell lysis. The use of vectors containing this insertion facilitates the purification of secreted proteins by avoiding the solubilization of host proteins. The system has been used for the high-level overproduction of cytoplasmic protein βGal, secretary protein PenPC β-lactamase (Thornewell *et al.*, 1993) and its mutants (Leung *et al.*, 1994).

The high levels of protein synthesis obtained with ϕ105 MU331derivatives have been attributed to the presence of a powerful inducible phage promoter upstream form from the cloning site in the prophage. The existence of two adjacent

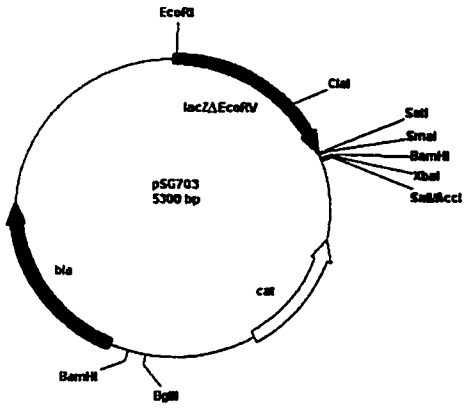
promoters was demonstrated by primer extension analysis (Leung and Errington, 1995). The two promoters are in an equivalent position to the strong late promoter of phage λ and are likely to contribute to the high-level transcription of genes inserted in the expression vectors.

In our laboratory, *B. subtilis* 1A304 ϕ105MU331 has been successfully used to facilitate the expression and purification of PenPC, PenP β-lactamase and their hybrid derivatives (Cheung, 2000; Leung, 1994). Considering the advantages of the expression system, *B. subtilis* is the first choice for the expression of β-lactamases. While the HybridF β-lactamase can be expressed by use of the system for sure, the efforts to express PC1 β-lactamase and its hybrid derivatives with PenPC β-lactamase in the system will be made in this study.

4.2 Construction of the pSG703 derivatives containing various β-lactamase genes

To facilitate the introduction of heterologous genes for expression into the ϕ105MU331 prophage, plasmid pSG703 was used. pSG703 was constructed by Thronewell *et al.* (1993), with a truncated *lacZ* gene, followed by a MCS containing several unique restriction sites, and an intact *cat* gene (Figure 4.1). Primers LHB5 (5'-cggca**CCCGGG**tgattgtctattatgtgtacg-3') and LHB8 (5'-cggct **GTCGAC**ttttaatactttcatgttaaaaatg-3') were used to amplify the *penPC* gene from pYCL18; Primers LHB6 (5'-cggca**CCCGGG**tattgacaccgatattacaattg-3') and LHB7 (5'-cggac**GTCGAC**gtcaacgataatacaaaatataatac-3') were used to amplify the *pc1* gene from pHBL1; Primers LHB5 and LHB9 were used to amplify *hybridF* gene from pHBL14; Primers LHB5 and LHB7 were used to amplify different hybrid genes of *penPC* and *pc1* from plasmids containing these genes, respectively. The PCR conditions for the amplification are described in Chapter 2. Promoter, RBS, whole β-

lactamase gene and transcriptional terminator were included in these PCR products. These PCR products were digested by restriction enzymes *Sma*I and *Sal*I, and then DNA fragments were purified and ligated with similarly digested pSG703 (Figure 4.1). After the ligation products were transformed into *E. coli* DH5α competent cells, some colonies were picked from each plate for the preparation of plasmid DNAs. The correct recombinant plasmids were screened by *Bam*HI digestion. While pSG703 contains two *Bam*HI sites, only one *Bam*HI site exists for the recombinant plasmid. One DNA band of 6.2 kb was seen when recombinant plasmid was digested by *Bam*HI (Figure 4.2). All these recombinant plasmids containing different β-lactamase genes in an appropriate orientation were confirmed by DNA sequencing.



PCR product of

penPC/pc1/hybrid gene

Digested by *Sma*I and *Sal*I, and ligated together, and then transformed into competent *E. coli* DH5α cells

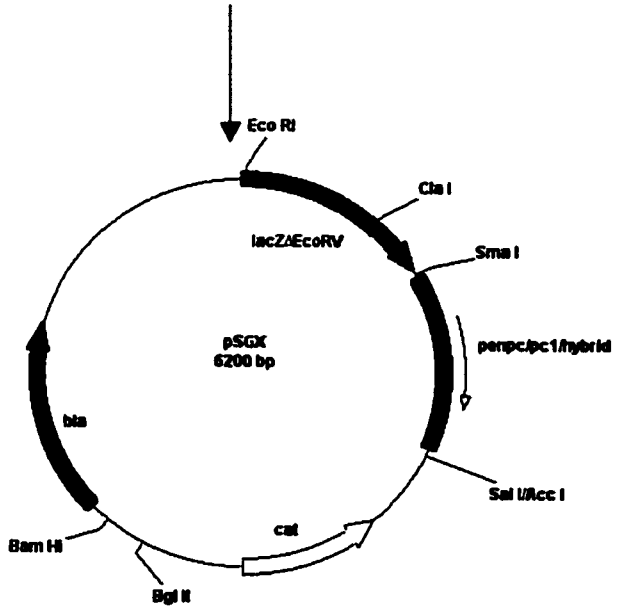


Figure 4.1 Construction of pSG703 derivatives containing various β-lactamase genes. Plasmid pSG703 and PCR products of different β-lactamase genes were digested by *Sma*I and *Sal*I. Digested fragments were purified and ligated together. The ligation product was then transformed into *E. coli* DH5α. After the preparation of plasmid DNAs from some selected transformants, the right recombinant plasmids were screened by restriction enzyme digestions and further confirmed by DNA sequencing.

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

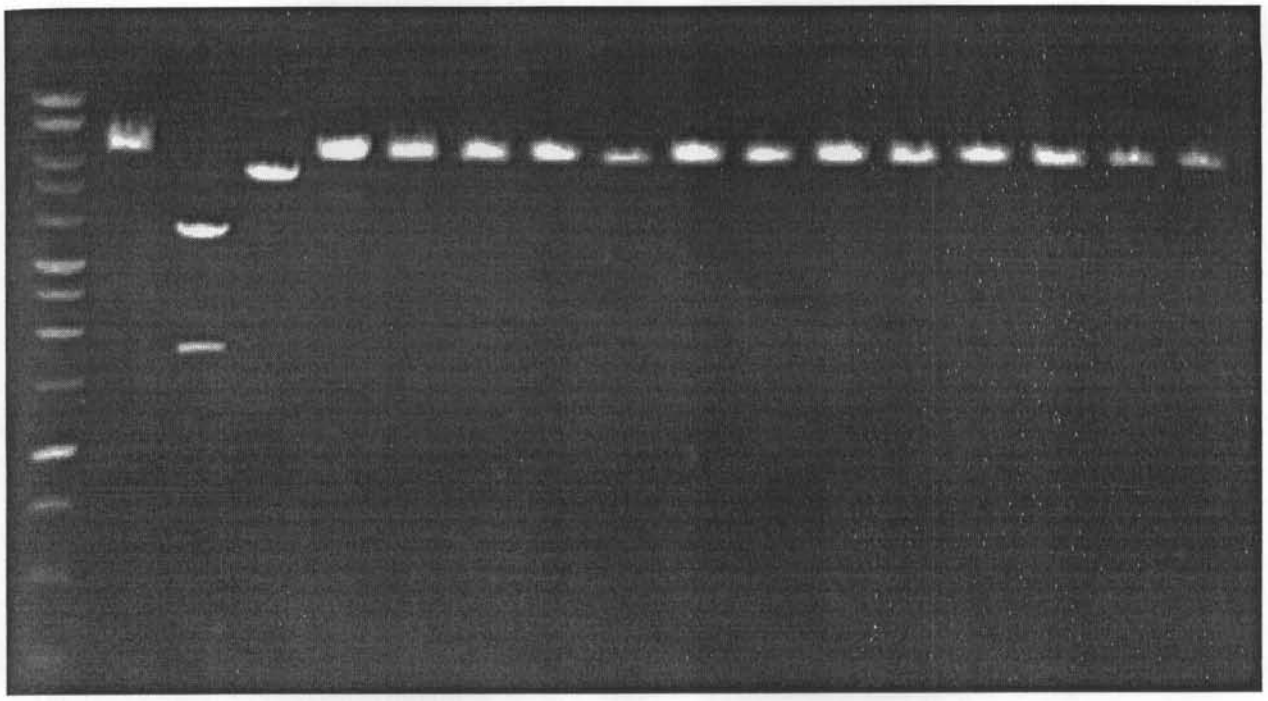


Figure 4.2 Analysis of recombinant plasmids derived from pSG703 by restriction enzyme digestion. While 1 kb DNA ladder (Promega) was loaded as marker (M) to estimate the sizes of DNA fragments, digested DNA samples were analyzed in 0.8% agarose gel. Lane 1 was pSG703 digested by *Eco*RI. Lane 2-16 were *Bam*HI-digested samples of pSG703, pSGPC1, pSGpenPC, pSGhybridF, pSGhybrid1, pSGhybrid2, pSGhybrid3, pSGhybrid4, pSGhybrid5, pSGhybrid6, pSGhybrid7, pSGhybrid8, pSGhybrid9, pSGhybrid10, and pSGhybrid11, respectively.

4.3 Transformation of the constructed pSG703 derivatives into *B. subtilis* 1A304 (ϕ105 MU331)

For protein expression, the pSG703 derivatives were transformed into *B. subtilis* strain 1A304(ϕ105MU331). According to previous study (Thornewell *et al.*, 1993), transformation of 1A304(ϕ105MU331) with undigested plasmid pSG704 gave both Cm^REr^R and Cm^REr^S colonies. While the Cm^REr^R colonies were resulted from single-crossover between plasmid and prophage, the Cm^REr^S colonies were resulted from double-crossover between plasmid and prophage (Figure 4.3). Normally, the latter class of transformants is used for protein expression, and linearized plasmid favors double-crossover events.

Because there are two crossover sites between plasmid and prophage (Figure 4.3), the Cm^REr^R transformants may be resulted form single crossover between the truncated *lacZ* gene of plasmid and the prophage (Figure 4.4) or between the complete *cat* gene of plasmid and the incomplete *cat* gene of the prophage (Figure 4.5). When the single crossover occurred at the truncated *lacZ* gene, the expressed gene was inserted into the prophage downstream from the *lacZ* gene. Similar to the situation in double crossover (Figure 4.3), the transcription of the expressed gene is placed under the control of the strong phage promoter. The expression level of the gene may be expected to be identical to the double crossover case in view that the location or distance between the expressed gene and the strong phage promoter was same with that in double crossover. However, for some unknown reasons, the protein expression level of Cm^REr^S transformants was found to be about 10-folder higher than that of the Cm^REr^R transformants when PenPC and some of its mutant β-lactamases were expressed in this system (data not shown). When the single crossover event occurred at the *cat* gene, even though the gene to be expressed was

also integrated into the prophage, the distance between the gene and the strong phage promoter was much longer. Thus, the expressed gene is too far away from the thermo-inducible promoter and probably is not under the control of the strong promoter. The transcription due to the thermo-inducible promoter may be terminated at the terminator sequence of the *ermC* gene. Therefore, thermo-induction may have no or much less effect on the expression of protein of interest.

In order to locate the crossover site after transformation, primer LHB10 (5'-ggcttgagtgcgggggca-3') was designed to carry out PCR amplification with LHB7, LHB8 or LHB9. The sequence of primer LHB10 was designed based on the DNA sequence located at the downstream of the prophage promoter. When prophage DNA prepared from a Cm^R transformant was used as the template, PCR reaction would result in the amplification of a DNA fragment of about 2.0 kb if a crossover even has occurred at the *lacZ* gene. For those Cm^R transformants that were used to express β-lactamases, since the 2.0 kb PCR products were observed after amplification for all the samples (Figure 4.6), all β-lactamase genes were confirmed to be near to as well as downstream of the strong promoter. To perform the PCR reactions, chromosomal DNAs from different transformants were prepared by the freeze-thaw method (Chapter 2) and were subjected to PCR reaction. For PCR reaction, thirty cycles of amplification were performed with the following temperatures: 30 s at 94°C, 30 s at 52°C and 2 min at 72°C.

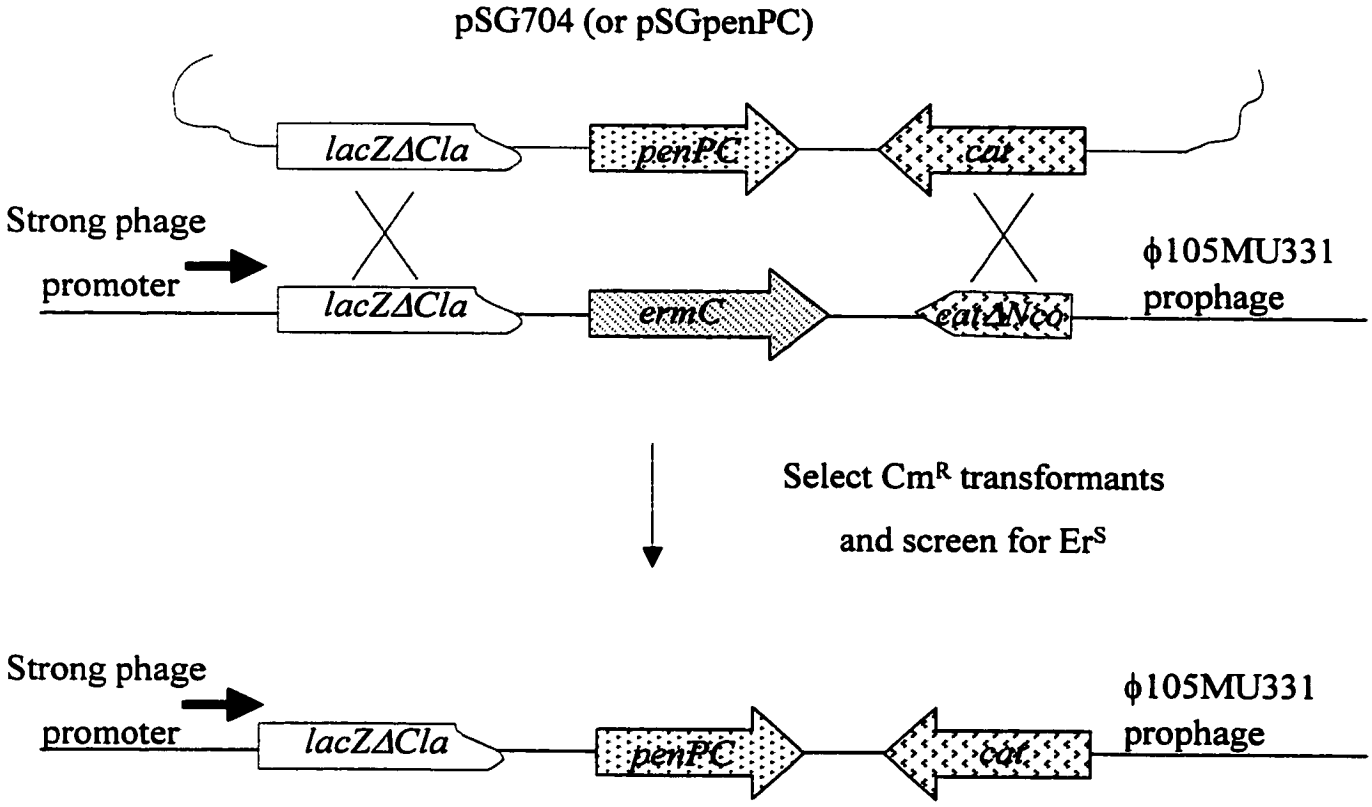


Figure 4.3 Schematic drawing of the construction of a prophage allowing expression of PenPC. Linearised pSG704 (or pSGpenPC) was transfromed into strain *B. subtilis* 1A304(ϕ105MU331) with selection for the Cm^R marker, and the transformants were screened for Er^S phenotype. Such transformants should have arisen from a double crossover event, as shown, placing transcription of the *penPC* gene under the control of the strong phage promoter. Plasmid pSGpenPC was constructed by inserting the *penPC* gene into pSG703 between *Sma*I and *Sal*I sites.

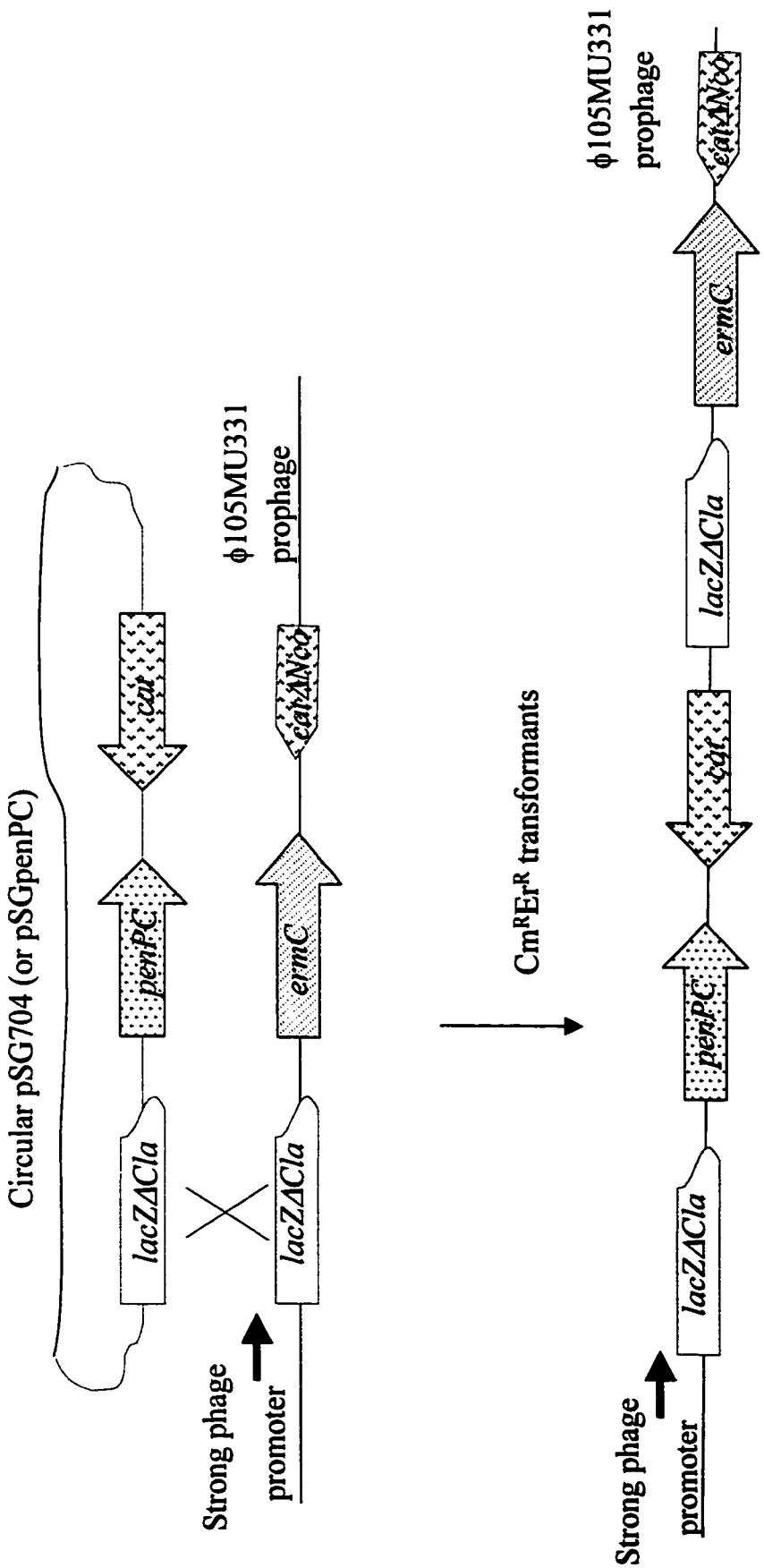


Figure 4.4 Schematic drawing of the formation of $Cm^{R}Er^{R}$ transformants by single crossover between the intact *lacZ* gene of plasmid and the truncated *lacZ* gene of prophage. Plasmid pSGpenPC was constructed by inserting the *penPC* gene into pSG703 between *SmaI* and *SalI* sites.

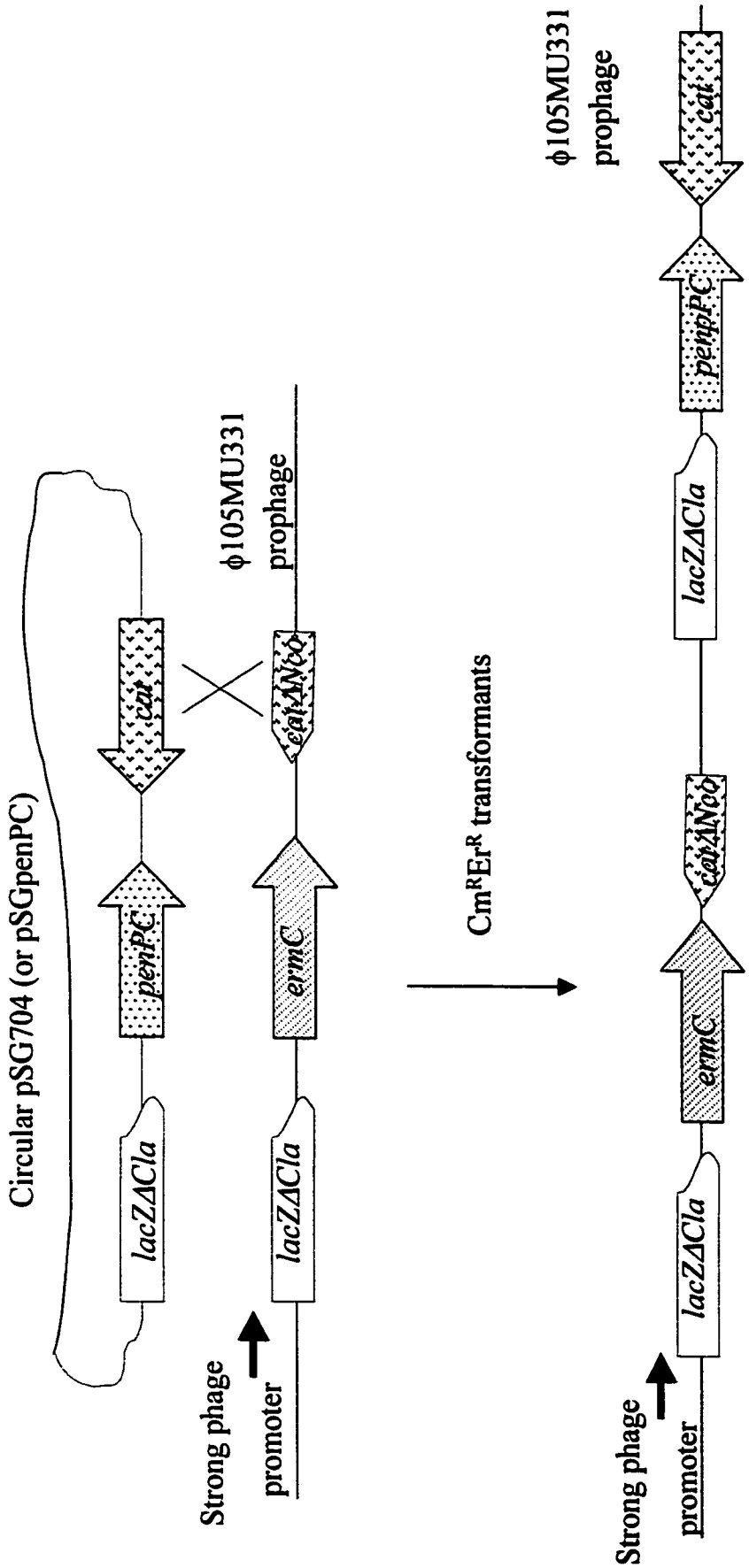


Figure 4.5 Schematic drawing of the formation of $Cm^{R}Er^{R}$ transformants by single crossover between the complete cat gene of plasmid and the incomplete *cat* gene of the prophage. Plasmid pSGpenPC was constructed by inserting the *penPC* gene into pSG703 between *SmaI* and *SalI* sites.

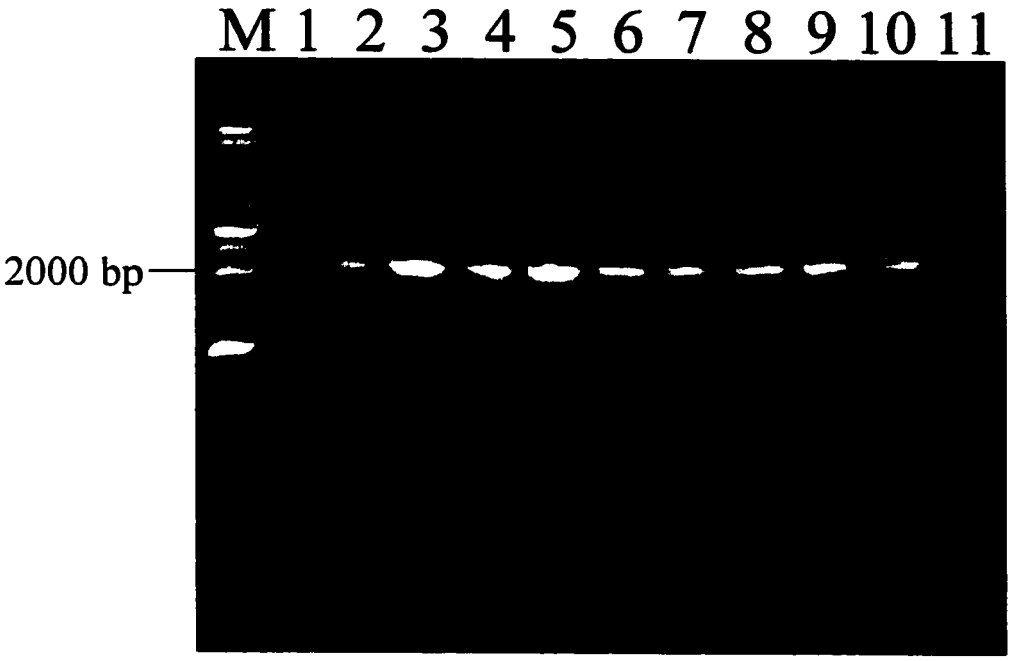


Figure 4.6 Determination of the crossover location of Cm^R transformants by PCR reaction. With 1kb DNA Ladder (M) as size marker, the PCR products from different chromosomal DNAs were analyzed in 0.8% agarose gel. Lanes 1-11 contain the the PCR products from ϕ105MU331, ϕ105HB1 (*penPC*), ϕ105HB2 (*pc1*), ϕ105HB3 (*hybridF*), ϕ105HB4 (*hybrid1*), ϕ105HB7 (*hybrid4*), ϕ105HB8 (*hybrid5*), ϕ105HB9 (*hybrid6*), ϕ105HB10 (*hybrid7*), ϕ105HB12 (*hybrid9*), and ϕ105HB14 (*hybrid11*), respectively. For PCR reaction, thirty cycles of amplification were performed with the following temperatures: 30 s at 94°C, 30 s at 52°C and 2 min at 72°C.

4.4 Small scale expression of β-lactamases from *B. subtilis*

For protein expression, as mentioned in Chapter 2, the transformants from different pSG703 derivatives were separately picked and grown on a small scale (5ml) firstly and then 1 ml of it was inoculated into 15 ml. Induction was done at 50°C for 5 min, and growth was continued at 37°C. Strain 1A304(ϕ105MU331) was similarly treated as a control. Samples of the culture were taken hourly after induction and stored at -20°C for further analysis.

4.5 SDS/PAGE analysis and activity assay of the expressed protein samples

The supernatants of culture samples at the fourth hour after thermo-induction were analyzed by SDS/PAGE analysis. We found that there was no β-lactamase expression for the strains containing the *pc1* or hybrid genes generated from crossing *penPC* and *pc1*, while the PenPC and HybridF β-lactamase (PenPC x PenP) were successfully expressed in *B. subtilis* (Figure 4.7). To detect the time-course of protein production of PenPC and HybridF β-lactamase, at hourly intervals for 8 h after thermoinduction, 10 μl samples of the culture were loaded directly onto SDS/PAGE for electrophoresis (Figure 4.8). The amount of β-lactamase present at each hour after thermoinduction was also assayed enzymatically with penicillin G as the substrate (Figure 4.9).

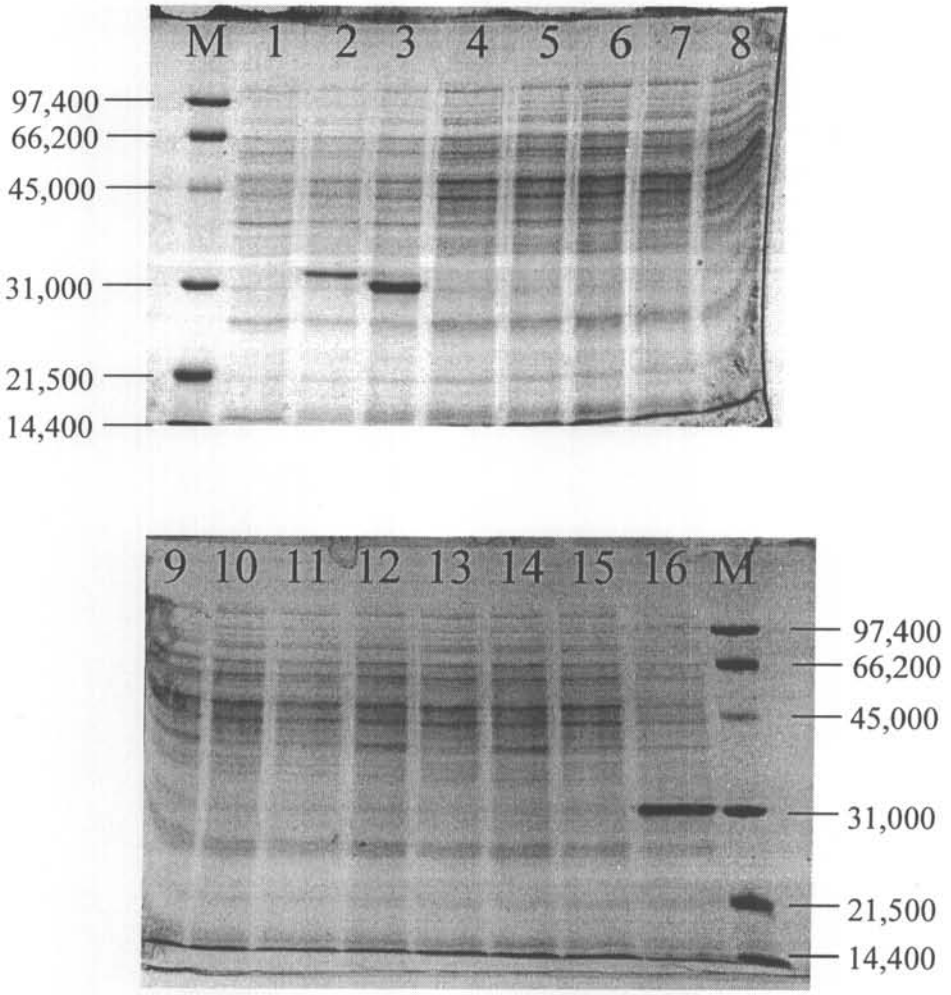


Figure 4.7 The production of β-lactamases from 1A304(ϕ105MU331) and its derivatives. With low range SDS/PAGE standard (M) for the size estimation, protein samples in the culture supernatants were analyzed by SDS/PAGE. Lanes 1-16 contain the samples from the cultures of 1A304(ϕ105MU331) and its derivatives containing the gene *hybridF*, *penPC*, *pc1*, *hybrid1*, hybrid2, *hybrid3*, *hybrid4*, *hybrid5*, *hybrid6*, *hybrid7*, *hybrid8*, *hybrid9*, *hybrid10*, *hybrid11*, or *penPC*, respectively.

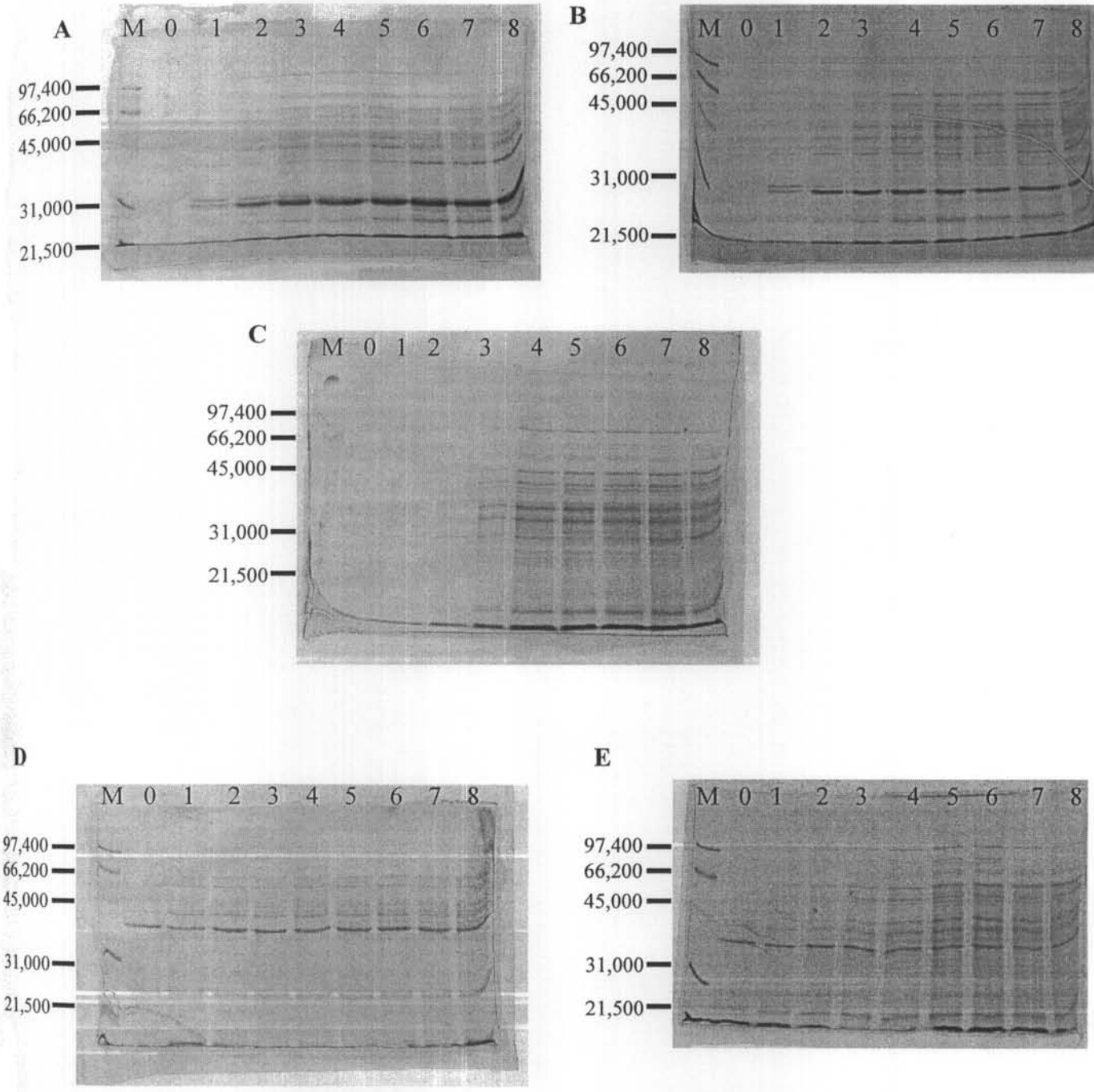


Figure 4.8 SDS/PAGE analysis of the production of β-lactamases. Culture supernatant samples of 1A304 (ϕ105MU331) derivatives containing the gene of *penPC* (A), *hybridF* (B), *pc1* (C), as well as the whole culture of 1A304(ϕ105MU331) (D) and its derivative containing the *pc1* gene (E), at different time points after thermo-induction were analyzed by SDS/PAGE. M is the protein marker; 0,1,2,3,4,5,6,7 and 8 indicate the number of h after thermo-induction when the samples were taken.

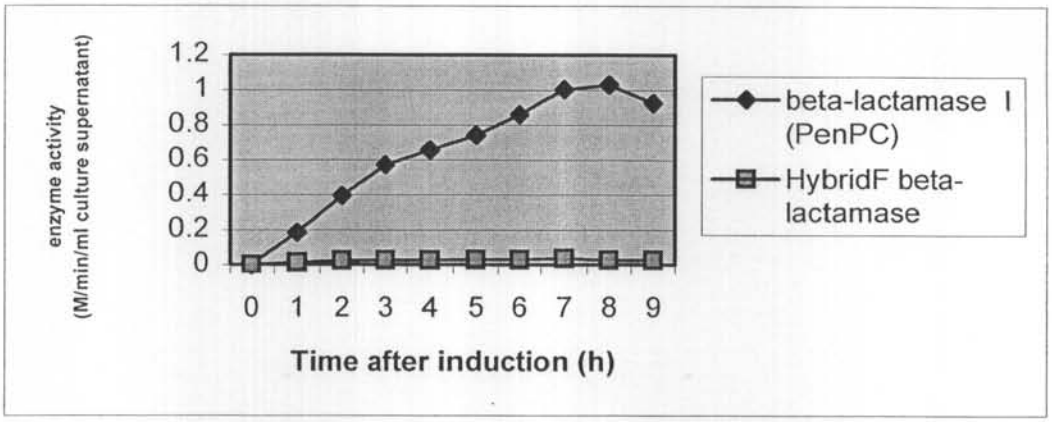


Figure 4.9 The enzyme activity of PenPC and HybridF (with Penicillin G as substrate) at different time points after thermo-induction in small-scale expression (15 ml).

In 1984, Saunders *et al.* (1984) suggested that the *pc1* promoter should be active *in vivo* in *B. subtilis* and the behavior of *pc1* in *B. subtilis* could parallel the behavior of the *penP* gene in the same host. It is surprising that PC1 and hybrid β-lactamases of PenPC and PC1 cannot be expressed in the ϕ105MU331 expression system. One possibility was that PC1 and its hybrid β-lactamases might have been expressed but could not secreted into the extracellular medium. However, when the samples of the culture including cells were taken and applied to SDS/PAGE, still no protein bands corresponding to β-lactamases were observed (Figure 4.8). Whether there was no β-lactamase expression at all or the expressed β-lactamase was degraded by proteases is still unclear.

B. subtilis is a promising host for the production of foreign protein, however, protease released by the host organism can often cause the breakdown of secreted heterologous proteins (Hemila *et al.*, 1992). A growth medium rich in succinate (0.5 M) has been suggested as a means of increasing the yield of *E. coli* β-lactamase in *B. subtilis* (Nakamura *et al.*, 1985). It was also reported that the addition of 6% glucose

and 100 mM potassium phosphate to the growth medium significantly reduces the degradation of *E. coli* TEM β-lactamase secreted form *B. subtilis* (Hemila *et al.*, 1992). However, the addition of 6% glucose and 100 mM potassium phosphate to BHY medium was proved to have no effect on the expression of PC1 and its hybrid derivatives (data not shown).

4.6 Effect of the transcriptional terminator from the gene *penPC* on the expression of PC1 in *B. subtilis*

The DNA sequence of hybrid11 β-lactamase gene is highly identical to that of *penPC* with the exception of about 120 bp-length of 3' coding sequence and transcriptional terminator sequence, which are from *pc1*. Codon usage, translation and secretion of Hybrid11 β-lactamase were expected to be very similar to those of PenPC. However, while PenPC was highly expressed and secreted, no expression of the Hybrid11 β-lactamase and the PC1 β-lactamase were observed in the cultured cells and culture supernatant. The lack of transcription of *pc1* and its hybrid derivatives in the ϕ105MU331 expression system may be the cause of the expression failure. So far, no sequence characteristic of typical Rho-independent transcription terminators (Terry, 1983) was found in the 170 bp sequence following the translation stop codon of *pc1* (Wang *et al.*, 1987). The important manner in which termination influences the expression of heterologous genes is in the stabilization of the message (Hess and Graham, 1990). Stable stem-loop structures associated with termination structures in *E. coli* have been shown to prolong message half-life through discouraging ribonuclease activity (Guarneros *et al.*, 1982). If the transcription were to blame for, replacement of transcriptional terminator sequences of *pc1* with those

of *penPC* would improve the protein expression of PC1 β-lactamase and its hybrid derivatives.

Two single-stranded oligonucleotides (LHB23 5'-**TCGA**TATTTTAGTT TTCGCATTTTTAACATGAAAGTATTAAAAG-3' and LHB24 5'-**TCGA**CTTTT AATACTTTCATGTTAAAAATGCGAAAACTAAAATA-3') were designed according to the 3' region of the *penPC* gene with *Sal*I site at 5' end. Same amount of the two oligonucleotides were added together and heated to 100°C for several minutes and then gradually cooled to room temperature. After annealing, the double-stranded DNA fragment was phosphorylated by T4 Polynucleotide Kinase (New England Biolabs) for 1 h at 37°C. The phosphorylated DNA was than ligated with SalI-digested pSGpc1. Several plasmids were prepared from transformants and their sequences were determinated by DNA sequencing. One plasmid was found with successful addition of the *penPC* transcriptional terminator downstream of the *pc1* gene. The plasmid was then transformed into *B. subtilis* for testing protein expression. However, no protein band corresponding to the size of PC1 was noted by SDS/PAGE analysis (data not shown). In addition, no β-lactamase activity was found in culture medium. It seems that the addition of the *penPC* transcriptional terminator to the 3' of the *pc1* gene did not improve the expression of PC1 in *B. subtilis*.

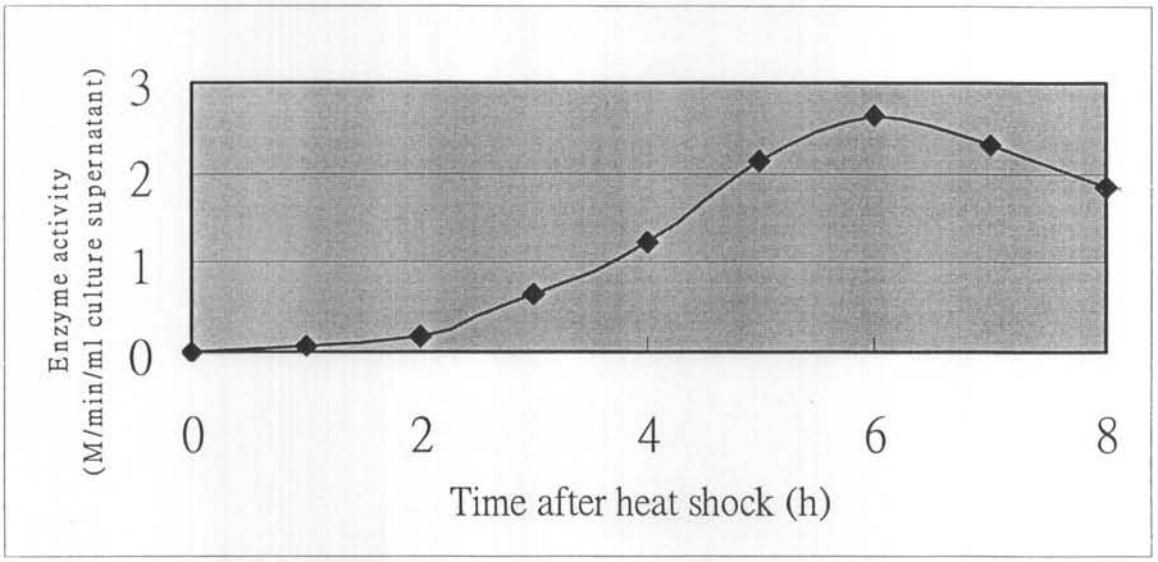
4.7 Large scale expression of PenPC, HybridB, and HybridF β-lactamases

Oxygen supply is critical for the growth of *B. subtilis*. In order to supply sufficient oxygen for large scale expression, 1-litre flask is used to culture only 100 ml of cells. However, it was found that too much oxygen supply (with higher ratio of flask to culture volume and the use of baffled flasks) would decrease the expression

of proteins, which was also observed when we recently expressed the β-lactamase inhibitory protein (BLIP) in the same system (see Chapter 7). It suggests that DO (dissolved oxygen) level may play an important role on cell growth and β-lactamase (and other enzymes) production, which is consistent with the report on *B. subtilis* fermentations (Sargantanis and Karim, 1998). Low DO levels result in limited growth conditions for the microorganism, but favor both long-term production and lower degradation rates. DO levels may be an important factor that influences maximum β-lactamase activity. Therefore, 1-litre flasks without baffles were used for the large scale expression of β-lactamases.

The expression of PenPC, HybridB and HybridF shown similar expression pattern but that the expression of PenPC was in a much higher level. Before heat shock, no β-lactamase was expressed. After heat shock, the β-lactamase concentration in culture media increased and reached the maximum at the sixth h (for PenPC) or the seventh h (for HybridB, HybridF). The activity of β-lactamase was also the highest at the sixth h after thermo-induction for PenPC or at the seventh h for HybridF (Figure 4.10).

A



B

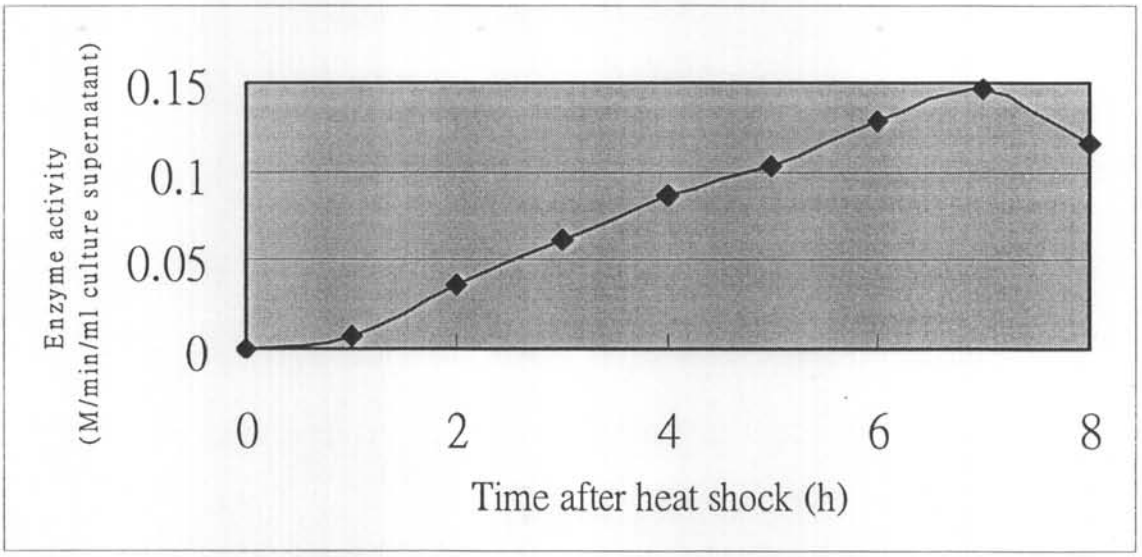


Figure 4.10 Enzyme activity of PenPC (A) and HybridF (B) at different time points after thermo-induction when they were expressed in large scale (100 ml). The enzyme assay was carried out with Penicllin G as substrate.

4.8 Purification of the β-lactamases expressed from *B. subtilis*

Celite (diatomaceous earth) was successfully used to purify β-lactamase I (PenPC) and β-lactamase II from *Bacillus cereus* 569/H/9 as early as 1974 (Davies and Abraham, 1974). It has also been proved to be an effective and simple method to purify HybridB, HybridC, HybridD and HybridE β-lactamases, but Celite cannot be used to purify PenP and Hybrid A β-lactamase (Cheung, 2000; Leung, 1994). However, HybridF β-lactamase was purified by Celite successfully, even though the binding affinity of HybridF β-lactamase to Celite was lower. The purification steps of PenPC, HybridB and HybridF were summarized in Table 4.1, Table 4.2 and Table 4.3, respectively. The β-lactamase activity of all the samples was measured using penicillin G as the substrate. HybridC, HybridD and HybridE were purified by the same method (Cheung, 2000). PenP and HybridA, which cannot be purified by Celite, were purified by a CM Sepharose column (cation exchange) (Cheung, 2002). After expression, the culture supernatant was collected and dialyzed against 20 mM sodium acetate buffer (pH 4.8). The sample was then loaded onto the column and eluted with a linear sodium chloride gradient (0-0.25 M). The fractions containing β-lactamase were confirmed by SDS/PAGE analysis and enzyme assay. Figure 4.11 shows the purity of all these purified β-lactamases.

Table 4.1 Purification of PenPC

Sample	Total Volume (ml)	Total protein (mg)	Total enzyme activity (M/min)	Specific activity (M/min/mg)	Recovery (%)	Purification fold
Culture supernatant	500	196	602	3.1	100	1
Culture supernatant (pH7.0)	500	192	599	3.1	99.5	1
After elution	310	27.8	409	14.7	67.9	4.8
After ultrafiltration	10.5	19.6	279	15	46.2	4.9

Table 4.2 Purification of the HybridB β-lactamase

Sample	Total Volume (ml)	Total protein (mg)	Total enzyme activity (M/min)	Specific activity (M/min/mg)	Recovery (%)	Purification fold
Supernatant	600	208	90	0.43	100	1
After elution	380	4.4	40.8	9.3	45.3	21.6
After ultrafiltration	18.2	3.2	33.2	10.2	36.8	23..7

Table 4.3 Purification of the HybridF β-lactamase

Sample	Total Volume (ml)	Total protein activity (mg)	Total enzyme (M/min)	Specific activity (M/min/mg)	Recovery (%)	Purification fold
Culture supernatant	500	97.4	3.86	0.0397	100	1
Culture supernatant (pH7.0)	500	95.1	4.11	0.433	106	1.1
After elution	350	3.96	2.03	0.515	52.5	13
After ultrafiltration	7.5	3.86	1.97	0.511	51	12.9

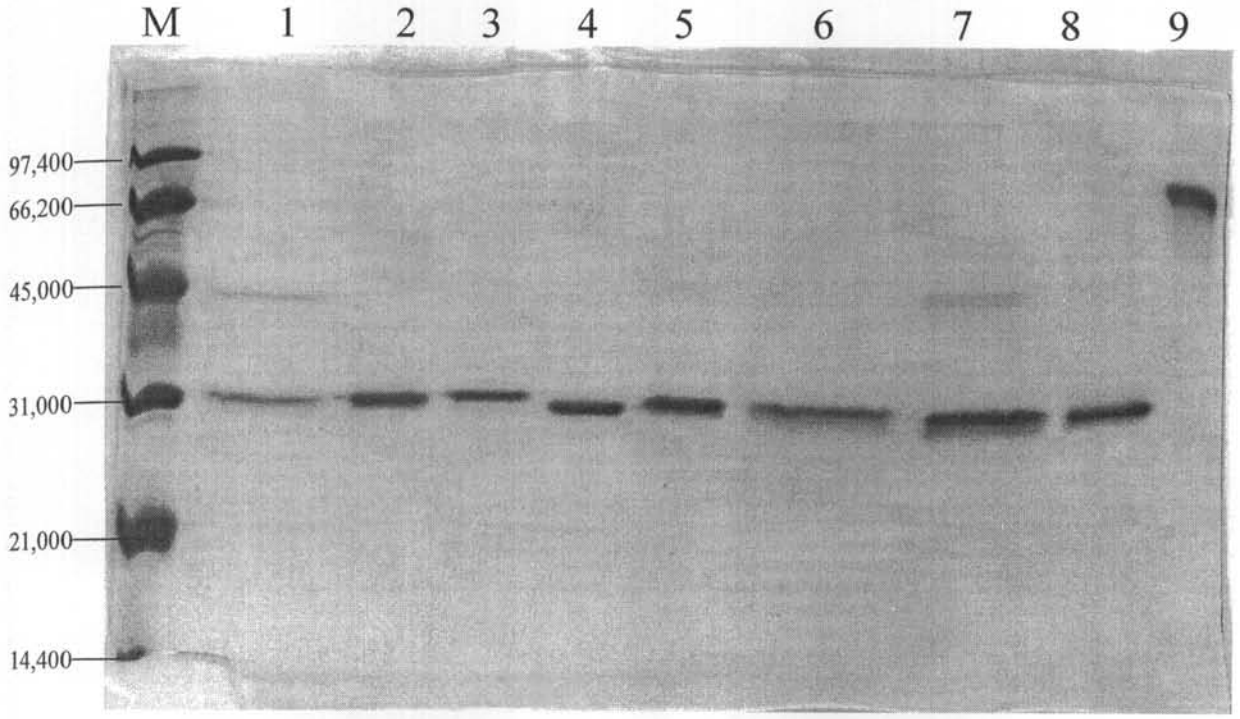


Figure 4.11 Analysis of the purified β-lactamases by SDS/PAGE. With low range SDS/PAGE standard (Bio-Rad)(M) for the size estimation and 0.5 μg BSA (Lane 9) for protein amount measurement, the purity of purified PenP (Lane 1), HybridA (Lane 2), HybridF (Lane 3), HybridB (Lane 4), HybridC (Lane 5), HybridD (Lane 6), HybridE (Lane 7) and PenPC (Lane 8) were analyzed by 15% SDS/PAGE.

4.6 Discussion

When linearized pSG703 derivatives containing various β-lactamase genes were transformed into *B. subtilis* 1A304(ϕ105MU331), the Er^R transformants with β-lactamase genes exactly located downstream of the strong phage promoter were selected for proteins expression.

PenPC, PenP as well as all their hybrid β-lactamases were successfully expressed in *B. subtilis* by the use of prophage vector ϕ105MU331. Much higher DO in culture (with higher ratio of flask to culture volume, higher shaking rate and the use of baffled flasks) may decrease the protein expression level in the cases of PenPC and HybridF. The decrease was more severe in HybridF. The growth rate of *B. subtilis* depends on the dissolved oxygen concentration, which exerts its aerobic influence on cellular activity by its effects on cellular energy regeneration through respiratory processes. At the same time, the DO level also affects the catabolic pathway used by bacteria. Therefore, low DO is detrimental to the cell growth. On the other hand, for recombinant protein expression, high DO levels are detrimental. A low aeration rate was beneficial for β-lactamase synthesis by a plasmid-containing *E. coli*. (Ryan *et al.*, 1989). Park (1993) studies the effect of three different dissolved oxygen ranges (>70%, <20%, and <7% of air saturation) on the production of β-lactamase using recombinant *Bacillus subtilis* in 1-liter fermenter. The fermentation with DO<20% was found to yield the best β-lactamase activity and productivity. While high DO level favor the growth of *B. subtilis*, it may also cause more secretion of protease and the higher degradation of expressed proteins. The foaming in baffled flasks may also cause the inactivation of secreted proteins, especially the less stable ones. Therefore, it can be deduced that there exists an optimum DO level for the production of β-lactamase.

While PenPC can be purified by Celite and PenP can not be purified by Celite, Hybrid F was proved to bind Celite with low affinity. The binding affinity of β-lactamase with Celite varies significantly among different β-lactamases, which may also be one of the properties determined by structure. For some unknown reasons, PenPC binds Celite very specifically and it can be purified by Celite binding. It was proposed that the binding might be due to ionic bonds (Davies and Abraham, 1974c; Mezes *et al.*, 1985). The binding of Celite to PenPC and hybrids (HybridB-E) might be due to the positive charges carried by these proteins at experimental conditions (Cheung, 2000). The isoelectric points of PenP, HybridA, HybridF, HybridB, HybridC, HybridD, HybridE and PenPC are calculated to be 5.08, 5.47, 5.47, 7.43, 8.57, 8.81, 8.17 and 8.17, respectively. The lower affinity of HybridF to bind Celite might due to fewer positive charges. However, HybridA and HybridF, which are expected to possess similar charge characteristics (same pI values), show a great difference in Celite binding. Other interactions as well as ionic bonds may be attributed to the binding between β-lactamase and Celite. The exact mechanism involved in their binding is still unclear.

Large-scale expression and purification were performed to get sufficient amount of pure β-lactamases for the further study of their enzyme properties. By comparing the enzyme properties of these β-lactamases (HybridA-F as well as the two parent β-lactamases, some important information about the structure-function relationships have been obtained (see Chapter 6).

The failure to express the PC1 β-lactamase and its hybrid derivatives with PenPC might result from the proteolytic degradation of the expressed proteins by host proteases. The development of heterologous protein production system based on *B. subtilis* has been hampered by the extracellular proteases, which may cause

degradation and result in a very low yield of foreign proteins (Hemila *et al.*, 1992). Inactivation of the two major protease genes of *B. subtilis* increased the yield of the *E. coli* β-lactamase (Nakamura *et al.*, 1985; Wong *et al.*, 1986), even though the degradation was not completely abolished. Several minor proteases have thereafter been inactivated, which has further increased the stability of the β-lactamase produced (Wu *et al.*, 1991). The extracellular *Staphylococcal aureus* β-lactamase is very sensitive to proteases (Saunders *et al.*, 1984), which was well demonstrated by Wang and Novick (1987). In their work, the structural gene encoding for β-lactamase in the *Staphylococcus aureus* plasmid pI258 was cloned into a *Staphylococcus aureus-Bacillus subtilis-Escherichia coli* shuttle vector, pWN101. pWN101 was structurally stable and the β-lactamase gene was expressed efficiently from its native promoter and ribosome-binding site in all three hosts. The results of Wang and Novick's work were summarized in Figure 4.12. As shown in Figure 4.12, β-lactamase specific activity peaked in the mid-exponential phase for *S. aureus* and *B. subtilis* and in the early exponential phase for *E. coli*. Under the condition of these experiments, in *S. aureus* about 50 to 60% of the β-lactamase remained bound to the cells and about 40 to 50% was secreted into the medium. However, in *B. subtilis* more than 90% of the β-lactamase was bound to the cells, and only very small amount of the enzyme was detected in the supernatant fluid. This difference was due to proteolytic degradation of the secreted enzyme. Therefore, PC1 was most probably be degraded by a minor protease in *B. subtilis*. The problem of proteolytic degradation may be overcome by the development of protease-deficient host strains. Quite a different approach to the protease problem was taken by Udaka's group, which has studied the possibility of using extracellular protease deficient *Bacillus brevis* as a host (Udaks *et al.*, 1989; Yamagata *et al.*, 1989)

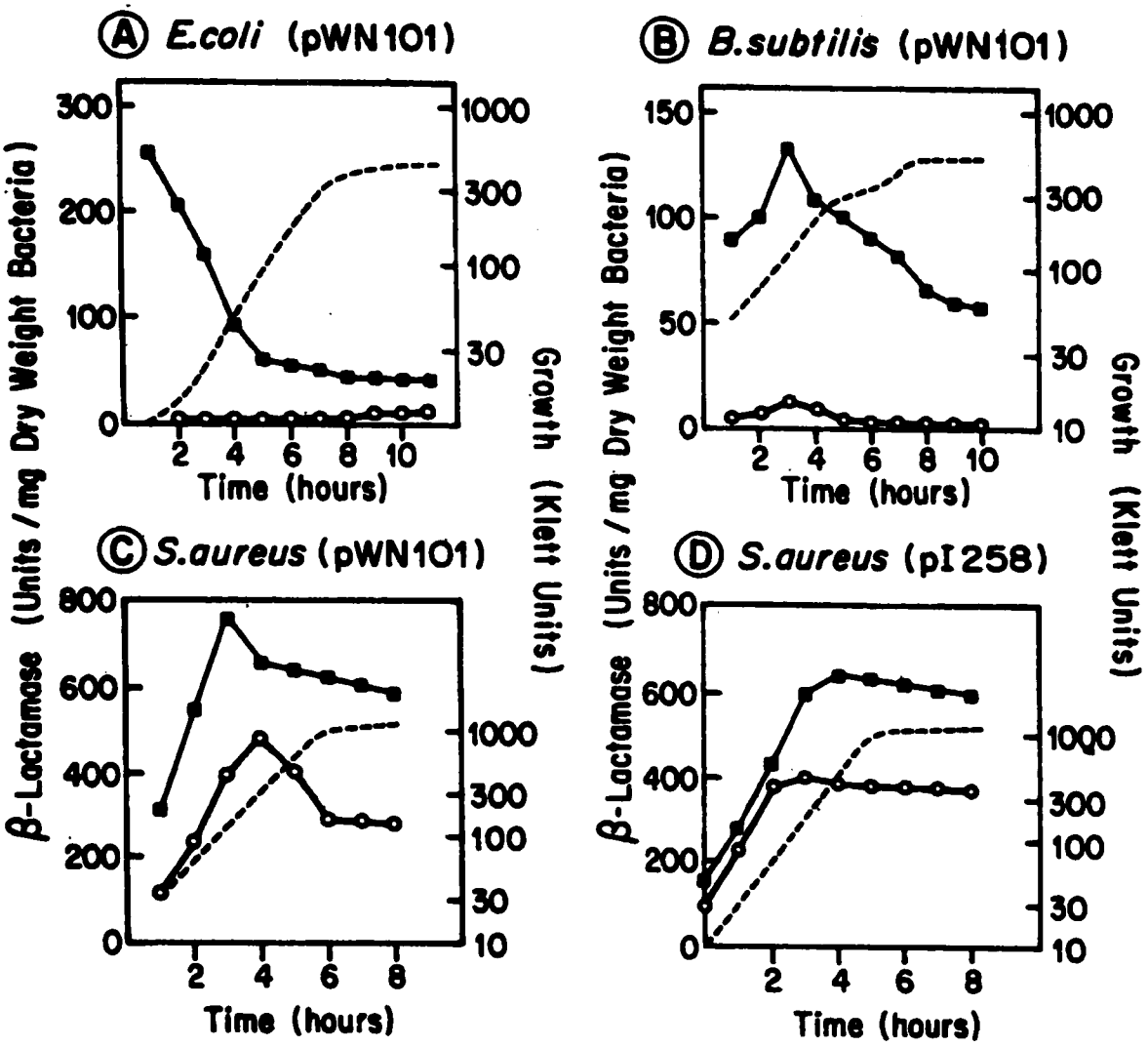


Figure 4.12 Expression of β-lactamase from pWN101 and pI258 (after Wang and Novick, 1987). (A) *E. coli* (pWN101); (B) *B. subtilis* (pWN101); (C) *S. aureus* (pWN101); (D) *S. aureus* (pI258). Cultures were sampled at various stages of growth (-----), and both the whole culture (■) and supernatant (O) were assayed for β-lactamase activity at pH 5.8 spectrophotometrically.

To summarize, while PenPC, PenP and all their hybrid β-lactamases were successfully expressed in *B. subtilis*, *B. subtilis* may not be an ideal host for the protein expression of PC1 and its hybrid derivatives, even though the *pc1* promoter is active *in vivo* in *B. subtilis*. The failure of the expression of PC1 and its hybrid derivatives might result from proteolytic degradation of secreted enzymes by host proteases. In order to get sufficient proteins for our studies, efforts to express PC1 and its hybrid derivatives in other hosts, such as *E. coli,* have been made (see Chapter 5).

Chapter Five

Expression of β-lactamases in *Escherichia coli*

5.1 Introduction

Among the many systems available for heterologous protein production, the Gram-negative bacterium *Escherichia coli* remains most attractive (Baneyx, 1999). The advantages of using *E. coli* for heterologous gene expression include the ease of growth and manipulation of this organism using simple laboratory equipment, the availability of an increasingly large number of vectors and host strains that have been developed for maximizing expression, a wealth of well understood knowledge about the genetics and physiology or biochemistry of *E. coli*, and the influence of specifically genetic and environmental factors on expression of heterologous proteins. Moreover, expression can often be achieved quite rapidly: beginning with a DNA clone, it is possible to express a protein in *E. coli* and purify it in milligram quantities in less than two weeks. Shaken-flask cultures can produce many tens of milligrams of heterologous protein per liter of culture. In fermenters, where much higher cell density can be achieved, it is possible to produce more than a gram of heterologous protein from per liter of culture. Recombinant proteins can be accumulated at levels up to 50% of total cell protein.

Nevertheless, there are situations where other expression systems, such as those based on yeast, insect, or mammalian cells, are more likely to be successful. The most important limitation is the inability of *E. coli* to carry out complex

post-translational modifications typical of eukaryotes, such as glycosylation, myristylation, phosphorylation, specific proteolytic processing, etc., and its limited ability to carry out extensive disulfide bond formation and assembly of heterologous proteins into multi-subunit assemblies. Another limitation of *E. coli*, which still requires a considerable effort to overcome, is that some proteins are expressed in insoluble form, a consequence of protein misfolding, aggregation, and sequestration into "inclusion body". In addition, codon usage in *E. coli* displays a bias, that is, it shows a nonrandom usage of synonymous codons. Heterologous genes that contain a substantial number of codons that are rarely used in *E. coli* may thus be expressed inefficiently. Finally, it is sometimes difficult to achieve a sufficiently high level of expression of full-length protein because of protein degradation, which would often happen when recombinant proteins were expressed in the cytoplasm.

Although there is no guarantee that a recombinant gene product will accumulate in *E. coli* at high levels in a full-length and biologically active form, a considerable amount of effort has been directed at improving the performance and versatility of this workhouse microorganism.

The development of sophisticated protein-fusion systems has facilitated high-level production and purification of recombinant proteins in *E. coli* (Makrides, 1996). Fusion partners offer several advantages, such as increased production of the

desired protein, prevention of inclusion-body formation, improved folding characteristic, limited proteolysis and simple detection and purification of proteins by use of affinity tags. The most probable reason for affinity tags to improve the folding (and/or reduced degradation) of desired proteins is that the fusion partner may efficiently and rapidly reaches a native conformation as it emerges from the ribosome (or soon at its release), and promotes the acquisition of correct structure in downstream folding units by favoring on-pathway isomerization reactions (Baneyx, 1999).

A variety of different gene fusion systems in order to use affinity chromatography have been developed since the first example was presented (Uhlen *et al.*, 1983). Different proteins show very divergent characteristics, regarding stability, solubility, size and secretability. Therefore, proteins need individual treatment and no ideal fusion system exists that is applicable on all proteins. Fusion protein purification systems can be used on several kinds of interactions. Examples of interaction are protein-protein interaction, enzyme-substrate interactions, protein-carbohydrate interactions and protein-metal interactions. Some of the most frequently used fusion protein systems for affinity purification are glutathione-S-transferase (GST) (Smith and Johnson, 1988), protein A (SPA) (Stahl and Nygren, 1997; Stahl *et al.*, 1997), maltose binding protein (MBP) (di Guan *et al.*, 1988; Maina *et al.*, 1988) and

thioredoxin (TR) (LaVallie and McCoy, 1995) (Table 5.1). There are also a number of short peptides used for selective purification, such as poly-histidine (Hochuli, 1990), poly-arginine (Smith *et al.*, 1984), and tryptophan tags (Kohler *et al.*, 1991) (Table 5.1). Specific excision sites are usually added to the junction of a protein with the fusion partner to allow eventual enzymatic or chemical removal (LaVallie and McCoy, 1995; Makrides, 1996). These enzymes are commercially available and are fairly specific for their recognition sequences, often allowing specific cleavage without protein degradation under gentle conditions where protein solubility and activity are preserved.

Table 5.1 Examples on available proteins or peptides for selective purification (After Hober and Uhlen, 2000)

Fusion partner	Ligand	Interaction	Elution condition	References
Protein A (SPA)	hIgG/Fc	protein-protein	low pH	(Stahl *et al.*, 1997)
Z	hIgG/Fc	protein-protein	low pH	(Stahl *et al.*, 1997)
Protein G (SPG)	hIgG/Fc	protein-protein	low pH	(Stahl *et al.*, 1997)
ABP	HAS	protein-protein	low pH	(Stahl *et al.*, 1997)
Glutathione-S-transferase (GST)	glutathione	protein-substrate	reduced glutathione	(Smith and Johnson, 1988)
Maltose-binding protein (MBP)	amylose	protein-carbohydrate	maltose	(di Guan *et al.*, 1988)
FLAG peptide	mAb	peptide-protein	low pH/FLAG peptide	(Hopp, 1988)
Biotinylate	monoavidin	biotin-avidin	biotin	(Wilchek and Bayer, 1990)
Polyhistidine	Me2+	peptide-IMAC	low pH/imidazole	(Hochuli, 1990)
Polyarginine	Anion	peptide-ionexchange	salt	(Smith *et al.*, 1984)
polytrytophan	-	2-phase system	-	(Kohler *et al.*, 1991)

Currently, GST and MBP were widely chosen as fusion partners because of the combined advantage of high-level expression and affinity purification. There are many commercially available vectors for the expression of GST (Pharmacial) or MBP (New England Biolabs) fusion protein. Protein expression from a pGEX plasmid or pMAL vector is under the control of the *tac* promoter, which is induced using the lactose analog isopropyl β-D-thiogalatoside (IPTG). The synthetic (hybrid) *tac* promoter, which consists of the -35 region of the *trp* promoter and the -10 region of the *lac* promoter, is very strong and routinely allow the accumulation of proteins to about 13-30% of the total cell protein. Full induction of *tac* with IPTG has been reported to yield fivefold greater expression of cloned genes than the *lac* promoter. For these plasmids, which also feature the $lacI^q$ gene, the leakiness of promoter can be efficiently repressed by the $lacI^q$ gene product. This single nucleotide mutation in the -35 hexamer of the *lacI* promoter leads to an increase in the number of LacI repressor molecules from 10-20 to over 100 per cell.

The use of host strains carrying mutations that eliminate production of cellular proteases can sometimes enhance product accumulation by reducing degradation (Makrides, 1996). For recombinant proteins expressed in the cytoplasm, mutations in the *lon*, *clp*, and *rpo*H (htpR) genes have reportedly been helpful. The *lon* and *clp* genes encode cytoplasmic proteases. Lon is the most highly characterized *E. coli*

protease, which acts on abnormal proteins but also cleaves normal proteins in the regulation of capsular synthesis and cellular stress responses. Clp has been shown to have its highest activity during late exponential and early stationary growth phases. The *rpoh* (htpR) gene encodes a sigma factor that stimulates production of heat shock proteins, some of which are proteases. Both pGEX vectors and pMAL vectors carry the $lacI^q$ gene, so there are no specific host requirements for propagation of the plasmids or for expression of fusion proteins. However, *E. coli* BL21 [F-, *ompT*, *hsdS*(r_B^-, m_B^-), *gal*] is recommended for expression of GST fusion proteins and MBP fusion proteins. As an *E. coli* strain, BL21 is deficient in the Lon protease and lacks the ompT outer membrane protease that can degrade proteins during purification. Thus, at least some target proteins should be more stable in BL21 than in host strains that contain these proteases. BL21 does not transform well and an alternate strain is recommended for maintenance for the plasmids.

Staphylococcus aureus PC1 β-lactamase has been successfully expressed in two different host systems, *S. aureus* (Robson and Pain, 1976) and *E. coli* (Zawadzke *et al.*, 1995), to high level. Considering the advantages and the availability of *E. coli* expression system, efforts have been made to express *B. cereus* β-lactamase I (PenPC), *S. aureus* PC1 β-lactamase (PC1), and their hybrid β-lactamases as fusion proteins in *E. coli* in the present studies.

5.2 Construction of the pGEX-6P-1 derivatives containing various β-lactamase genes

To facilitate the use of PreScission protease for the cleavage of the purified fusion proteins, PGEX-6P-1 vector (Figure 5.1) was chosen, on which the recognition sequence of PreScission protease is located immediately upstream from the multiple cloning sites.

In order to amplify the β-lactamase genes coding for mature enzymes only, the signal peptide sequences of various β-lactamases have to be identified. The signal peptide sequences of many class A β-lactamases have been determined, but unlike the homology seen in the mature forms of the enzymes, no apparent conservation is observed for the signal sequences (Wang and Novick, 1987). According to nucleotide sequence of *pc1* gene and deduced amino acid sequence of *S. aureus* β-lactamase precursor (Figure 5.2), Primer LHB12 (5'-cggca**GGATCC**aaagagtta aatgatttag-3') was designed to amplify the mature PC1 β-lactamase coding sequence with primer LHB7 from pHBL1 plasmid.

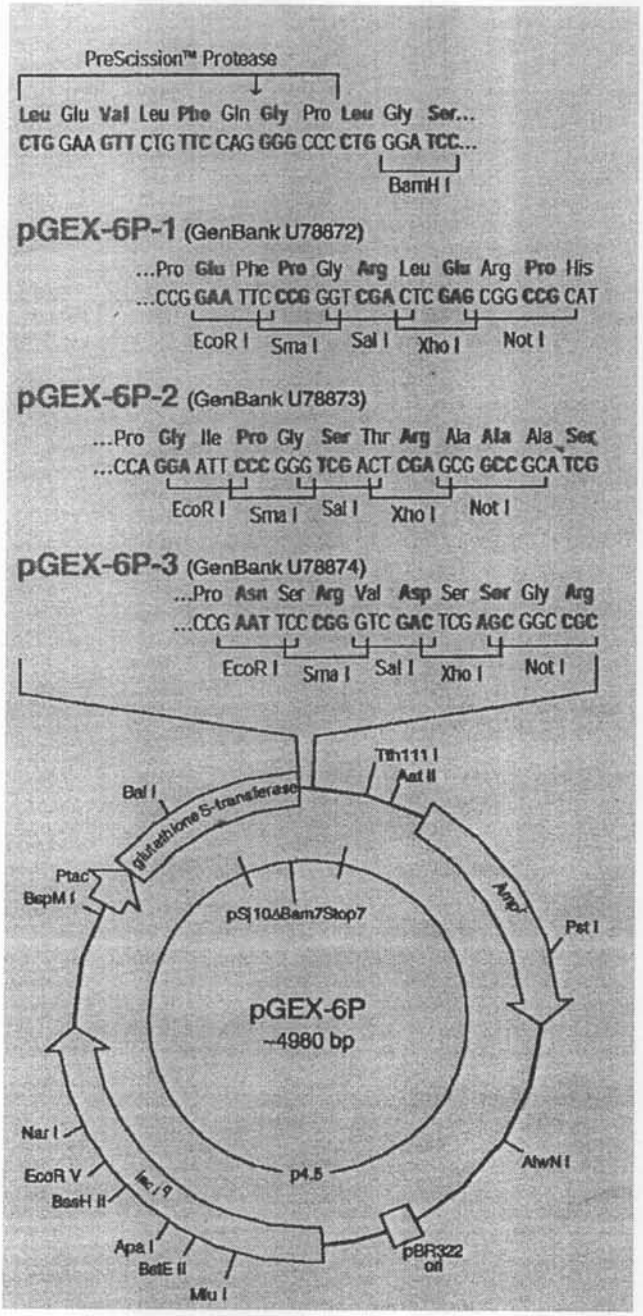


Figure 5.1 Map of pGEX-6 vectors showing reading frames and main features

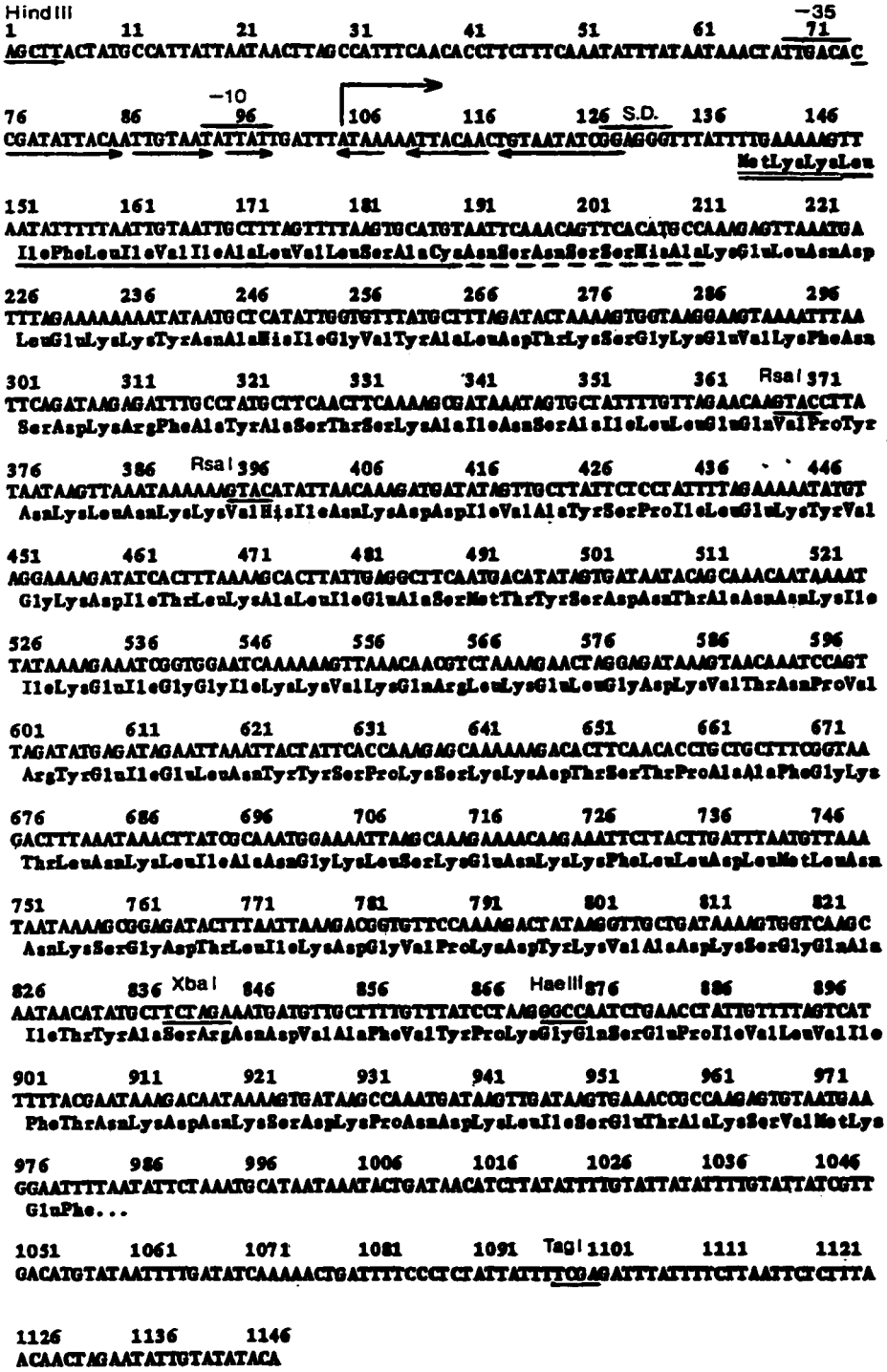


Figure 5.2 Nucleotide sequence of the *pc1* gene and the deduced amino acid sequence of the *S. aureus* β-lactamase precursor (after Wang and Novick., 1985). The -35 and -10 transcriptional initiation signals and the Shine-Dalgarno (S.D.) sequence are indicated. A prominent inverted repeat, which may be involved in regulation, is indicated by the horizontal arrows below the sequence. The transcription initiation site is marked with an arrow above the sequence. The regions of the signal peptide are underlined as follows: ═, positively charged residues adjacent to the initiator; —, hydrophobic stretches; -----, hydrophilic region between the N terminus of the membrane-bound enzyme and the stable secreted form.

However, the N-terminal amino acid sequence of PenPC purified form *E. coli* was different from those from *B. subtilis* or *B. cereus* (Figure 5.3; Mezes *et al.*, 1985). While the form of PenPC purified from *E. coli* RR1/pRWY22 has been processed at a single site after Ala 25 in the signal peptide, the two processed forms of PenPC purified from *B. subtilis* carrying *penPC* were both smaller that the *E. coli* product. The amino acid sequence from Phenylalanine 26 to Valine 42 of PenPC signal peptide may be regarded as a part of the mature enzyme in *E. coli*. Therefore, primer LHB11 (5'-cggca**GGATCC** acaagcctagaagcttttac-3') was designed to amplify the *penPC* gene encoding mature enzyme with primer LHB8 from pYCL18; Primers LHB11 and LHB7 were used to amplify the different hybrid genes generated from crossing *penPC* and *pcI* from the plasmids containing these genes. The PCR conditions for the amplification were described in Chapter 2. These PCR products were digested with restriction enzymes *Bam*HI and *Sal*I, and DNA fragments were purified and ligated with similarly digested pGEX-6P-1 (Figure 5.4). A 1.0 kb DNA fragment was seen from the agarose gel when the recombinant plasmid was digested by *Bam*HI and *Xho*I (Figure 5.5). Finally, two primers specific for the pGEX vector (5' pGEX sequencing primer 5'-gggctggcaagccacgtttggtg-3' and 3' pGEX sequencing primer 5'-ccgggagctgcatgtgtcagagg-3') were used for DNA sequencing.

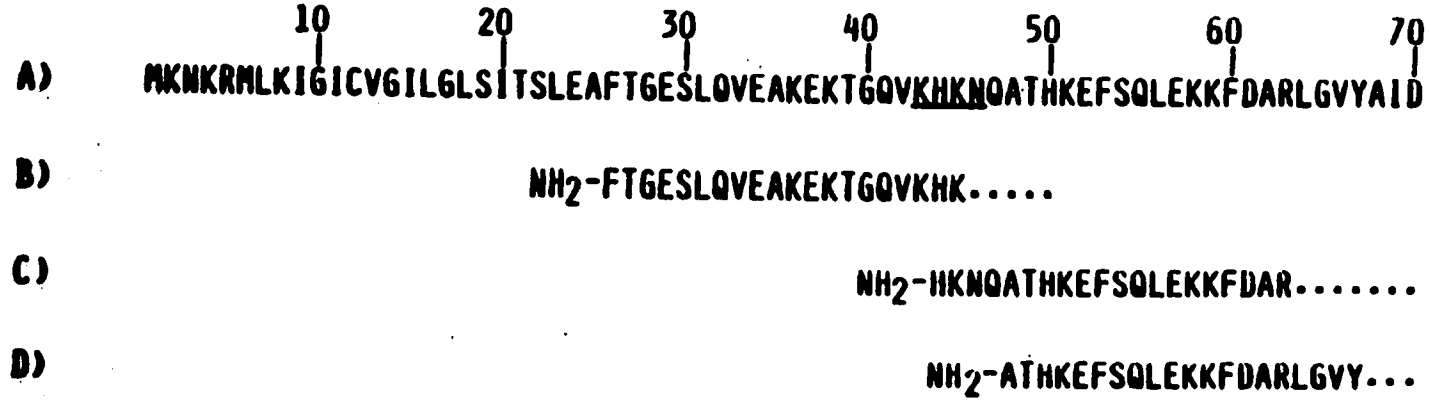
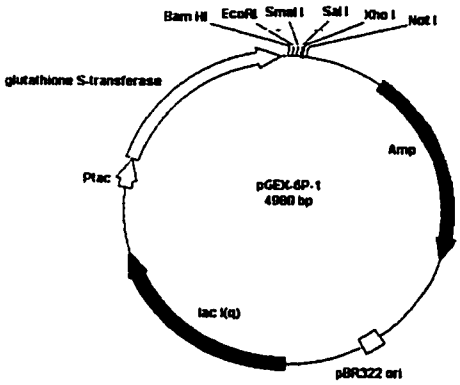


Figure 5.3 N-terminal amino acid sequences of PenPC purified from *E. coli*/pRWY22 and *B. subtilis*/pRWM5.
A) amino acid sequence of the first 70 residues of penPC predicted from DNA sequence data. Previously known N termini of processed forms of the enzyme (Ambler, 1980) are underlined;
B) N terminus and sequence of the PenPC from *E. coli*;
C) N terminus sequence of the large form of PenPC from *B. subtilis*;
D) N terminus sequence of the small form of PenPC from B. *subtilis*.



PCR product of beta-lactamase

Digested by *Bam*HI and *Sal* I, and ligated together, and then transformed into competent *E. coli* DH5α cells

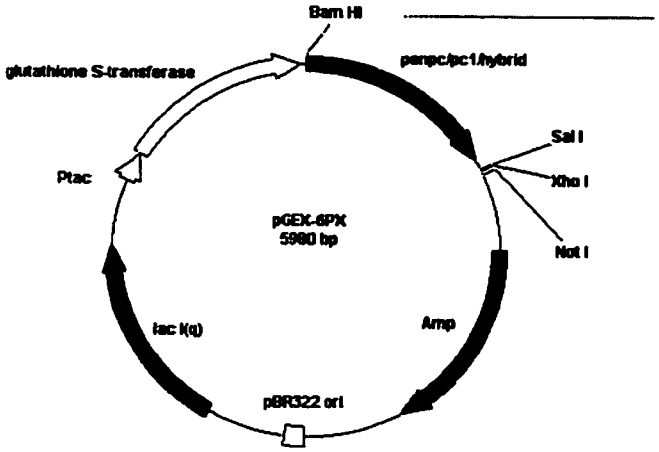


Figure 5.4 Construction of pGEX-6P-1 derivatives containing various β-lactamase genes

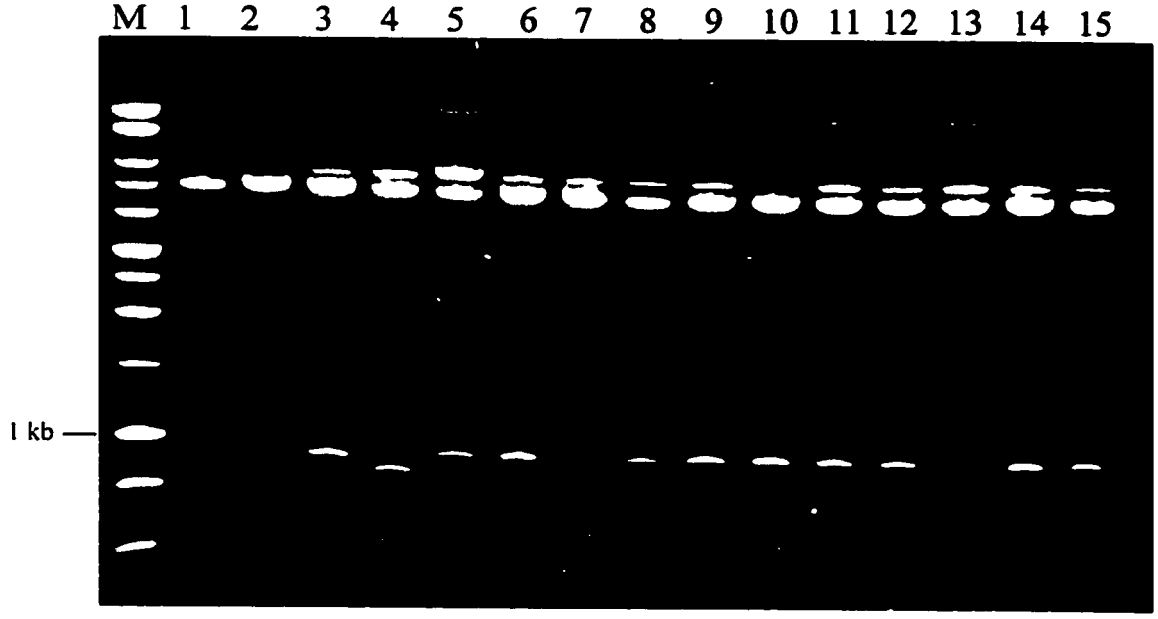


Figure 5.5 Analysis of recombinant plasmids by digestion with restriction enzymes. With 1kb DNA Ladder as marker (M), different digestion samples were analyzed in 0.8% agarose gel. Lane 1 is pGEX-6P-1digested with *Bam*HI, Lanes 2-15 are the *Bam*HI-*Xho*I-digested samples of pGEX-6P-1, pGEXpenPC, pGEXpc1, pGEXhybrid1, pGEXhybrid2, pGEXhybrid3, pGEXhybrid4, pGEXhybrid5, pGEXhybrid6, pGEXhybrid7, pGEXhybrid8, pGEXhybrid9, pGEXhybrid10, and pGEXhybrid11, respectively.

5.3 Expression of GST-β-lactamases fusion proteins in *E. coli*

For protein expression, as mentioned in Chapter2, the DH5α cells carrying different pGEX-6P-1 derivatives were grown in 2 x TY medium at 37°C with vigorous shaking. When the OD_{600} of the cultures was about 0.6-0.8, IPTG was added to a final concentration of 0.1 mM, and growth was continued at 37°C for 3 h. BL21 cells transformed with pGEX-6P-1 were similarly treated as a control. As shown in Figure 5.6, GST and GST fusion proteins with various β-lactamases were successfully expressed at high levels from *E. coli*. For Hybrid9 and Hybird10, the sizes of their GST fusion proteins are smaller than the other fusion proteins, due to the fact that a stop codon was generated downstream of the crossover site by homologous recombination in these two cases.

5.4 Solubility of expressed GST-β-lactamase fusion proteins

High-level heterologous protein expression in *E. coli* often results in the formation of inclusion body. Before protein purification is carried out, the solubility of the expressed GST-β-lactamase has to be confirmed. Therefore, after protein expression, the cells were harvested and resuspended in PBS solution, and subjected to sonication. For each expressed protein, same volume of total cell lysate and supernatant after centrifugation were collected and applied to SDS/PAGE to estimate

the solubility of the proteins. As shown in Figure 5.7, while GST and GST fusion proteins with PenPC, PC1, Hybrid1, Hybrid2, Hybrid3, and Hybrid4 were highly soluble, the expressed GST-Hybrid5 was partially soluble, the other GST fusion proteins were found to be expressed as inclusion bodies.

Several parameters have been shown to influence inclusion body formation in *E. coli*. They include the transcription rate of the gene of interest, the growth temperature, the composition and pH of the culture medium, and the cellular localization of the overexpressed protein (Hockney, 1994). However, even many efforts were made to alter growth conditions, such as lowering the growth temperature during induction to 20°C, 25°C or 30°C, decreasing IPTG concentration to 0.05 mM, expressing fusion protein in different host stains [BL21, BL21(DE3)], no improvement on the solubility of these insoluble fusion proteins was achieved (data not shown).

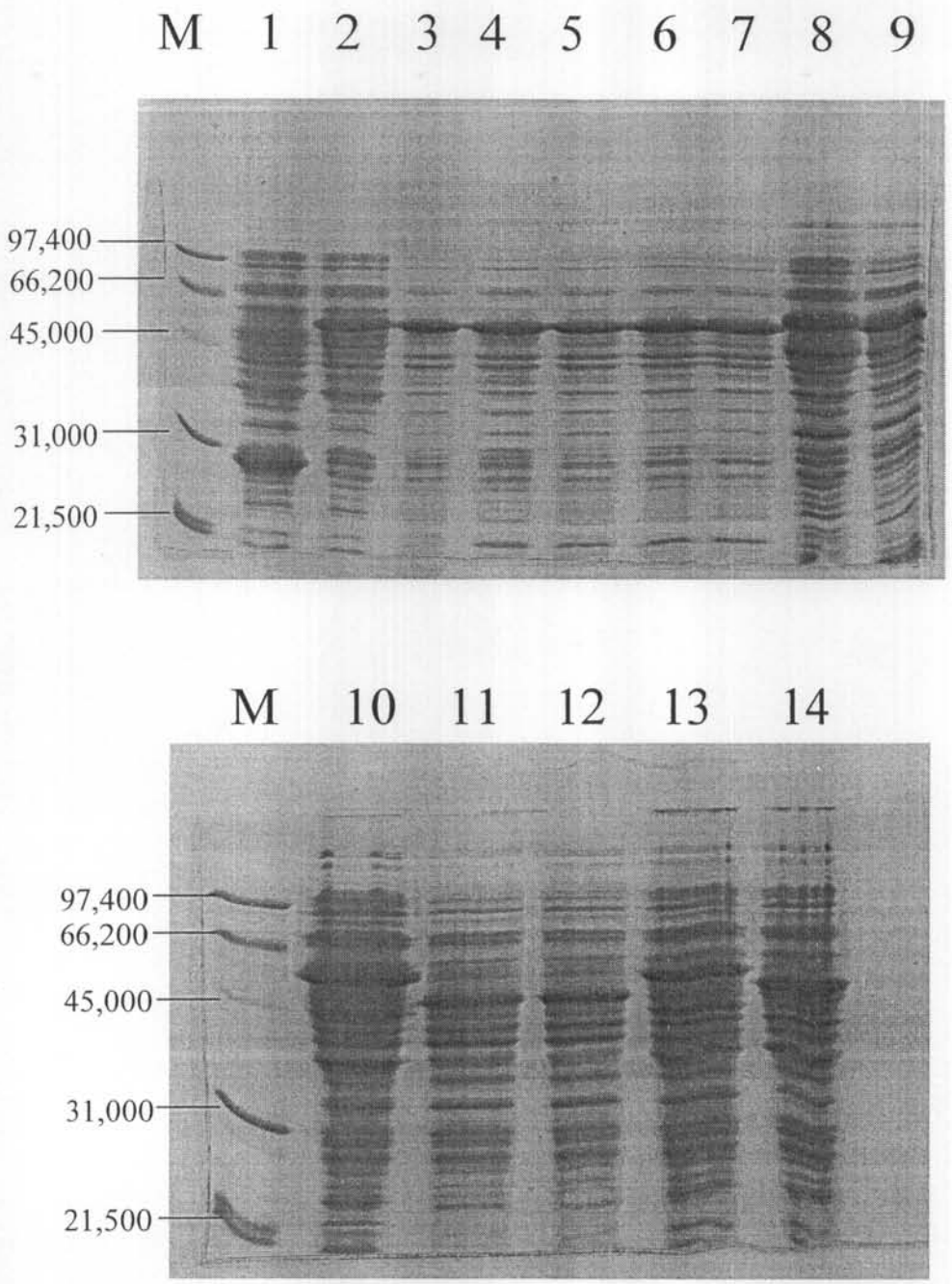


Figure 5.6 SDS/PAGE analysis of the expression of GST fusion proteins in *E. coli* DH5α cells. With low range proteins standard as marker (M), cell samples were analyzed by 12% SDS/PAGE. Lanes 1-14 are the samples from the cells expressing GST, GST-PenPC, GST-Hybrid1, GST-Hybrid, GST-Hybrid3, GST-Hybrid4, GST-Hybrid5, GST-Hybrid6, GST-Hybrid7, GST-Hybrid8, GST-Hybrid9, GST-Hybrid10, GST-Hybrid11, and GST-PC1, respectively.

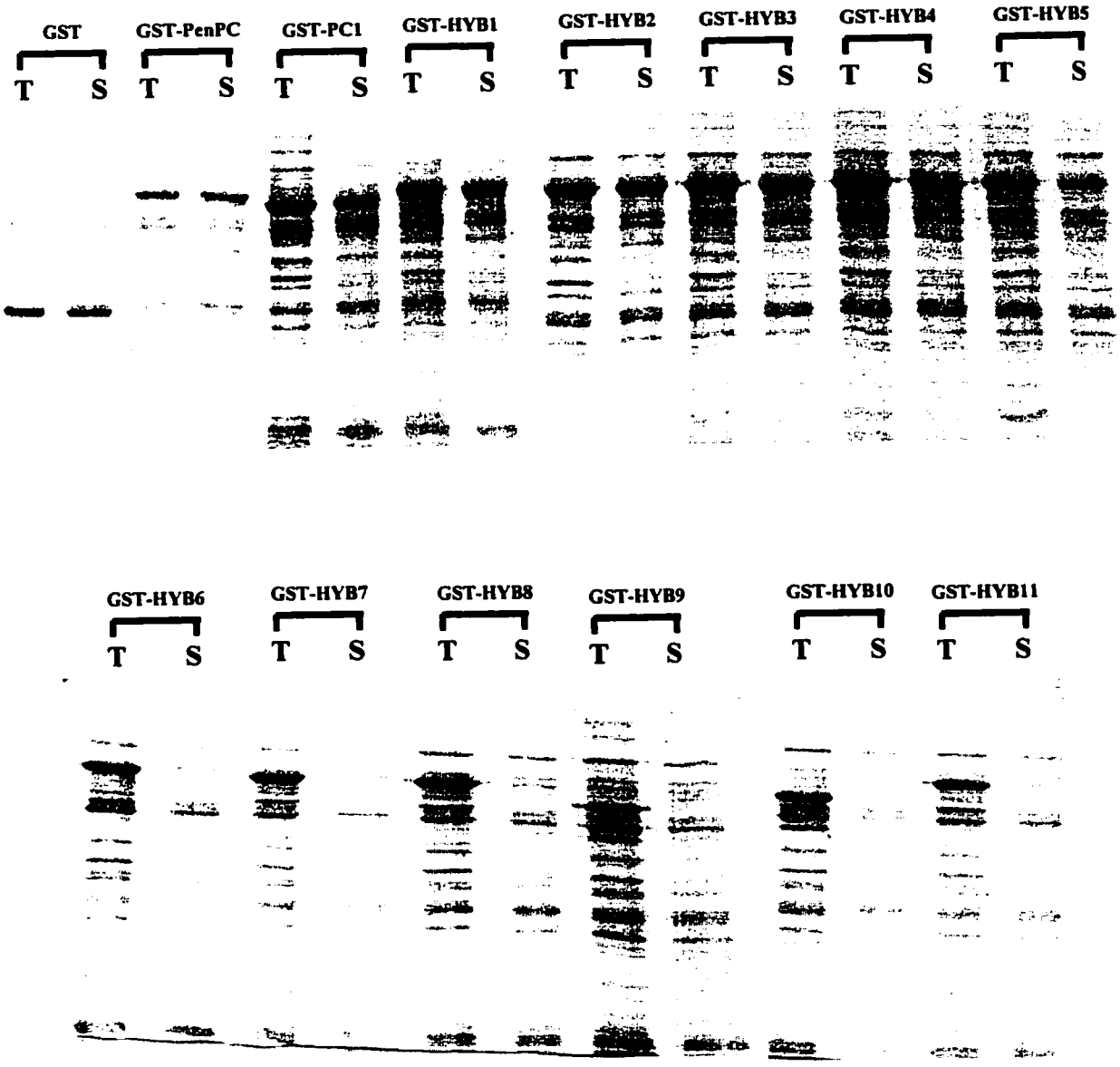


Figure 5.7 Solubility of the GST-β-lactamase fusion proteins produced in *E. coli* DH5α. Samples of the total (T) and soluble (S) intracellular protein fractions from cells producing each GST-β-lactamase fusion protein were prepared and analyzed by 12% SDS-PAGE.

It has become clear that alteration of the intracellular concentration of folding modulators can have a significant impact on the folding of many recombinant gene products (Georgiou and Valax, 1996; Thomas *et al.*, 1997; Thomas and Baneyx, 1997; Wall and Pluckthun, 1995). When *E. coli* is subjected to a variety of stresses, including temperature upshift, exposure to organic solvents and the accumulation of misfolded proteins, the synthesis of 20-30 heat-shock proteins (hsps) is transiently upregulated in order to repair cellular damage. The increased transcription of hsps results from higher concentrations of the heat-shock transcription factor δ^{32}, which directs the RNA polymerase core enzyme to heat-shock gene promoters (Gorss, 1996). Although the cellular function of many hsps remains unclear, several are known to be ATP-dependent proteases and molecular chaperones. The role of the latter proteins is to help nascent and partially folded polypeptides to reach a proper conformation or cellularlocation. The two major chaperone systems present in the cytoplasm of *E. coli* are the DnaK-DnaJ-GrpE and GroEL-GroES folding machines (Hartl, 1996). Co-overexpression of components of either set of chaperones can significantly improve the solubility and/or secretion of many structurally and functionally unrelated recombinant polypeptides. In addition, indirect methods leading to an increase in the concentration of chromosomal hsps, including growth of the cells at high temperatures, mutations in negative regulators of the heat-shock response, and co-overexpression of

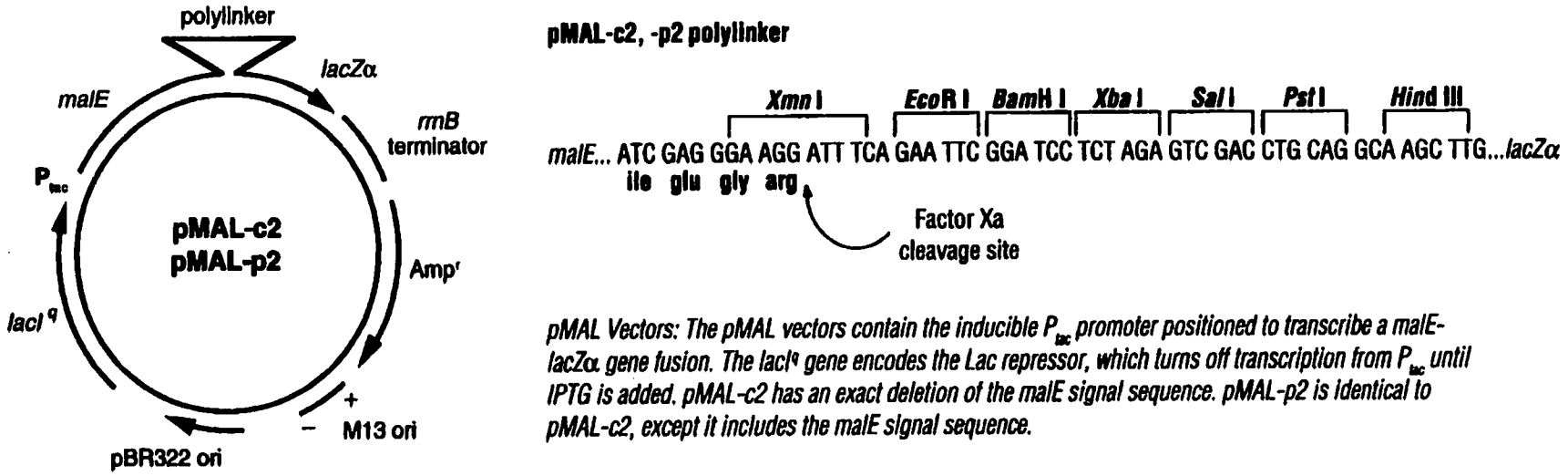
plasmid-encoded δ^{32}, have also proven beneficial to the folding and secretion of certain overexpressed proteins (Thomas and Baneyx, 1997). Ethanol is one of the most powerful elicitors of the heat-shock response in *E. coli*. Its addition to the growth medium has been shown to increase the recovery yield of many overexpressed proteins (Thomas and Baneyx, 1997). However, the addition of ethanol (3%) to 2 x TY medium or the heat-shock of cells at 42°C for 2 min after the addition of IPTG was proved to have no impact on the solubility of these GST fusion β-lactamases (data not shown).

Therefore, it seems that the low solubility or insolubility of these GST fusion β-lactamases might not be related to the expression conditions but might be due to the expressed proteins themselves. There is no direct correlation between the propensity of the inclusion body formation of a certain protein and its intrinsic properties, such as molecular weight, hydrophobicity and folding pathways (Mitraki, 1991). The formation of inclusion body might result from the incorrect protein folding when these hybrid β-lactamases were expressed in the cytosol.

5.5 Expression of β-lactamases as fusion with MBP

It is proposed that *E. coli* maltose-binding protein (MBP) is particularly effective in promoting the solubility of polypeptides with which it is fused (Kapust and Waugh,

1999). The solubility of many proteins was enhanced when they were fused with MBP (Kapust and Waugh, 1999; Nomine *et al.*, 2001; Nomine *et al.*, 2001; Wang *et al.*, 1999). In order to improve the solubility of β-lactamases, the different β-lactamase genes were subcloned into the pMAL-c2 vector (Figure 5.8). The different β-lactamase genes were cut by restriction enzymes from their individual pGEX-6P-1 derivatives and ligated with similarly digested pMAL-c2. The recombinant plasmids were screened by restriction enzyme digestion and further confirmed by DNA sequencing. Two primers for the pMAL vectors (*malE* primer 5'-GGTCGTCAGACTGTCGATGAAGCC -3' for sequencing downstream from the *malE* gene across the polylinker and M13/pUC sequencing primer 5'-CGCCA GGGTTTTCCCAGTCACGAC-3' for sequencing upstream from the *lacZα* gene across the polylinker) were used for DNA sequencing. When expressed in DH5α, all expressed MBP-β-lactamases were found to be highly soluble (Figure 5.9). According to the crossover site distribution of the hybrid enzymes (Figure 5-10), MBP-Hybrid1, MBP-Hybrid4, MBP-Hybrid5, MBP-Hybrid6, MBP-Hybrid7, MBP-Hybrid8, MBP-Hybrid11, as well as MBP-PenPC, and MBP-PC1, were transformed into BL21 (DE3), expressed in a large scale and used for further studies.



pMAL Vectors: The pMAL vectors contain the inducible P_{tac} promoter positioned to transcribe a malE-lacZα gene fusion. The lacI^q gene encodes the Lac repressor, which turns off transcription from P_{tac} until IPTG is added. pMAL-c2 has an exact deletion of the malE signal sequence. pMAL-p2 is identical to pMAL-c2, except it includes the malE signal sequence.

Figure 5.8 Map of the pMAL fusion protein vectors showing reading frames and main features

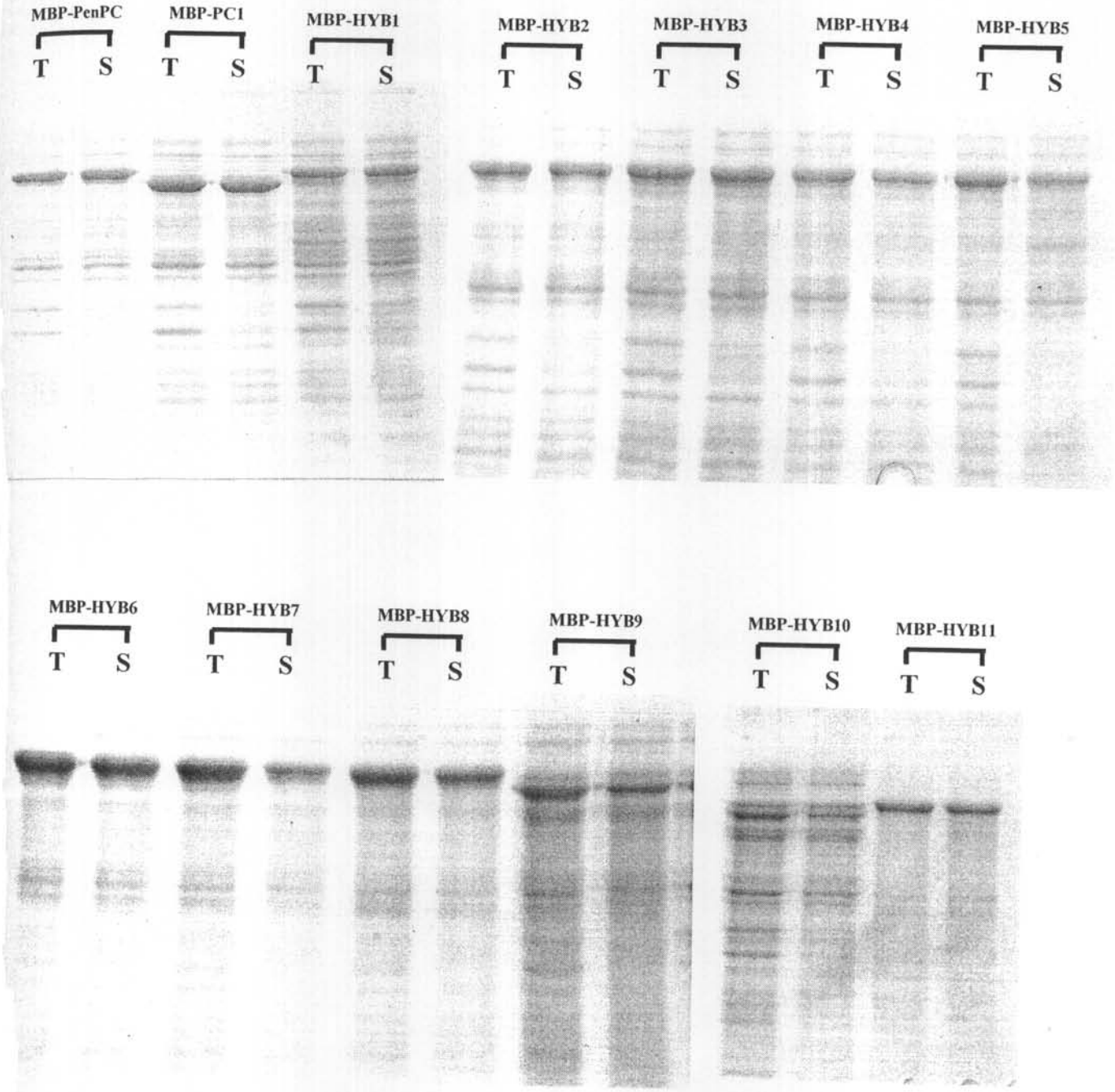


Figure 5.9 Solubility of MBP- β-lactamase fusion proteins produced in *E. coli* DH5α. Samples of the total (T) and soluble (S) intracellular protein fractions from cells producing each MBP-β-lactamase fusion protein were prepared and analyzed by 10% SDS-PAGE. HYB is the abbreviation of Hybrid.

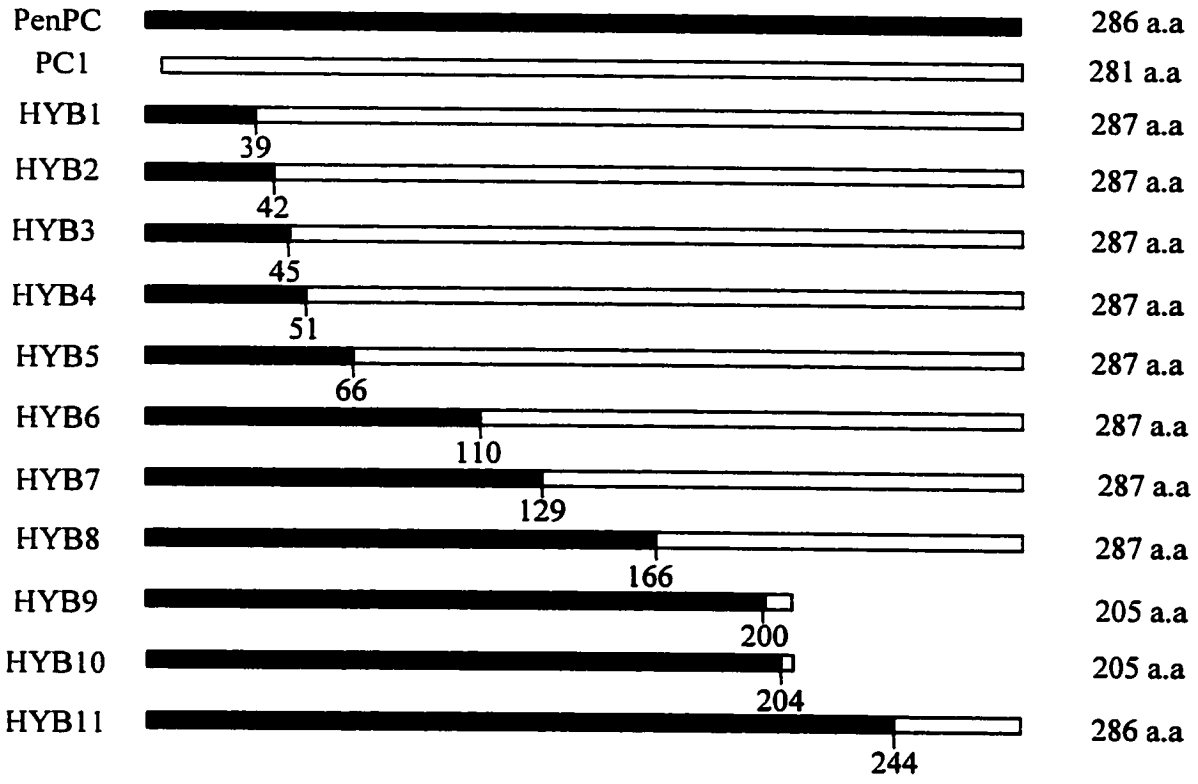


Figure 5.10 Schematic alignment of the primary structures of various hybrid β-lactamases after translation from the generated hybrid genes. The black regions represent the N-terminal sequence derived from *B. cereus* 569/H β-lactamase I while the white region represents the C-terminal sequence derived from *S. aureus* PC1 β-lactamase .The length of the whole enzyme and the length of the fragment from PenPC of each hybrid are also shown on the right and under the fusion point, respectively. HYB is the abbreviation of Hybrid.

5.6 Expression and purification of MBP-β-lactamase fusion proteins

The detail of the expression and purification was described in Chapter 2. Figure 5.11 shows the purification profile of the MBP-Hybrid6 fusion protein in amylose affinity column. The samples in different fractions were also analyzed by SDS/PAGE (Figure 5.12), very pure fusion protein was found in the fraction 60 to fraction 70, the impurities in other fractions within the elution peak may be due to protein degradation by host proteases. SDS/PAGE analysis of the purification of other MBP fusion proteins was also outlined in Figure 5.13. More than 90% pure MBP fusion proteins were obtained by one-step affinity chromatography (Figure 5.14), the yield for different fusion proteins varies from 50-100 mg/L.

Many studies in which proteins have been expressed using the pGEX vectors have failed to find significant differences in antigenicity, enzyme activity, or binding properties between the GST fusion protein and the cleaved product (Smith, 2000). The attachment of MBP to the N-terminus does not interfere with the function of some proteins, and several groups have used fusion proteins without cleavage of the MBP for structure-function studies (Sachdev and Chirgwin, 2000). Two proteins essential for nucleotide excision repair in human were expressed solubly in *E. coli* as fusion to MBP and complemented excision repair activity in mutant cell-free extracts (Park and Sancar, 1993). A MBP-DnaJ fusion protein expressed in *E. coli* and purified to

homogeneity was able to carry out the functions of DnaJ, such as stimulating ATPase activity of DnaK and preventing aggregation of denatured protein structure (Ishii *et al.*, 1998), indicating that fusion of MBP did not affect the functions of DnaJ. From a practical point of view, the cleavage of fusion proteins *in vitro* and their repurification to remove the bacterial partner are rate-limiting steps in terms of both time and expense. Because the structure and folding of MBP are well characterized, many experiments can be carried out with purified but uncleaved MBP fusions (Sachdev and Chirgwin, 2000). β-Lactamases have never been expressed as MBP fusions. In order to examine whether fusion to MBP will affect the functions of β-lactamases, the purified MBP-β-lactamases were cleaved with factor Xa into two parts: MBP and β-lactamase (Figure 5.15). The specific activities of all MBP-β-lactamases with penicillin G, penicillin V, ampicillin and nirtrocefin as substrates were found to be almost identical to those of the cleaved proteins. This indicated that fusion to MBP did not have any significant effect on the catalytic functions of β-lactamases.

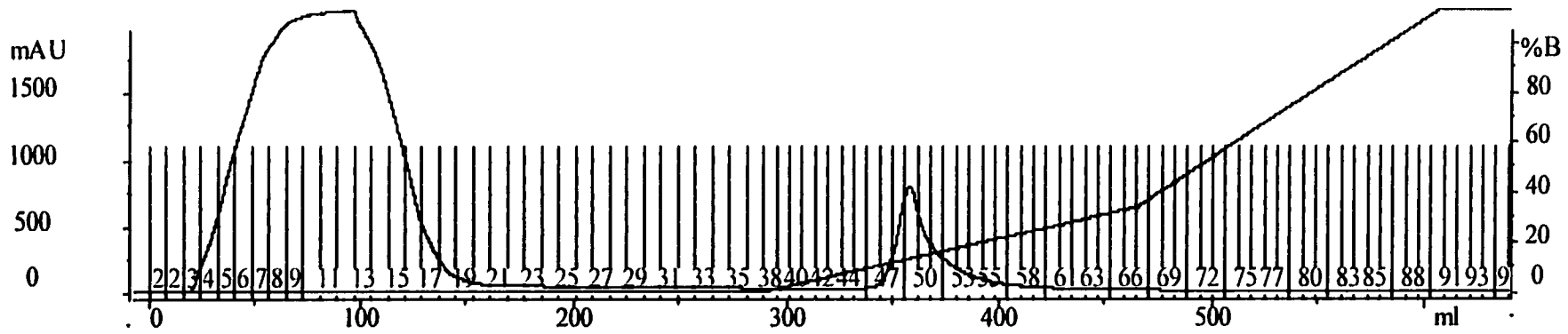


Figure 5.11 The purification profile of MBP-Hybrid6 fusion protein from an amylose affinity column. Extracted protein solution was applied to a 10 mm x 60 cm column packed with amylose affinity gel(New England Biolabs), which was pre-equilibrated with MBP column buffer (pH 7.4). After washed with the column buffer, the column was eluted with a gradient of two segments (0 – 4 mM and 4 – 10 mM maltose) and MBP-Hybrid6 was eluted from the column in the first segment. 100% B is equal to 10 mM maltose, and 1 mAU is approximately equal to protein concentration of 1 mg/ml.

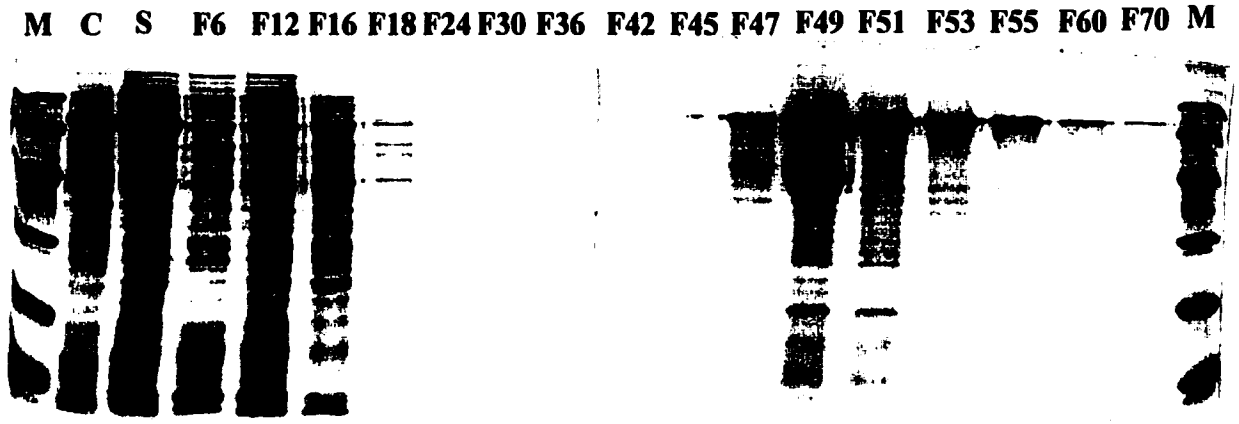


Figure 5.12 SDS/PAGE analysis of protein samples in different fractions from the purification of MBP-Hybird6. Where M indicates the low range protein molecular weight standard, C is the protein sample from the cell cultures before harvest, S is the protein sample before chromatography, and F6 to F70 indicate the different fraction samples after chromatography.

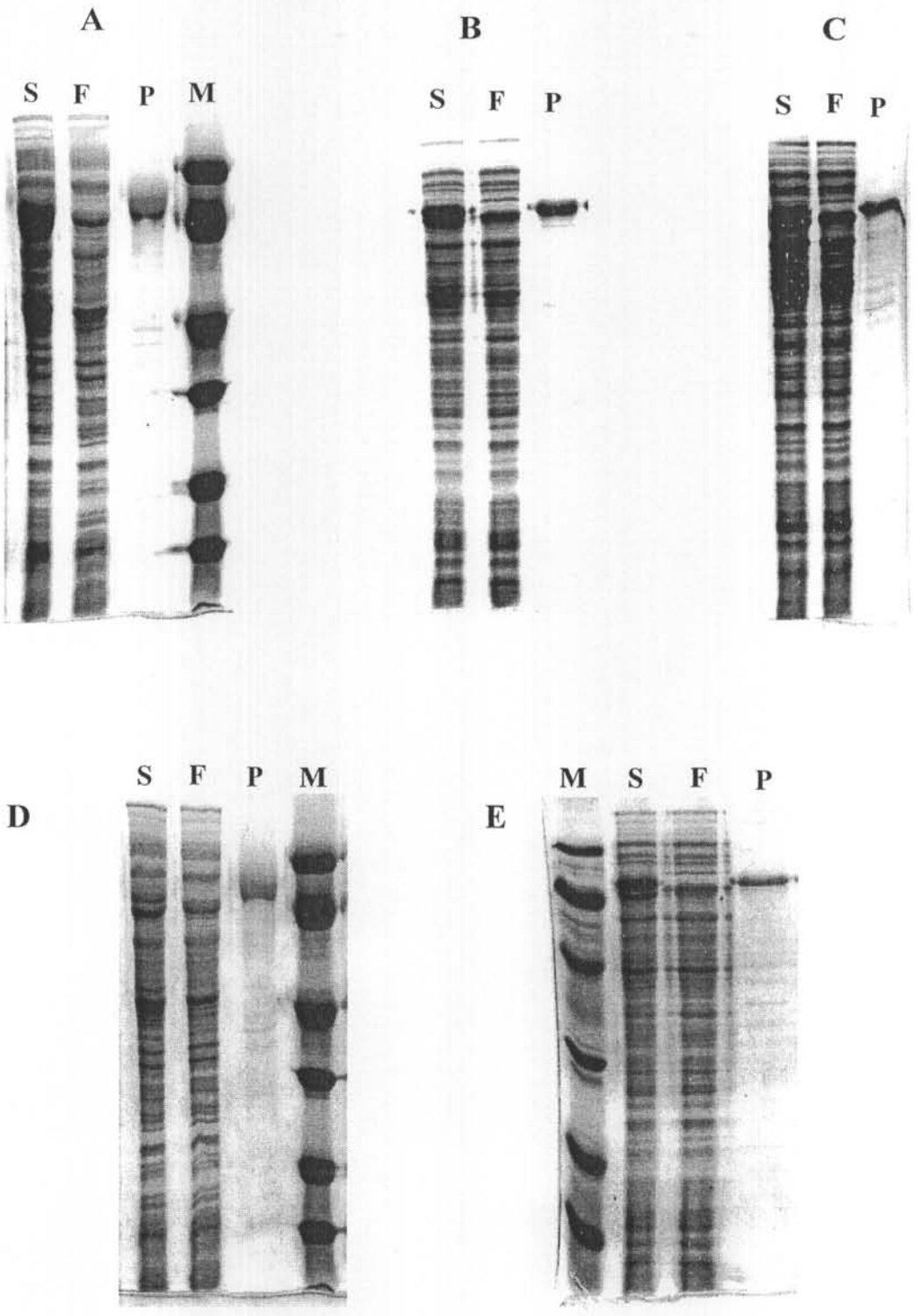


Figure 5.13 Analysis of purifications of MBP-Hybrid1 (A), MBP-Hybrid4 (B), MBP-Hybrid5 (C), MBP-Hybrid7 (D), MBP-Hybrid11 (E) fusion protein by SDS/PAGE. Proteins were stained with Coomasie Blue. Lanes show protein electrophoresis of cell lysate supernatant (S), unbound proteins in flow-through (F) and eluted pure fusion protein (P). The sizes of purified proteins were estimated by comparing with the low rang protein standard (M).

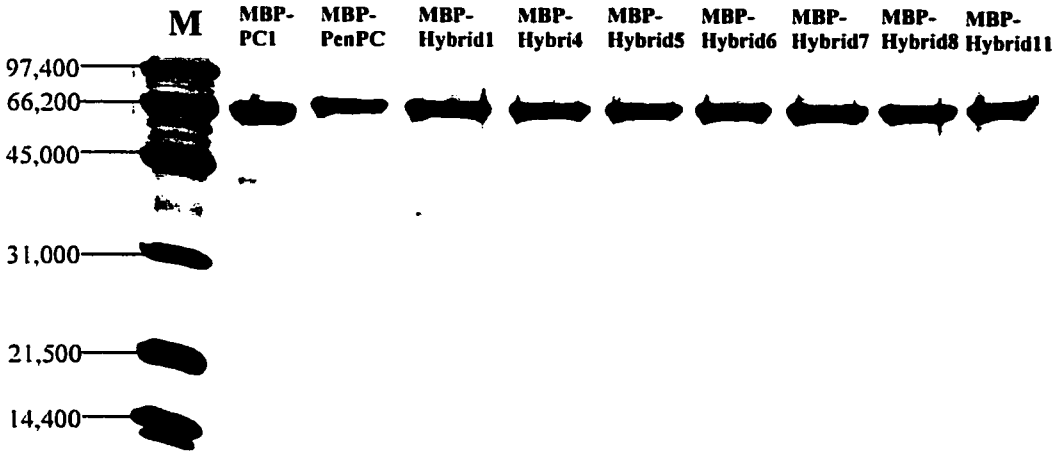


Figure 5.14 Analysis of purified MBP fusion proteins by SDS/PAGE

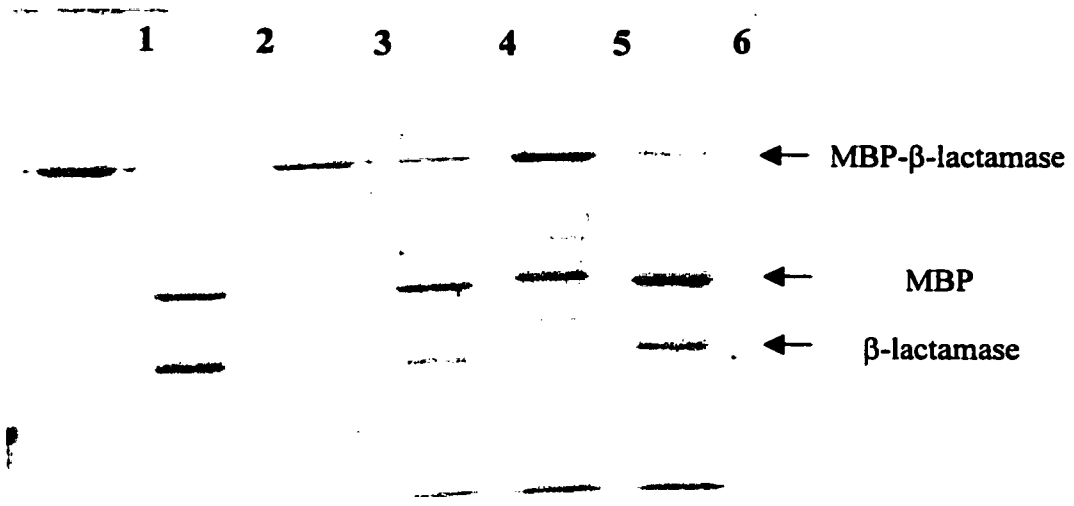


Figure 5.15 SDS/PAGE analysis of the cleavage of MBP-β-lactamases by factor Xa. Lane 1, MBP-Hybrid11; lane 2, MBP-Hybrid11 cleaved by factor Xa; lane 3, MBP-Hybrid8; lane 4, MBP-Hybrid8 cleaved by factor Xa; lane 5, MBP-Hybrid5; lane 6, MBP-Hybrid5 cleaved by factor Xa.

5.7 Signal peptidase contaminants during fusion protein purification

When the purified GST-PenPC and GST-PC1 were stored at 4°C in the elution buffer for two weeks, we were surprised to find that two protein bands of small sizes were seen from SDS/PAGE, which corresponded to GST and β-lactamase. The same phenomenon was also found in the purified MBP-PenPC, two protein bands corresponding to MBP and β-lactamase were visualized from SDS/PAGE. It seems that there were some signal peptidase activities in these samples. Signal peptidases remove targeting peptides from pre-proteins and play central role in the secretary pathway, as well as in the delivery of proteins to the mitochondrial intermembrane space and to the luman of thylakoids. The catalytic mechanism of pre-protein cleavage has long been an enigma, but data from site-directed mutagenesis and sequence alignment studies suggest that signal peptidases may constitute a new type of serine protease, mechanistically related to the β-lactamases (Dalbey and Von Heijne, 1992). β-Lactamases were also found to be able to hydrolyze peptides, esters and S-esters (Damblon *et al.*, 1995; Rhazi *et al.*, 1999). It would be very interesting to learn whether β-lactamases themselves or signal peptidases contaminants were contributing to the signal peptidase activities.

On-column cleavage was carried out to test the signal peptidase activity. After expression, the cell pellet was resuspended in PBS lysis buffer and subjected to

sonication. The suspension was centrifuged and supernatant was collected and loaded to affinity column (see Chapter 2 for detail). Following washing, the column was closed at both ends, sealed and kept at room temperature (about 20°C). For every 12-16 hours, one fraction was collected and fresh PBS was added to the column. After a total of 4-5 fractions were collected, the residual proteins were eluted from the column by the addition of Glutathione Elution Buffer (Chapter 2). All the samples from different stages of GST-PC1 purification were analyzed by SDS/PAGE (Figure 5.16) and enzyme activity assay. The pure PC1 enzyme was found in some fractions. The same result was observed during purification of GST-PenPC. It suggests that β-lactamase is cleaved and released during incubation. The band corresponding to PC1 was then excised and subjected to N-terminal protein sequencing, the first 9 amino acids of the N-terminal region of the protein were determined to be "KELNDLEKK". According to the deduced primary sequence of the GST-PC1 fusion protein (Figure 5.17), the PC1 was surprisingly cleaved exactly without any other amino acids from its fusion partner. It is obvious that there is the existence of some peptidases.

In order to see whether the peptidase activity is from β-lactamase, lithium clavunate, a β-lactamase mechanism-based inhibitor, was added to PBS during protein cleavage (Figure 5.18). Even β-lactamase activity was totally diminished by the

inhibitor (the specific activity of PC1 in PBS without inhibitor is about 0.48 mM/min/μg), the cleavage still occurred. It suggests that the cleavage is not due to β-lactamase itself, but the contaminant of host signal peptidases. The signal peptidases from *E. coli* are membrane–bound endopeptidases, less clarified may contain some membrane components with signal peptidases that result in the cleavage of fusion protein. To decrease the contaminant of membrane components, the cell lysate was centrifuged at 39,191g (maximum RCF for JA20 rotor) for 3 h (the normally used time is 30 min) and further clarified by filtration through a 0.45 μM filter before applying it to the column. From the result shown in Figure 5.19, the cleavage of the fusion was drastically alleviated by using more clarified lysate, which further support the suggestion that the cleavage of fusion proteins may be due to the existence of signal peptidases in the lysate.

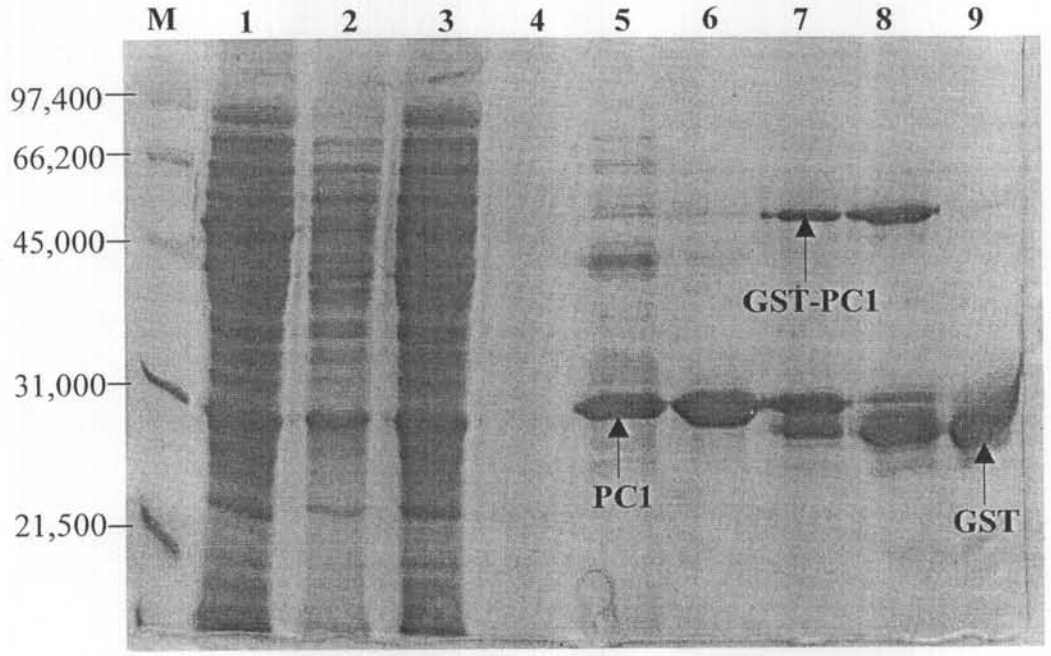


Figure 5.16 SDS/PAGE analysis of the steps in the purification strategy. Samples were separated on a 12% polyacrylamide gel. Lane 1, supernatant of total protein extract of induced culture after centrifugation; lane 2, the first collection of flow-through from Glutathione 4 B column; lane 3, the third collection of flow-through from Glutathione 4 B column; lane 4, the collection of wash from the column; lane 5, the first collection of cleaved protein; lane 6, the third collection of cleaved protein; lane 7, the first collection of eluted protein by Glutathione Elution Buffer; lane 8, the second collection of eluted protein; lane 9, the third collection of eluted protein.

MSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFE
LGLEFPNLPYYIDGDVKLTQSMAIIRYIADKHNMLGGCPKERAEISMLE
GAVLDIRYGVSRIAYSKDFETLKVDFLSKLPEMLKMFEDRLCHKTYLN
GDHVTHPDFMLYDALDVVLYMDPMCLDAFPKLVCFKKRIEAIPQIDK
YLKSSKYIAWPLQGWQATFGGGDHPPKSDLEVLFQGPLGSKELNDLE
KKYNAHIGVYALDTKSGKEVKFNSDKRFAYASTSKAINSAILLEQVPY
NKLNKKVHINKDDIVAYSPILEKYVGKDITLKALIEASMTYSDNTANN
KIIKEIGGIKKVKQRLKELGDKVTNPVRYEIELNYYSPKSKKDTSTPAA
FGKTLNKLIANGKLSKENKKFLLDLMLNNKSGDTLIKDGVPKDYKVA
DKSGQAITYASRNDVAFVYPKGQSEPIVLVIFTNKDNKSDKPNDKLISE
TAKSVMKEF

Figure 5.17 The primary structure of the expressed GST-PC1 fusion protein. The green region is the N-terminal sequence derived from GST; the blue region indicated the PreScission protease recognition site, Leu-Phe-Gln/Gly-Pro; the red region is encoded by restriction sites from the vector; and the black region is the mature PC1 protein sequence, the sequence confirmed by N-terminal sequencing is underlined.

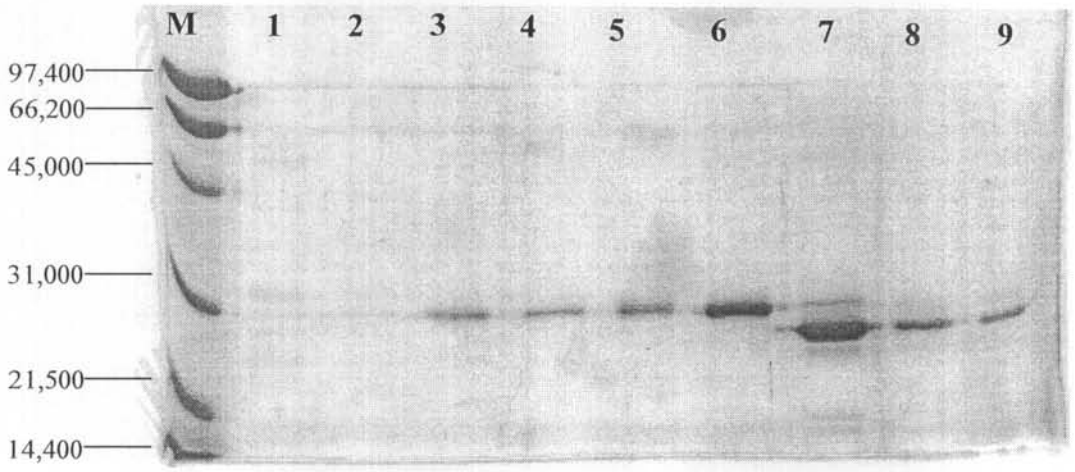


Figure 5.18. SDS/PAGE analysis of the influence of β-lactamase mechanism-based inhibitor, lithium clavulanate, on the cleavage of GST-PC1 fusion protein. Samples were separated on a 12% polyacrylamide gel. Lane 1, first fraction of cleaved protein in PBS; lane 2, second fraction of cleaved protein in PBS; lane 3, first fraction of cleaved protein in PBS with 1 mg/ml inhibitor; lane 4, the fourth fraction of cleaved protein in PBS with 1 mg/ml inhibitor; lane 5, the fifth fraction of cleaved protein in PBS with 1 mg/ml inhibitor; lane 6, the sixth fraction of cleaved protein in PBS with 1 mg/ml inhibitor; lane 7, the first fraction of eluted protein; lane 8, the second fraction of eluted protein; lane 9, the third fraction of eluted protein.

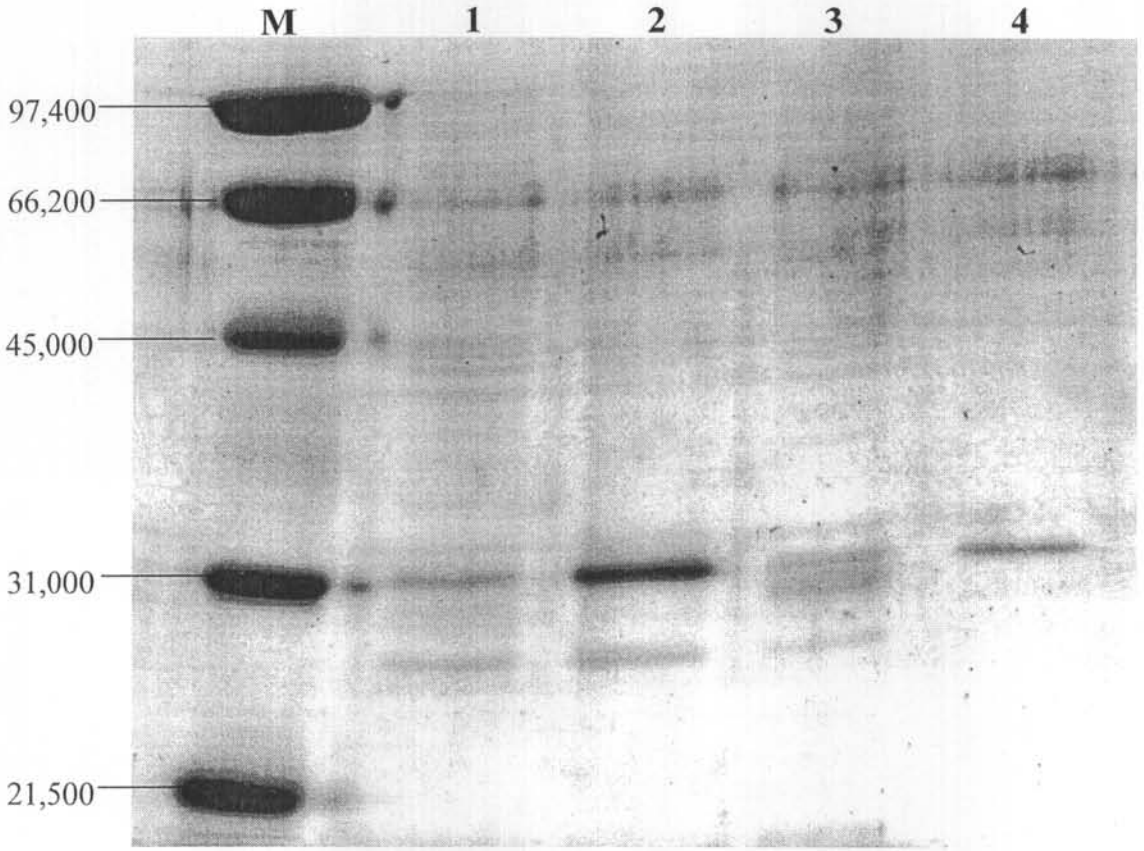


Figure 5.19 SDS/PAGE analysis of the influence of the clarity of lysate on the cleavage of fusion protein on-column. Samples were separated on a 12% polyacrylamide gel. Lane 1, first fraction of cleaved protein in PBS; lane 2, second fraction of cleaved protein in PBS; lane 3, third fraction of cleaved protein in PBS; lane 4, the fourth fraction of cleaved protein in PBS.

5.5 Discussion

Various β-lactamases (PenPC, PC1 and their hybrid β-lactamases) were expressed at high levels in the form of GST fusion proteins in *E. coli*. However, while GST fusion proteins with PenPC, PC1, Hybrid1, Hybrid2, Hybrid3 and Hybrid4 were highly soluble, the expressed GST-Hybrid5 was partially soluble, and other fusion β-lactamases were proved to be expressed mainly in inclusion bodies. Many efforts made to improve the solubility of these fusion proteins were demonstrated to be unsuccessful, including lowering the growth temperature during induction from 37°C to 20°C, 25°C or 30°C, decreasing IPTG concentration to 0.05 mM, expressing fusion protein in different host stains [BL21, BL21(DE3)] and the addition of ethanol (to a final concentration of 3%) to the growth medium. Therefore it seems that the low solubility or insolubility of these GST fusion β-lactamases is not related to the expression conditions but their protein sequences.

Point mutations that reduce the solubility of proteins were identified at the protein level about 30 years ago (Nathan, 1973), and their frequency emphasizes the fragile relationship between structure and solubility. Mutations that improve solubility are more interesting, especially if they can serve as a paradigm for how to alter other proteins to improve solubility. Several papers have described single point mutation that can completely alter activity and structure, decrease thermal stability without

affecting activity at lower temperatures or increase thermostability. One may draw on these examples when attempting to explain increases or decreases in the solubility of proteins produced in *E. coli* as a result of single amino acid changes. However, it is difficult to derive empirical rules from the examples at hand. Luck *et al.* (1992), for example, have observed that cysteine to serine mutants of bovine prolactin facilitate the extraction of protein into the sodium-deoxycholate buffer used to wash the pellet fraction after cell lysis. In other cases, cysteine to serine mutations have been shown to actually decrease protein solubility (Rinas *et al.*, 1992).

The amorphous appearance of most protein aggregates has led biochemists to consider folding-related aggregation to be a nonspecific process of little fundamental interest. The conventional model for this aggregation is that it is dominated by hydrophobic interactions of side chains that are normally buried in the native state, but that are more exposed in an unfolded, non-native state. Based on such a model, the existence of dramatic effects of single-residue replacements would not be predicted, given their small contribution to the net hydrophobicity (or other properties) of the polypeptide. However, the underlying structural order and dramatic mutational effects that can be observed in both amyloid and inclusion body formation suggest that aggregate formation may sometimes involve specific interactions of some kind that control the folding and stability of globular proteins.

In our studies, a sequence consisted of 12 amino acids is different between GST-Hybrid4 and GST-Hybrid5 with the comparison of protein sequences, but the solubility of the expressed GST-Hybrid5 is lower than that of the expressed GST-Hybrid4. Since the sequence of GST-Hybrid6 is more different from that of GST-Hybrid4, the expressed fusion protein was found totally insoluble. It is hard to delineate the coherence between structure and solubility of hybrid β-lactamases at this moment, however, it is quite obvious that the aggregation of expressed GST fusion proteins may be due to the misfolding of these proteins by the loss of specific interactions of some kind that control the folding and stability of globular proteins.

It is amazing that all β-lactamases were functionally expressed in *E. coli* as MBP fusion proteins with high solubility. Although many investigators have exploited the solubility-enhancing property of MBP (Fox *et al.*, 2001; Kapust and Waugh, 1999; Pryor and Leiting, 1997; Smith, 2000), the mechanism by which MBP increases the solubility of its passenger proteins is not well understood. One possibility, which seems to be consistent with all the available experimental evidences, is that MBP possessed chaperone-like qualities. These properties may allow MBP to bind reversibly to folding intermediates of its fusion partners, termed passenger proteins, and temporarily sequester them in a conformation that prevents their self-association and aggregation. According to this mode (Kapust and Waugh, 1999), the tether that

joints the two interacting partners facilitates iterative cycles of binding and release. By preventing the accumulation of a high concentration of unfolded, unsequestered passenger protein, this process may eventually steer the passenger protein toward its native conformation. Actually, proper folding of aggregation-prone passenger proteins fused to MBP has been reported in several cases (Kapust and Waugh, 1999; Mottershead *et al.*, 1996; Rao and Bodley, 1996; Thomas *et al.*, 1996).

The studies presented here also suggests that the cleavage of purified GST-PC1 and other GST-β-lactamases during storage is not due to the β-lactamases themselves, but very likely to the contaminants of the signal peptidases of host cells. The signal peptidases from *E. coli* are membrane-bound endopeptidases. Less clarified lysate may contain some membrane components with signal peptidase, which result in the cleavage of fusion protein. The work carried out by Tschant *et al.* (1995) shown that the detergent Triton-X100 is essential for optimal activity of signal peptidase. This is consistent with our earlier observations that the MBP-PC1 (or MBP-PenPC) was less efficiently cleaved in solution and on-column than GST-PC1, since no Triton-X100 was included during purification of the MBP fusion proteins. Centrifugation at 47,000 g for 20 min is required to clarify the cell lysate. However, the maximum RCF of the available JA-20 rotor was less than 40,000 g, which might cause the incompletely removal of signal peptidases in cell lysate. Based on the study,

more powerful centrifugation or the addition of a very efficient signal peptidase inhibitor might be able to diminish the cleavage of fusion protein during protein purification. On the other hand, the finding in this study may be used to develop a simple and rapid method for the isolation of soluble "native" proteins without the use of the expensive Factor Xa.

Chapter Six

Characterization of Hybrid β-lactamases

6.1 Far-UV circular dichroism analysis of β-lactamases

Circular dichroism (CD) is a valuable spectroscopic technique for studying protein structure in solution because many common conformational motifs, including α-helixes, β-pleated sheets and turns have characteristic far-UV (178-250 nm) CD spectra. For example, α-helices display large CD bands with negative ellipticity at 222 and 208 nm, and positive ellipticity at 193 nm, β sheets exhibit a broad negative band near 218 nm and a large positive band near 195 nm, while disordered extended chains have a week broad positive CD band near 217 nm and a large negative band near 200 nm. The spectrum of a protein is basically the sum of the spectra of its conformational elements, and thus CD can be used to estimate secondary structure (Greenfield, 1999). Furthermore, CD has many more facets than simply being a tool to estimate protein structure. For example, it is an excellent technique for determining the thermodynamics and kinetics of protein folding and denaturation and is unsurpassed for following the effects of mutations on protein folding and stability. In addition, it can be an excellent tool for following protein-ligand interactions. As sophisticated mathematical programs for fitting nonlinear equations and deconvoluting sets of curves using personal computers have become increasingly available, the analysis of thermodynamic and kinetic experiments, which can be performed by following

changes in CD spectra, have become much simpler, increasing the usefulness of the technique.

Far-UV (190-250 nm) CD spectra of purified MBP-β-lactamases were measured and shown to be very similar (Figure 6.1). The concentrations of the enzymes varied from 77.3 μg/ml to 115.5 μg/ml. The calculated secondary structures of different MBP-β-lactamases are listed in Table 6.1.

Table 6.1 The secondary structures of different MBP-β-lactamases

	α-Helix (%)	β-Sheet (%)	Turn (%)	Random (%)
MBP-PC1	29.3	18.4	29.9	22.4
MBP-Hybrid1	26.4	26.1	26	21.6
MBP-Hybrid4	23.4	31.2	23.8	21.5
MBP-Hybrid5	24.5	25.8	24.9	24.8
MBP-Hybrid6	20.5	39.6	19.7	20.1
MBP-Hybrid7	25.9	27.8	24.3	22
MBP-Hybrid8	24.5	29.5	24.1	21.8
MBP-Hybrid11	22.7	29.4	24.8	23.1
MBP-PenPC	30.4	10.2	33	26.4

From the calculation, while all fusion proteins share very similar overall folding, hybrid β-lactamase fusion proteins have slightly less amount of α-helix (23-26 %) than that of wild type β-lactamase fusion proteins (about 30%), with the exception of

MBP-Hybrid6, which has a more significantly lower amount of α-helix (20%). Interestingly, for some unknown reasons, the lower amount of α-helix in hybrid proteins was compensated by higher amount of β-sheet. It seems that hybrid fusion proteins show subtle alterations in folding. These differences may underlie their different biological activities.

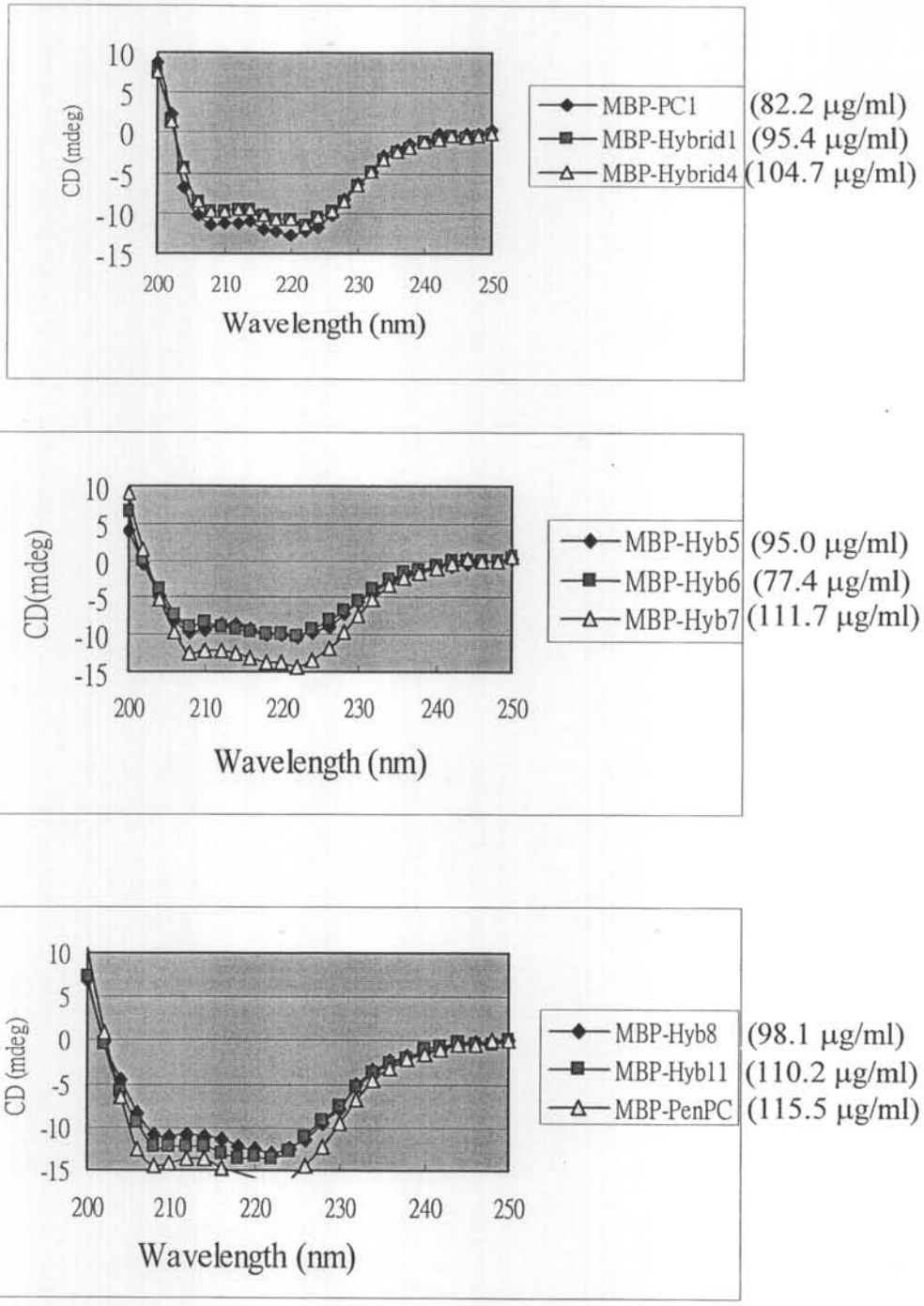


Figure 6.1 Far-UV circular dichroism spectra of MBP-β-lactamases. The protein samples were dissolved in the MBP column buffer (20 mM Tris-HCl, 0.2 M NaCl, 1 mM EDTA, pH 7.4) and their concentrations were 82.2 μg/ml, 95.4 μg/ml, 104.7 μg/ml, 95.0 μg/ml, 77.4 μg/ml, 111.7 μg/ml, 98.1 μg/ml, 110.2 μg/ml, 115.5 μg/ml for MBP-PC1, MBP-Hybrid1, MBP-Hybrid4, MBP-Hybrid5, MBP-Hybrid6, MBP-Hybrid7, MBP-Hybrid8, MBP-Hybrid11, MBP-PenPC, respectively.

6.2 Thermal denaturation of MBP-β-lactamases

Thermal denaturation of MBP-β-lactamases was monitored by measuring the far-UV molar ellipticity at 222 nm. Data were collected as a function of temperature with a scan rate of 1°C/min over the range of 20-90 °C in MBP column buffer (20 mM Tris-HCl, 0.2 M NaCl, 1 mM EDTA, pH 7.4). The protein concentrations for different samples varied from 178 μg/ml to 240 μg/ml. Melting curves obtained for some hybrid MBP fusion proteins, as well as MBP-PC1 and MBP-PenPC, are displayed in Figure 6.2A and 6.2B. Even though the midpoint temperatures of all these MBP-β-lactamases are very similar (57°C-62°C) (as shown in Figure 6.2C), the steepness of the unfolding curves is clearly different for all these proteins. The most cooperative transition on thermal denaturation is observed for MBP-PenPC. The denaturation curves for MBP-Hybrid4, MBP-Hybrid6 and MBP-Hybrid7 are not obviously different from those for other hybrid MBP-β-lactamases, therefore, they are not presented here.

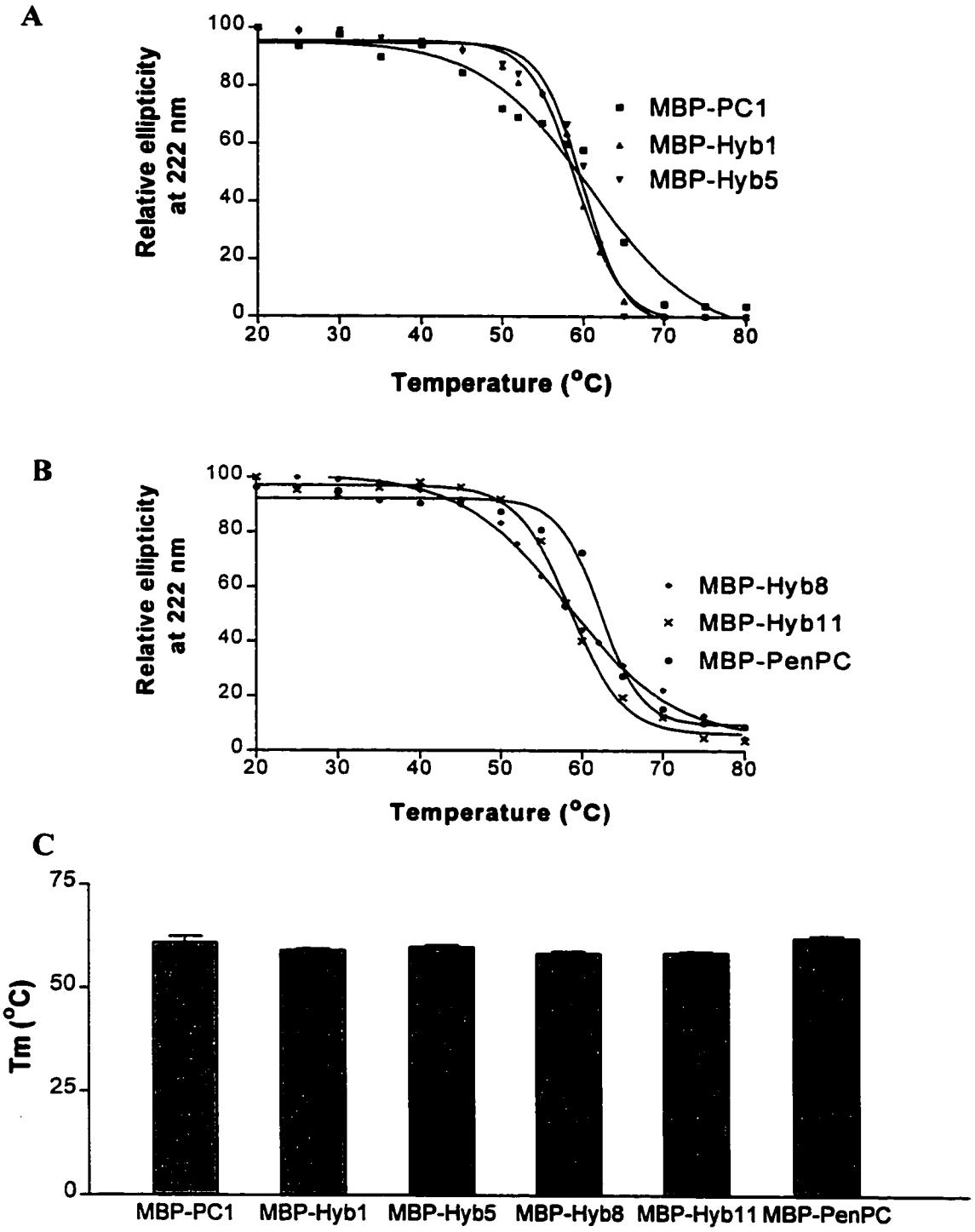


Figure 6.2 Thermal denaturation curves for MBP-β-lactamases (A) and (B), and the comparison of Tm values among different MBP-β-lactamases (C). The deviations of Tm value obtained from curve fitting are indicated by error bars.

6.3 Equilibrium unfolding

β-Lactamase activity measurement can be regarded as the most sensitive and unique conformational probe to study protein unfolding of the different MBP-β-lactamases. Using this criterion, guanidine hydrochloride (GdnHCl) inactivation experiments were carried out to determine the relative stability of different MBP-β-lactamases. GdnHCl-induced unfolding of β-lactamases was studied by measuring enzyme activity with penicillin G as substrate. The protein was denatured in 0-3 M GdnHCl in 50 mM potassium phosphate buffer (pH 7.0) at 25°C for 10-12 h to ensure equilibrium denaturation. The amount of GdnHCl carried over to the assay mixture never exceeded 50 mM and it had no inhibitory effect on the enzyme activity. As shown in Figure 6.3A, Boltzman sigmoidal fits of the individual curves yield inactivation concentration of the transitions, Cm, of 2.15 M, 0.80 M, 0.82 M, and 0.72 M of GdnHCl for MBP-PenPC, MBP-PC1, MBP-Hybrid1 and MBP-Hybrid4, respectively. MBP-Hybrid11 is very sensitive to GdnHCl, the enzyme lost all its activity at the presence of low concentration (<0.5 M) of GdnHCl. MBP-Hybrid5 is also very sensitive to GdnHCl inactivation, but its residual activities is hard to be calculated because of its unique behavior of substrate-induced activation. While the Cm values of MBP-PC1, MBP-Hybrid1 and MBP-Hybrid4 are very similar, MBP-PenPC shows much higher Cm value than other enzymes. For MBP-Hybrid6,

MBP-Hybrid7 and MBP-Hybrid8, we did not perform similar experiments due to their very low enzyme activities. Even if they were kept at 4 °C, obvious activity decreases were observed for the concentrated samples of these three purified fusion enzymes. We believe that these three proteins are much less stable than MBP-Hybrid11 if they were exposed to GdnHCl. GdnHCl-induced unfolding of PenPC and PC1 (without the MBP tag) was also investigated. It seems that both β-lactamases are slightly more stable than their MBP fusion counterparts, but the difference is not significant (Figure 6.3B).

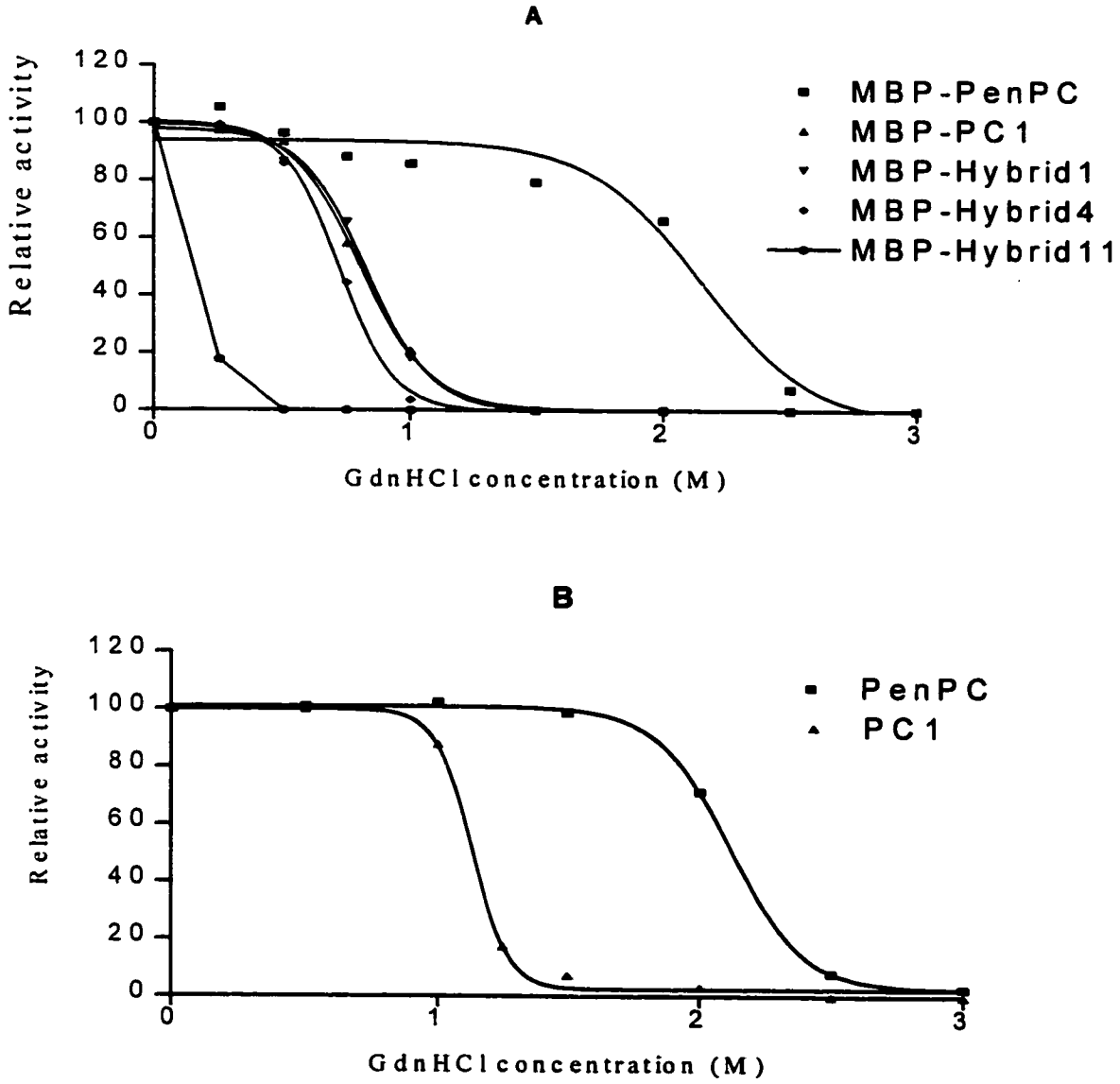
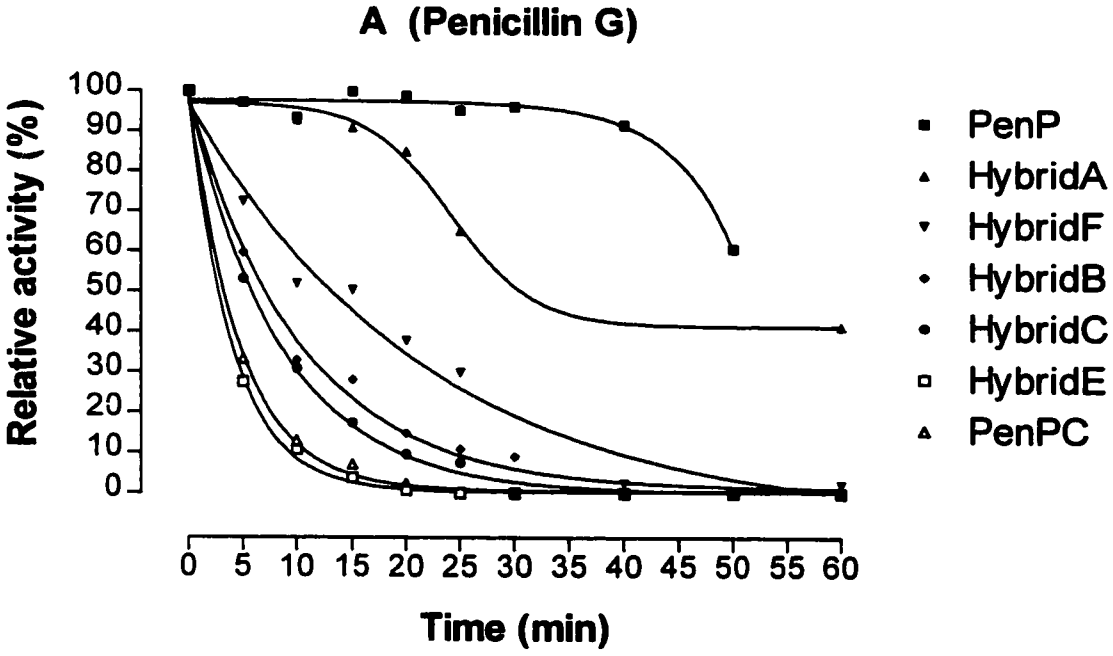
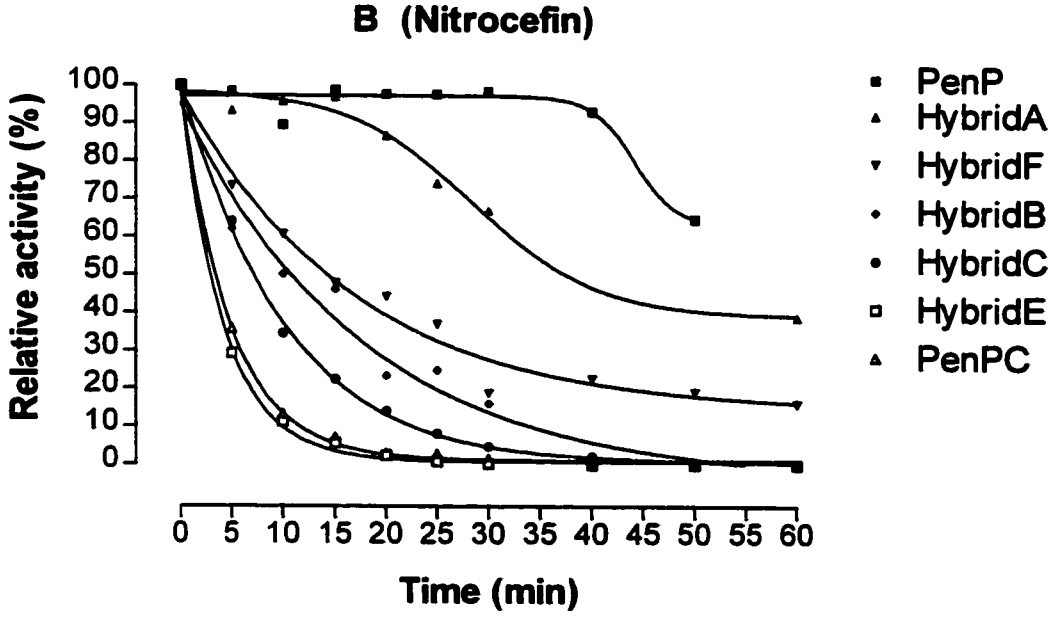


Figure 6.3 Equilibrium unfolding. The GdnHCl-induced unfolding transitions of different MBP-β-lactamases (A) or β-lactamases without the MBP tag (B) were assessed by measuring the β-lactamase activity with penicillin G as substrate after the enzymes were incubated in 0-3 M GdnHCl at 25°C for 10 h. Each single point is obtained from a single measurement.

6.4 Thermo-inactivation studies

For PenP, PenPC, and their hybrid enzymes, heat inactivation was performed at 55°C in assay buffer containing 0.1 mg/ml bovine serum albumin. Samples were withdrawn after various periods of time and rapidly cooled down on ice. The residual activity was determined with penicillin G, nitrocefin or cephaloridine as substrate at 25 °C and the results are shown in Figure 6.4. The time required for inactivating half of the enzyme activity of each enzyme at 55 °C was also calculated and compared to the others in Figure 6.5. The result indicates that the changes of enzyme activities as penicillinase (with penicillin G as substrate) or cephalosporinase (with nitrocefin or cephaloridine as substrates) show very similar pattern after heat inactivation. Under the conditions used in the present study, PenP shows much higher thermal stability than PenPC. For each hybrid enzyme, the more protein sequence is derived from PenP, the higher is its thermal stability. Schematic alignments of the primary structures of various hybrid β-lactamases are shown in Figure 6.6.


A (Penicillin G)
Relative activity (%)
100
90
80
70
60
50
40
30
20
10
0
0 5 10 15 20 25 30 35 40 45 50 55 60
Time (min)
PenP
HybridA
HybridF
HybridB
HybridC
HybridE
PenPC


B (Nitrocefin)
Relative activity (%)
100
90
80
70
60
50
40
30
20
10
0
0 5 10 15 20 25 30 35 40 45 50 55 60
Time (min)
PenP
HybridA
HybridF
HybridB
HybridC
HybridE
PenPC

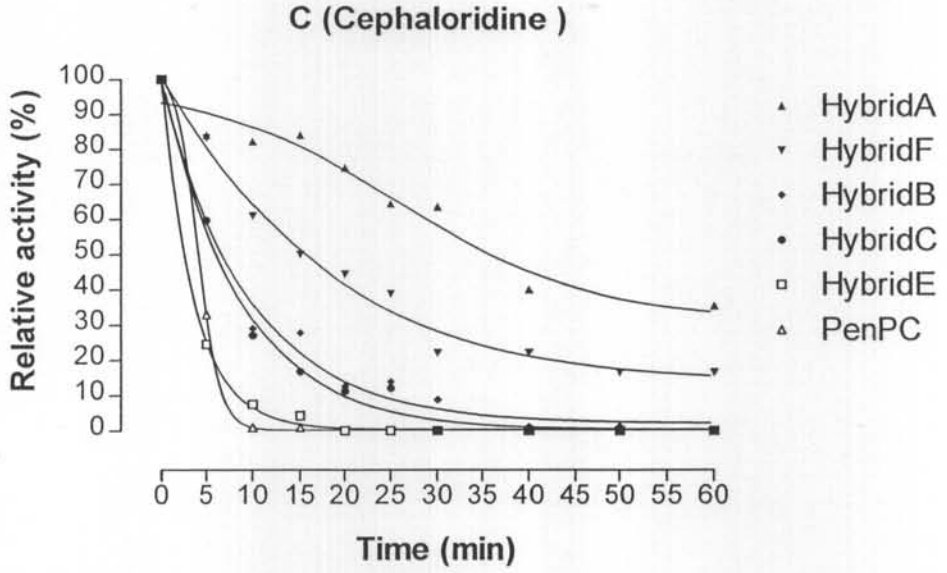


Figure 6.4 Relative activities as a function of time. The enzymes were incubated at 55°C in enzyme assay buffer (0.05 M sodium phosphate, 0.5 M NaCl, 0.1 mM EDTA, pH 7.0) containing 0.1 mg/ml bovine serum albumin. Samples were withdrawn after various periods of time and rapidly cooled down on ice, the residual activity was determined with penicillin G (A), nitrocefin (B), cephaloridine (C) as substrate at 25°C.

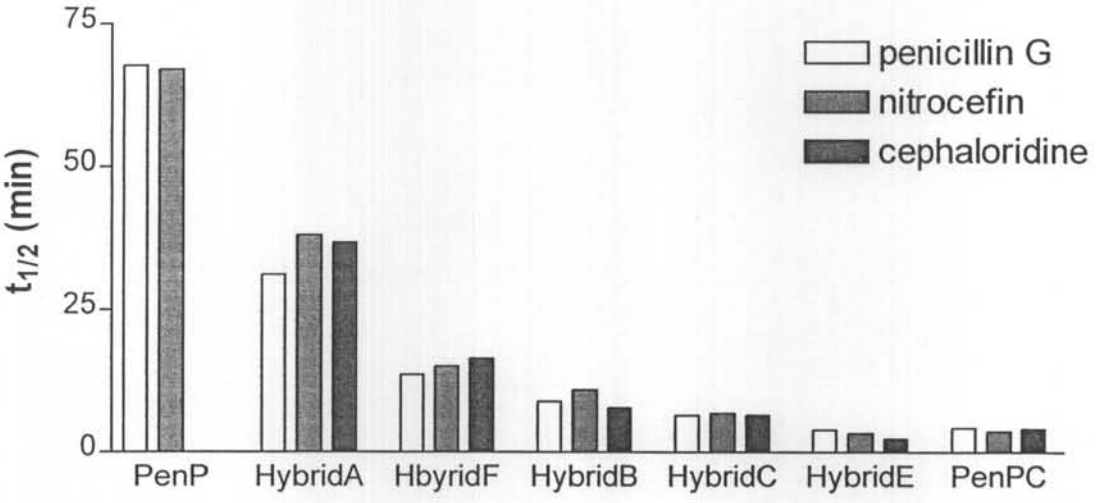


Figure 6.5 The comparison of half-life values of different β-lactamases at 55°C for the hydrolysis of penicillin G, nitrocefin or cephaloridine. The half-life time ($t_{1/2}$) is the time required to inactivate half of the enzyme activity at the experimental condition, which is derived from each of the curves in Figure 6.4.

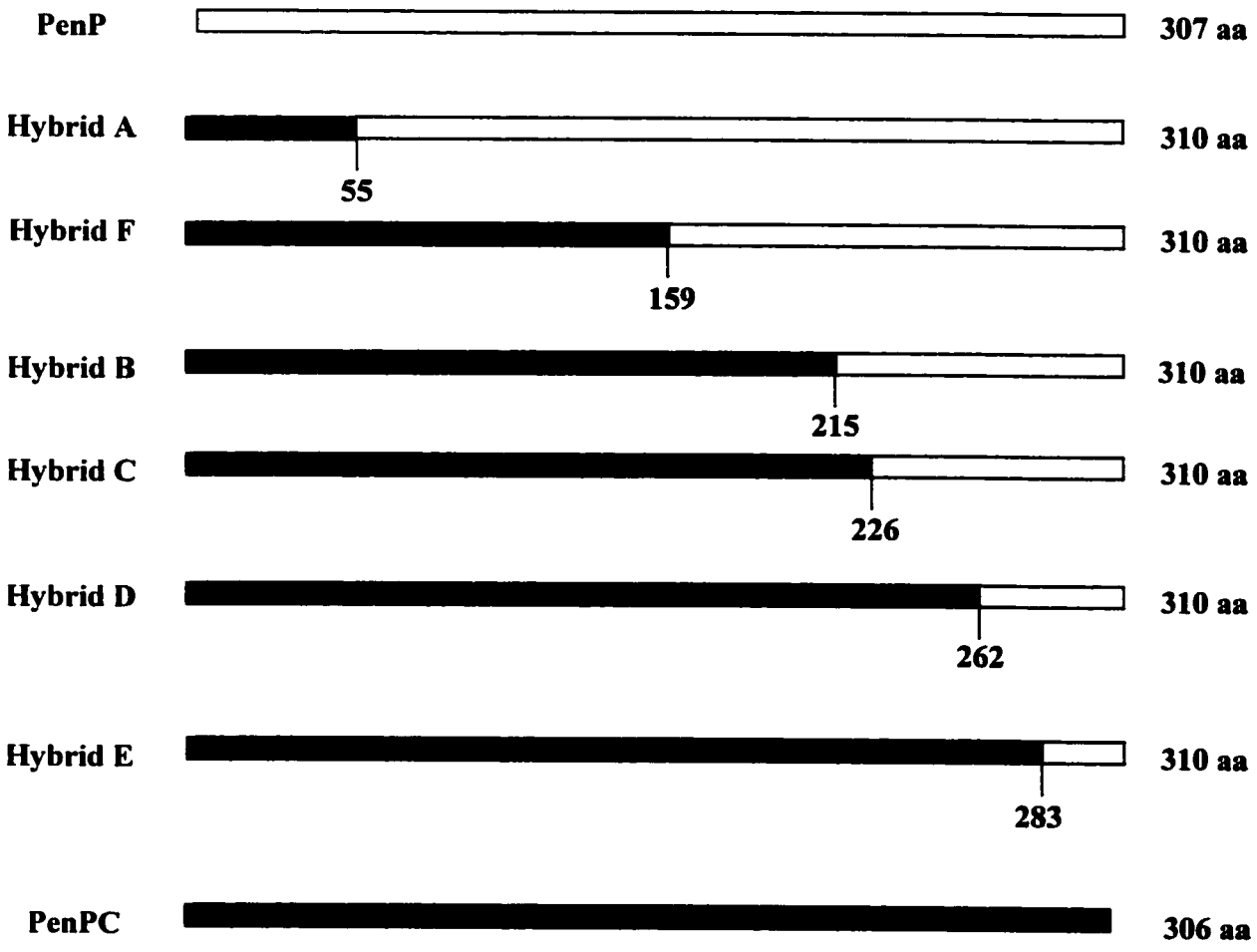


Figure 6.6 Schematic alignments of the primary structures of various hybrid β-lactamases by the translation from generated hybrid genes. The black regions represent the N-terminal sequence derived from *B. cereus* 569/H β-lactamase I (PenPC) while the white regions represent the C-terminal sequence derived from *B. licheniformis* PenP β-lactamase. The length of the whole enzyme and the length of the fragment derived from PenPC of each hybrid are shown on the right and under the fusion point, respectively.

6.5 K_m measurement as a K_i value with nitrocefin as the reporter substrate

As introduced in Chapter one, Class A β-lactamases utilize an active-site serine residue and function by the three-step mechanism (shown in Scheme 1).

$$E + C \underset{k_{-1}}{\overset{k_{+1}}{\longleftrightarrow}} EC \xrightarrow{k_2} EC^* \xrightarrow{k_3} E + P$$

Scheme 1

This mechanism assumes that the reaction passes through a non-covalent enzyme-substrate complex EC, that undergoes catalytic transformation to a covalent acyl-enzyme EC*, which then breaks down to form products. The mechanism is characterized by the following values of the steady-state kinetic parameters (Waley, 1992):

$$K_m = \frac{k_3 K'}{k_2 + k_3}$$

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3}$$

$$k_{cat}/K_m = \frac{k_2}{K'} \text{ where } K' = \frac{k_2 + k_{-1}}{k_{+1}}$$

By the measurement of initial rates of reaction in different concentrations of a substrate, these kinetic parameters can be conveniently determined using the Lineweaver-Burk plot (also called double-reciprocal plot) or non-linear regression. However, the commonly employed method, based on ultraviolet spectroscopy, cannot be used to measure the kinetic data of enzymes with larger Michaelis-Menten constant

(K_m) or very low turnover number (k_{cat}), because high concentration of substrate or β-lactamases in the reaction mixture may severely interfere the UV-absorbing detection. In addition, kinetic data of enzymes with very low K_m values (below 10 μM) are hard to be determined because it is very difficult to detect the initial rate at low concentration of substrate. In these cases, K_m was measured as a K_i value with nitrocefin as the reporter substrate. After the determination of K_m value of the studied enzyme for nitrocefin, the initial rates of the enzymes for nirtrocefin hydrolysis were measured in the absence or presence of different concentrations of the studied substrate, which was regarded as a competitive inhibitor. According to the Michaelis-Menten equation, in the absence of inhibitor, $V_0 = \frac{V_{max}[S]}{K_m + [S]}$; and in the presence of competitive inhibitor, $Vi = \frac{V_{max}[S]}{K_m(1+\frac{[I]}{K_i})+[S]}$. Therefore,

$$\frac{V_0}{V_i} = \frac{\frac{V_{max}[S]}{K_m+[S]}}{\frac{V_{max}[S]}{K_m(1+\frac{[I]}{K_i})+[S]}} = \frac{K_m(1+\frac{[I]}{K_i})+[S]}{K_m+[S]} = \frac{K_m+\frac{K_m[I]}{K_i}+[S]}{K_m+[S]}$$

so that, $\frac{V_0}{V_i} = 1 + \frac{K_m}{K_m+[S]}\frac{1}{K_i}[I]$

Where V_0 is the initial rate of the enzyme for nitrocefin hydrolysis in the absence of other β-lactam substrate, K_m is the Michaelis-Menten constant of the enzyme for nitrocefin, [S] is the concentration of nitrocefin used for assay; V_i is the initial rate of the enzyme for nitrocefin hydrolysis in the presence of other β-lactam substrate; [I] is

the concentration of the other substrate to be studied; K_i actually is the Michaelis-Menten constant of the enzyme towards the studied substrate. Therefore, the plot of V_0/V_i against [I] is expected to generate a straight-line, with a slope of $\frac{K_m}{K_m+[S]}\frac{1}{K_i}$. For example, kinetic parameters of MBP-PenPC for oxacillin are hard to be determined by normal method because of very low substrate binding affinity (high K_m) and catalytic activity (low k_{cat}). We measured the initial rates of nitrocefin hydrolysis (100 mM nitrocefin in assay mixture) in the presence of different concentrations of oxacillin, as shown in Figure 6.7. The slope of the plotted line is 0.2948. Therefore, $K_i = \frac{K_m}{K_m+[S]}\left(\frac{1}{0.2984}\right) = \frac{73.5}{73.5+100}\left(\frac{1}{0.2984}\right) = 1.42$ (mM).

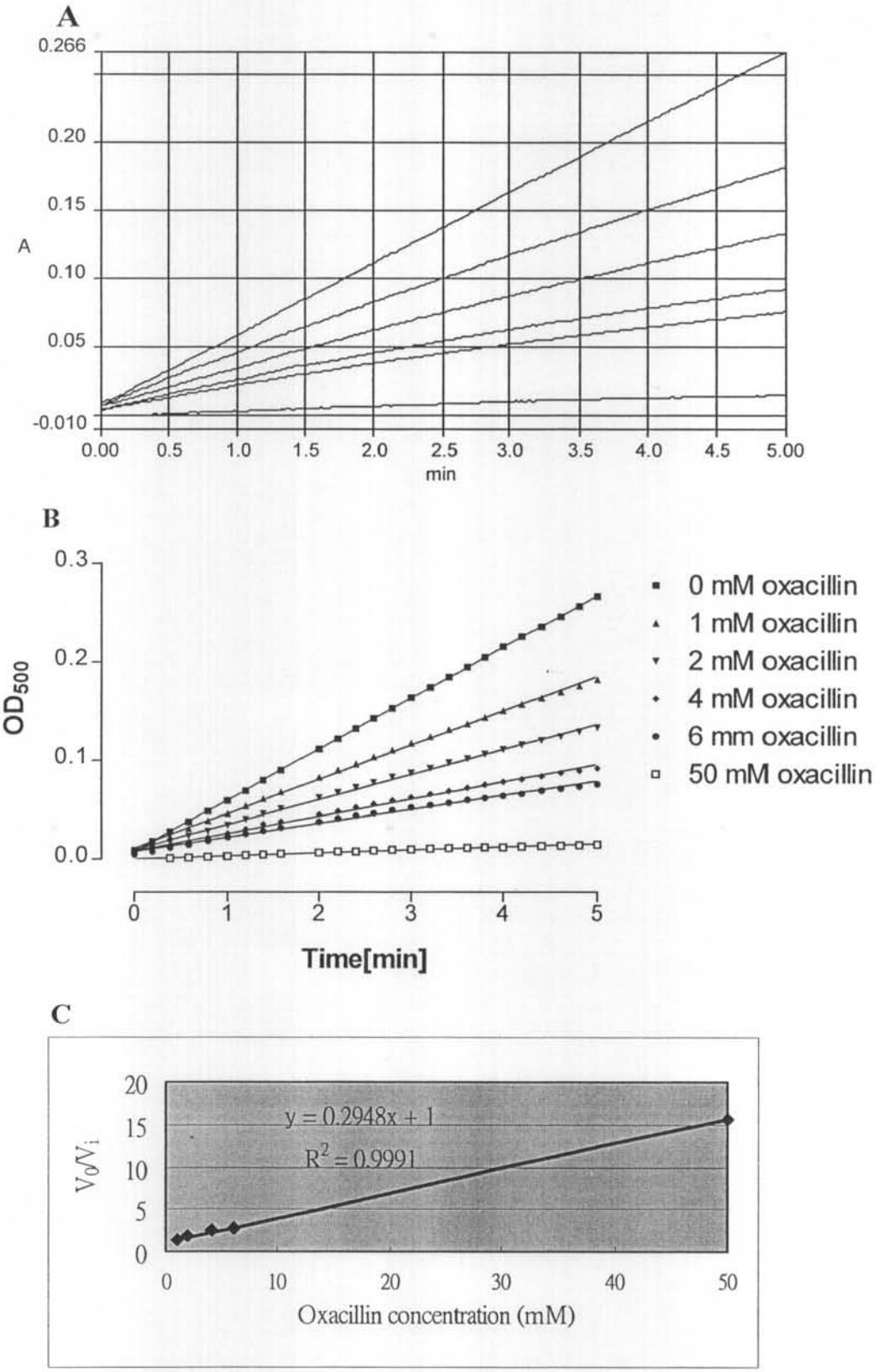


Figure 6.7 K_m determination as a K_i value with nitrocefin as the reporter substrate. With 100 μM nitrocefin in the assay buffer, initial rates of reactions in the absence and presence of different concentrations of oxacillin, as shown in the raw graph data (A) or plotted graph using the raw data (B). The plot of V_0/V_i against [I] (oxacillin concentration) is shown in (C).

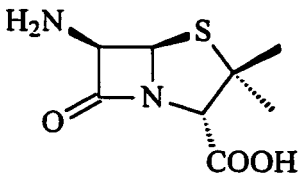
6.6 Kinetic characterization of PenP, PenPC and their hybrid enzymes

In the previous work in our laboratory (Cheung, 2000), kinetic parameters of PenP, HybridA, HybridB, HybridC, HybridD, HybridE, and PenPC were determined with penicillin G, penicllin V and cephaloridine as substrates. However, we obtained quite different values when we measured the kinetic parameters of PenPC and Hybrid B towards the same set of substrates, possibly because the conditions used for activity assay were different. Therefore, we cannot systemically compare the kinetic data of Hybrid F with those of the other hybrid enzymes that were measured by Cheung (Cheung, 2000). For better comparison in the future, we will measure the kinetic data of all these enzymes using the same conditions towards penicillin G, penicillin V and cephaloridine as substrates. However, as shown in Table 6.2, our present results indicated that the newly generated HybridF enzyme shows higher substrate binding affinity (lower K_m), much lower catalytic activity than PenPC (one of its parents), consequently, its catalytic efficiency (k_{cat}/K_m) is lower than that of PenPC.

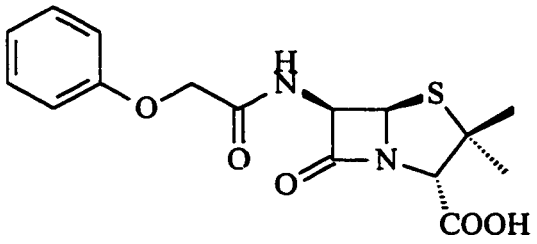
Table 6.2 The determined kinetic parameters of PenPC and HybridF

	Penicillin G			Cephaloridine		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}S^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	kcat/Km ($\mu M^{-1}S^{-1}$)
PenPC	112±7	2170±74	19±2	42±3	6.1±0.6	0.15±0.2
HybridF	37±4	132±3	3.6±0.4	28±2	2.3±0.4	0.08±0.009

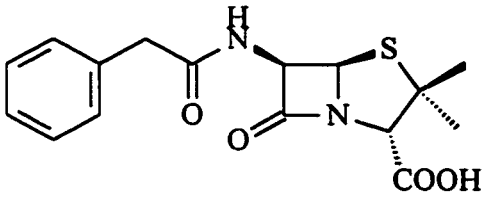
In this study, detailed kinetic properties of PenP, PenPC and all their hybrid enzymes were determined with nitrocefin, 6-aminopenicillanic acid (6-APA), methicillin, oxacillin and cefuroxime as substrates. For nitrocefin, PenP and PenPC show great differences in turnover number (k_{cat}), while their K_m values are quite similar. For using 6-APA as the substrate, PenPC and PenP behave dramatically different in both apparent substrate binding affinity (K_m) and substrate turnover. The K_m values of all these β-lactamases towards 6-APA, methicillin, oxacillin and cefuroxime were determined as K_i values with nitrocefin as the reporter substrate. The chemical structures of the antibiotics in the current study are shown in Figure 6.8. All the kinetic data obtained are summarized in Table 6.3.



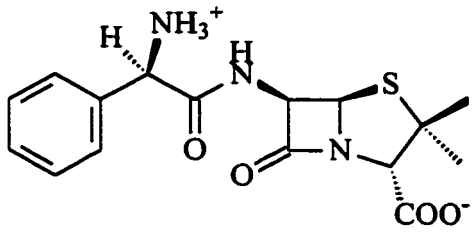
6-aminopenicillanic acid (6-APA)



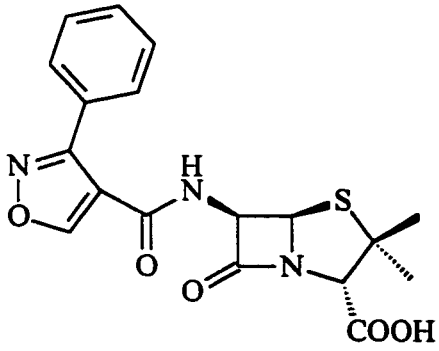
Penicillin V



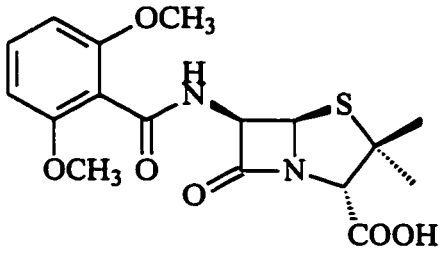
Benzylpenicillin (Penicillin G)



Ampicillin

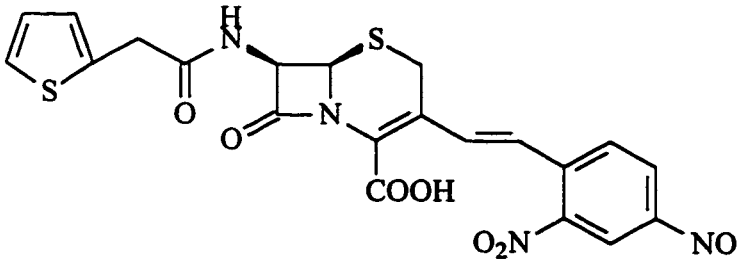


Oxacillin

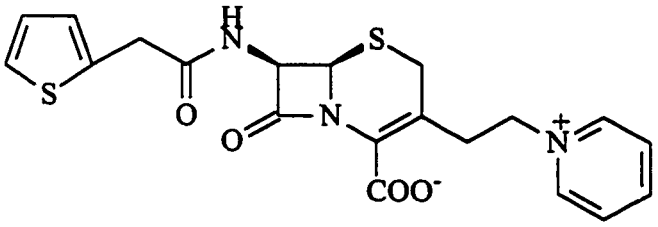


Methicillin

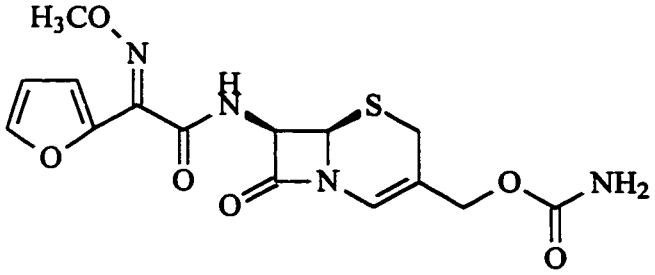
Figure 6.8A Structures of penicillin antibiotics used in this study



Nitrocefin



Cephaloridine



Cefuroxime

Figure 6.8B Structures of cephalosporin antibiotics used in this study

Table 6.3 Kinetic parameters of PenP, PenPC and their hybrid β-lactamases for nitrocefin, 6-APA, methicillin, oxacillin and cefuroxime

	Nitrocefin			6-APA			Methicillin	Oxacillin	Cerufoxime
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}S^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}S^{-1}$)	K_m (μM)	K_m (μM)	K_m (μM)
PenP	70.0±8.9	854±67	12.2±2.5	8.1±1.2	19.2±2.1	2.4±0.6	6.17±1.32	13.7±2.0	872±138
Hybrid A	37.4±4.1	598±56	16.0±3.2	5.8±0.8	25.5±3.5	4.4±1.0	5.32±0.78	10.4±1.6	634±93
Hybrid F	37.9±1.8	16.4±1.2	0.43±0.05	204±19	87.1±5.6	0.42±0.07	52.9±3.6	13.5±1.2	241±18
Hybrid B	53.5±2.5	120±16	2.2±0.4	2470±260	115±23	0.04±0.01	2872±169	3408±217	346±21
Hybrid C	80.0±2.9	101±15	1.2±0.2	2790±260	93.7±6.7	0.03±0.01	1349±72	1500±75	544±28
Hybrid D	61.4±2.8	24.7±5.6	0.40±0.11	1870±180	110±17	0.06±0.01	286±19	836±55	1063±64
Hybrid E	74.2±2.8	23.0±2.7	0.31±0.05	1280±140	85.0±6.6	0.07±0.01	318±20	664±38	1211±72
PenPC	77.2±2.2	25.5±2.6	0.34±0.04	1470±150	189±14	0.12±0.02	392±29	2319±183	1360±61

6.7 Kinetic characterization of MBP fusion enzymes of PC1, PenPC and their hybrids

In this study, detailed kinetic properties of MBP fusion enzymes of PC1, PenPC and their hybrids were determined with two S-type penicillins (penicillin G and ampicillin), two A-type penicillins (oxacillin and methicillin), two first-generation cephalosporins (nitrocefin and cephaloridine) and one oxyimino cephalosporin (cefuroxime) as substrates. The structures of these β-lactams are shown in Figure 6.8.

Penicillin G is a natural penicillin, which was the first β-lactam antibiotic that received widespread clinical application since 1940. Ampicillin, a D-α-aminobenzyl penicillin available since 1960, has the antibacterial activity of penicillin G against gram-positive species. Methicillin and oxacillin have been available since 1960 as anti-staphylococcal, penicillinase-resistant penicillins. The basis of the activity of these two penicillins against β-lactamase-producing isolates is the steric hindrance around the carbon atom attached to the C-6 side chain amide carbonyl group. Methicillin is a phenyl penicillin in which both of the *ortho* positions of the phenyl group are occupied by a methoxy group. The use of larger moieties in this position results in penicillinase resistance, but markedly lowers antimicrobial activity due to poor binding to penicillin-binding proteins. Combination of an isoxazole ring with a phenyl group on the acyl side chain produces β-lactamases stability for oxacillin. The first generation cephalosporins such as cephaloridine contained no substitutions on the

α-carbon of the C-7-acyl side chain and possessed a thienyl ring. A major improvement in antibacterial activity was produced by the introduction of a 2-aminothiazolyl side chain. Since the aminothiazolyl group did not provide any β-lactamase stability, other modifications of the 7-β-acyl side chain were made. Introduction of an α-iminomethoxy group provided β-lactamase stability without significant loss of *in vitro* activity. Cefuroxime was the first widely used agent to possess an α-oxyimino grouping. It has β-lactamase stability against many plasmid and chromosomal β-lactamases and the cephalosporinases found in *Pseudomonas* and *Enterobacter*. Cefuroxime demonstrates another aspect of changes in the 7-β-acyl side chain. A furyl ring replaces the phenyl and thienyl groups found in most of the first- and second-generation cephalosporins. The aminothiazol group and iminomethoxy groups have been utilized in what have been referred as third-generation cephalosporins – cefotaxime, ceftizoxime, cefmenoxime, ceftriaxone and cefodizime (Neu, 1992). The designation of β-lactam compounds into generations is historical.

Values of kinetic parameters obtained with the various MBP-β-lactamases for all these penicillins and cephalosporins are presented in Table 6.4 and Table 6.5, respectively.

Table 6.4 Kinetic parameters of MBP fusion enzymes of PC1, PenPC and their hybrids towards penicillin G, ampicillin, methicillin, and oxacillin.

	Penicillin G			Ampicillin			Methicillin	Oxacillin
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}S^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}S^{-1}$)	K_m (μM)	K_m (μM)
MBP-PC1	5.18±0.78	164±15	31.6±7.6	48.1±4.0	211±13	4.4±0.6	>200 mM	>100 mM
MBP-Hybrid1	4.95±0.14	104±7	21.0±2.0	49.6±7.4	46.4±9.5	0.93±0.33	>200 mM	>100 mM
MBP-Hybrid4	7.46±0.89	112±9	15.0±3.0	44.0±3.5	90.0±6.6	2.0±0.3	>200 mM	>100 mM
MBP-Hybrid5	4.28±0.43	11.9±0.7	2.8±0.4	67.5±3.4	9.73±0.12	0.14±0.01	>200 mM	>100 mM
MBP-Hybrid6	74.4±7.2	0.156±0.012	21±3 E-04	110±11	0.11±0.01	9.8±1.9 E-04	3.45±0.36	31.8±3.1
MBP-Hybrid7	41.1±4.3	0.044±0.003	10±2E-04	146±15	0.049±0.006	3.3±0.7 E-04	0.79±0.08	7.44±0.79
MBP-Hybrid8	108±23.	0.0076±0.0008	7.0±2.2E-05	131±26	0.026±0.003	2.0±0.4E-04	1.75±0.37	18.8±4.0
MBP-Hybrid11	155±7	44.7±0.6	0.29±0.02	345±26	22.3±0.6	0.064±0.006	64.2±4.3	286±21
MBP-PenPC	93.9±5.8	2034±365	21.6±5.2	279±15	2479±657	8.9±2.8	963±52	1298±67

Table 6.5 Kinetic parameters of MBP fusion enzymes of PC1, PenPC and their hybrids towards nitrocefin, cephaloridine and cefuroxime.

	Nitrocefin			Cephaloridine			Cefuroxime
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}S^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}S^{-1}$)	K_m (μM)
MBP-PC1	0.56±0.06	10.1±0.1	17.9±2.2	0.80±0.09	substrate-induced inactivation		>10 mM
MBP-Hybrid1	1.22±0.09	8.46±0.20	6.96±0.68	0.95±0.10	substrate-induced inactivation		>10 mM
MBP-Hybrid4	1.20±0.11	9.91±0.27	8.25±0.98	1.28±0.16	substrate-induced inactivation		>10 mM
MBP-Hybrid5	1.25±0.13	1.54±0.02	1.23±0.15	2.38±0.32	0.087±0.010		29.3±4.0
MBP-Hybrid6	89.5±3.8	0.11±0.002	117±4 E-05	2434±243	too low		8514±822
MBP-Hybrid7	79.1±6.3	0.03±0.001	38±4.5 E-05	910±95	too low		4490±475
MBP-Hybrid8	215±41.	0.011±0.001	5.1±1.5 E-05	2800±580	too low		927±196
MBP-Hybrid11	40.5±1.7	0.27±0.004	66±3.7 E-04	42.8±3.2	0.045±0.001	10.5±1.0 E-04	250±16
MBP-PenPC	73.5±2.4	22.6±0.2	0.31±0.01	35.0±3.3	5.11±0.16	0.14±0.02	433±23

When we measured the initial rate of PenPC and PC1 for penicillin G, the patterns of the progress curves of these two enzymes are found to be significantly different. As shown in Figure 6.9A and Figure 6.9B, while the slope of the PenPC curves (the slope indicates the activity of enzyme) decreased slowly, the slope of the PC1 curves almost kept constant for a period of time and then suddenly droped to zero, no matter how much the enzyme was used. As presented in Figure 6.9C and Figure 6.9D, when the slope becomes zero, very similar progress curve may be restored by the addition of the same amount of penicillin to the reaction mixtures, but not by the addition of enzymes. These results suggest that there is no enzyme inactivation and product inhibition occurred in both cases. The decrease of the curve slope to zero is due to the complete exhaustion of the substrate. By measurement, there is about 20-fold difference between the K_m values of these two enzymes (112 μM for PenPC and 5.7 μM for PC1). The low K_m value of PC1 may be the reason of its unique pattern of progress curve. For any enzyme reaction,

$$v = -\frac{ds}{dt} = \frac{V_{max} s}{K_m + s}$$

Where v is the rate, and s the substrate concentration. As the substrate is used up, and the reaction approaches completion, $s << K_m$, so that

$$-\frac{ds}{dt} = \frac{V_{max} s}{K_m}$$

Integration gives us the total time for given change in s

$$-\int_{s_0}^{s_1}\frac{ds}{s} = -\frac{V_{max}}{K_m}\int_0^t dt \qquad \text{or} \quad \ln\frac{s_0}{s_1} = \frac{V_{max}}{K_m}t$$

Suppose time T is needed for a decrease in s from 10% to 1%, the following expression must hold:

$$2.3\log\frac{10}{1} = \frac{V_{max}}{K_m}T \text{ or } T = \frac{2.3K_m}{V_{max}}$$

This tells us that the time for substrate decrease is proportional to K_m and inversely proportional to V_{max}. It completely supports our notion that the sudden drop of the progress curve slope to zero is the indicator of a low K_m. Throughout our studies, the progress curve pattern always happens in the case of K_m below 10 μM, even for hysteretic enzymes, such as MBP-Hybrid 5 (Figure 6.9E). We observe the phenomena without exception when we measured the K_m values of various β-lactamases with penicillin G, penicillin V, nitrocefin and 6-APA as substrates (Figure 6.10). The sudden drop of the progress curve slope to zero may be used as a rough estimation of the K_m value that should be below 10 μM.

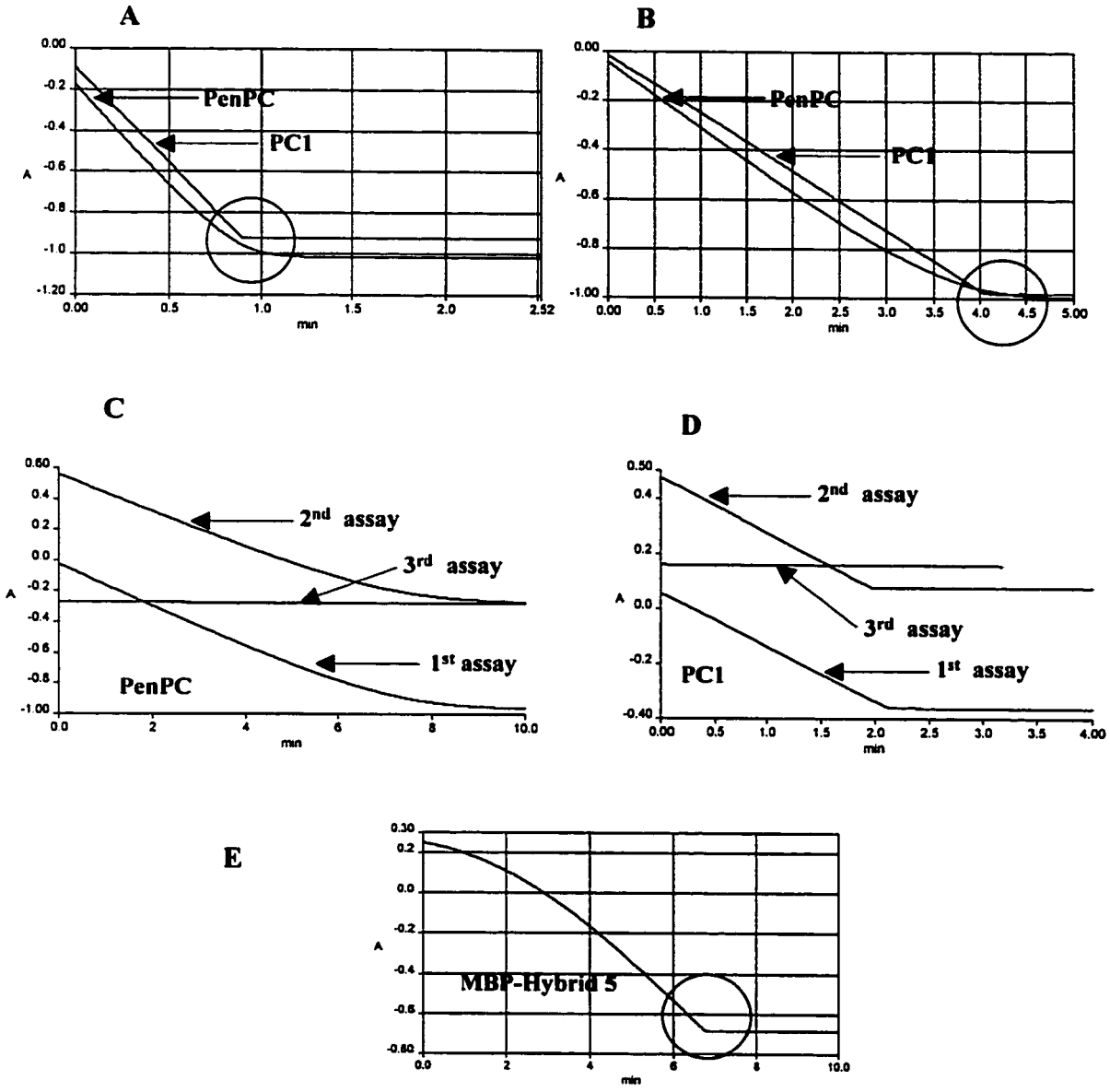


Figure 6.9 Progress curves of penicillin G hydrolysis by PenPC and PC1 (A and B), PenPC (C), PC1 (D), and MBP-Hybrid 5(E). The 1st assay means normal detection after mixing enzyme and substrate in assay buffer; the 2nd assay means the addition of the same amount of substrate as that used in the 1st assay when the slope of the 1st assay had dropped to zero. The 3rd assay means the addition of the same amount of enzyme as that used in the 1st assay when the slope of the 2nd assay had dropped to zero. The amount of enzymes used in (A) is more than in (B).

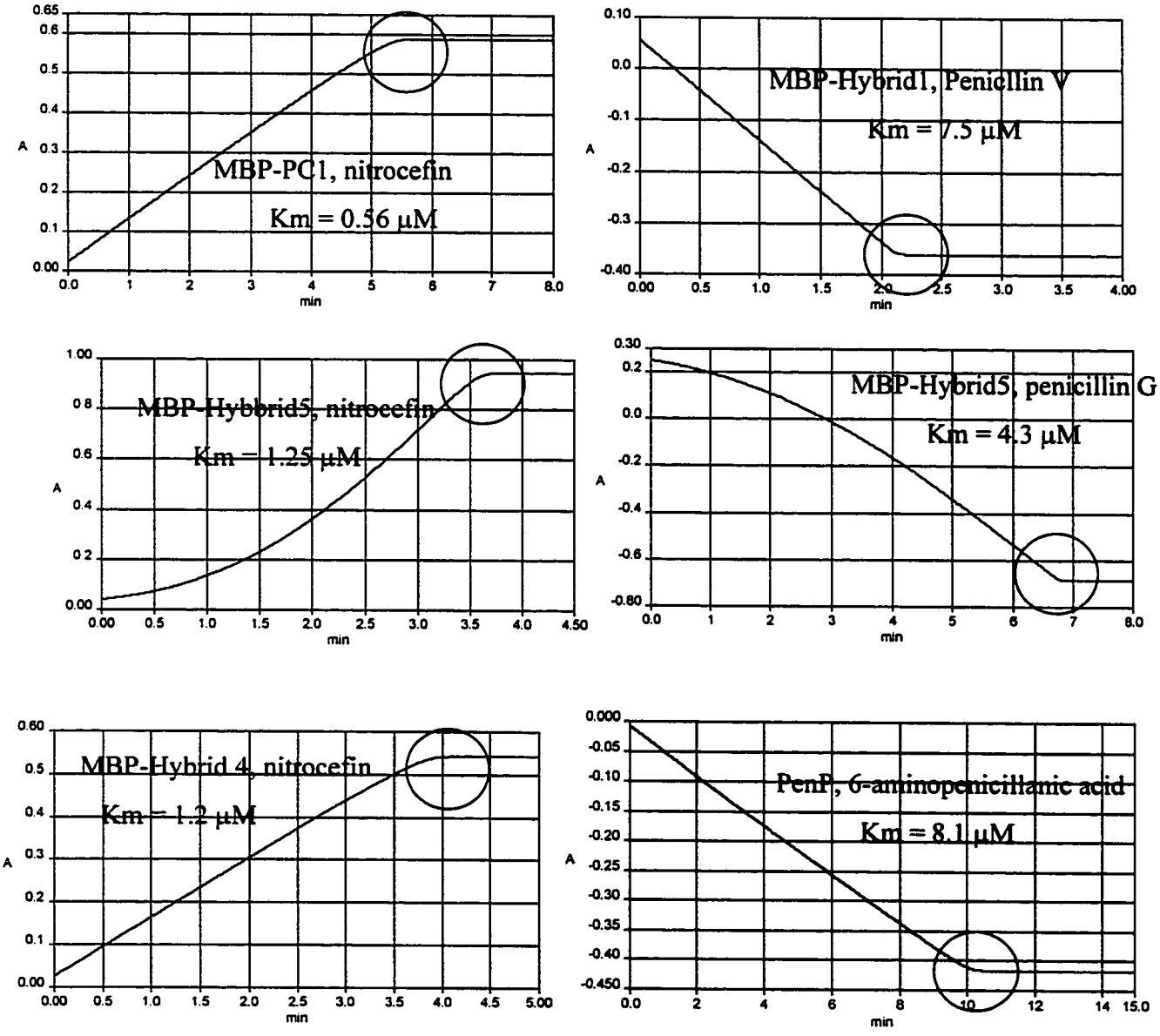


Figure 6.10 Progress curves of some samples with low Km values (below 10 μM). For assay, the concentration of nitrocefin was 100 μM, and the concentration of other substrates was 1 mM. The enzyme concentrations of MBP-PC1, MBP-Hybrid1, MBP-Hybrid4 and MBP-Hybrid5 used in the assay were153 nM, 182 nM, 156 nM, 1 μM and 73.2 nM, respectively.

Unlike the native enzymes, for penicillin G, penicillin V, ampicillin and nitrocefin, MBP-Hybrid5 exhibits 'lag'-type kinetics (Matagne *et al.*, 1990), also termed substrate-induced activation (Pain and Virden, 1979) or hysteresis (Neet and Ainslie, 1980), and its activity changes during the assay progress. As shown in Figure 6.11, the activity of MBP-hybrid5 for the hydrolysis of penicillin G or nitrocefin linearly increased with the time. This is very surprising. For hysteretic enzymes, the velocity v at time t normally changes exponentially from an initial value v_i to a steady-state value v_s and the following expression must hold (Frieden, 1970; Waley, 1991):

$$v = v_s(1\text{-}e^{-kt}) + v_i \cdot e^{-kt}$$

where k is the rate constant characterizing the change. However, for MBP-Hybrid5, linear but not exponential substrate-induced activation was observed during activity assay, and this unique kinetic property of MBP-Hybrid5 is worth further investigation.

The A238S:I239del PC1 mutant was also reported to exhibit substrate-induced activation (Zawadzke *et al.*, 1995). Its hydrolytic activity towards nitrocefin for the latter "faster" part of the reaction is similar to the native enzyme and the rate of hydrolysis of penicillin G is reduced by 23-fold. More interestingly, the A238S:I239del β-lactamase hydrolyzes third-generation cephalosporins much better than the native enzyme. When the native and the mutant PC1 β-lactamase structures

are superimposed together with the *E. coli* TEM-1 structure, the most notable structural difference in the active site is an opening of the active site gully in the double mutant structure association with residue 238-240. The mutant molecule exhibits the largest shift because of the larger side-chain of Ser 238, which in addition to its size is also positioned to form an electrostatic interaction between its O^{γ} atom and the O^{δ} atom of Asn 170. The ability of the mutant enzyme to hydrolyze third-generation cephalosporin is attributed to this structural feature, which enables the enzyme to accommodate the larger side-chain substituents.

For MBP-hybrid5, as shown in Table 6.4 and Table 6.5, while its apparent binding affinity (K_m value) to penicillin G, ampicillin, nitrocefin or cephaloridine is very similar to that of MBP-PC1, one of its parents, its hydrolysis activities towards these β-lactams are reduced to different extents. More importantly, it shows very high apparent binding affinity (K_m = 29.3 μM) to oxyimino β-lactam cefuroxime, which is stable to most class A β-lactamases (the Km values for MBP-PenPC and MBP-PC1 are 433 μM and >10 mM, respectively). Surprisingly, the hybrid enzyme had no significant effect on K_m values for oxacillin and methicillin, which are also characteristic of larger side chain. This suggests that structural changes in MBP-Hybrid5 may not be the same as in the A238S:I239del PC1 mutant.

Structure of a chromosomal extended-spectrum β-lactamase (ESBL) having the ability to hydrolyze cephalosporins including cefuroxime and ceftazidime has recently been determined by X-ray crystallography to 1.75 Å resolution (Nukaga *et al.*, 2002). The folding of the Class A β-lactamase from *Proteus vulgaris* K1 (K1 enzyme) is broadly similar to that of non-ESBL TEM-type β-lactamases (2 Å rmsd for C^{α}) and differs by only 0.35 Å for all atoms of six conserved residues in the catalytic site. Other residues promoting extended-spectrum activity in K1 include the side-chains of atypical residues Ser 237 and Lys 276 (Figure 6.12). While the non-ESBL TEM-1 β-lactamase has two opposing glutamic acid side-chains (Glu 104 and Glu 240) at the two lower corners of the binding site, the ESBL K1 enzyme is less electronegative in this region, having instead Ala 104 and a shorter Asp 240. These changes at positions 104 and 240 also widen the binding site at its base where large R1 substitutents of oxyimino-cephalosporins would lie. All class A β-lactamases contain an absolutely conserved Glu 166, which is required for deacylation of the Ser70-bound acyl intermediate. The glutamic acid residue activates a water molecule for attack on the acyl ester bond. In K1, a second water molecule hydrogen bonded (2.7 Å) to the same oxygen atom of the carboxylic acid group was found. This second water molecule also hydrogen bonds to the conserved Lys 73, producing in K1 a movement of the side-chain N^{ε} atom away from the conserved Ser130, relative to PC1 or TEM-1. The

distance between 73 N^{ε} and 130 O^{γ} varies significantly, being 3.6-3.7 Å in PC1 and TEM-1, but 4.1 Å in K1. The larger separation between Lys73 and Ser130 in K1 could change the character or orientation of Ser 130. The importance of Ser 130 in catalysis has been examined in many studies (Jacob *et al.*, 1990; Juteau *et al.*, 1992; Lamotte-Brasseur *et al.*, 1991), one of which indicated that hydrolysis of cephalosporins is more dependent on Ser 130 than is the hydrolysis of penicillins (Juteau *et al.*, 1992). Perhaps the subtle changes near Ser 130 promote the K1 enzyme's cephalosporinase activity. We speculate that the unique kinetic property may also result from the subtle changes near Ser 130. However, we still need the helps from the study of protein crystal structure to clarify the speculation.

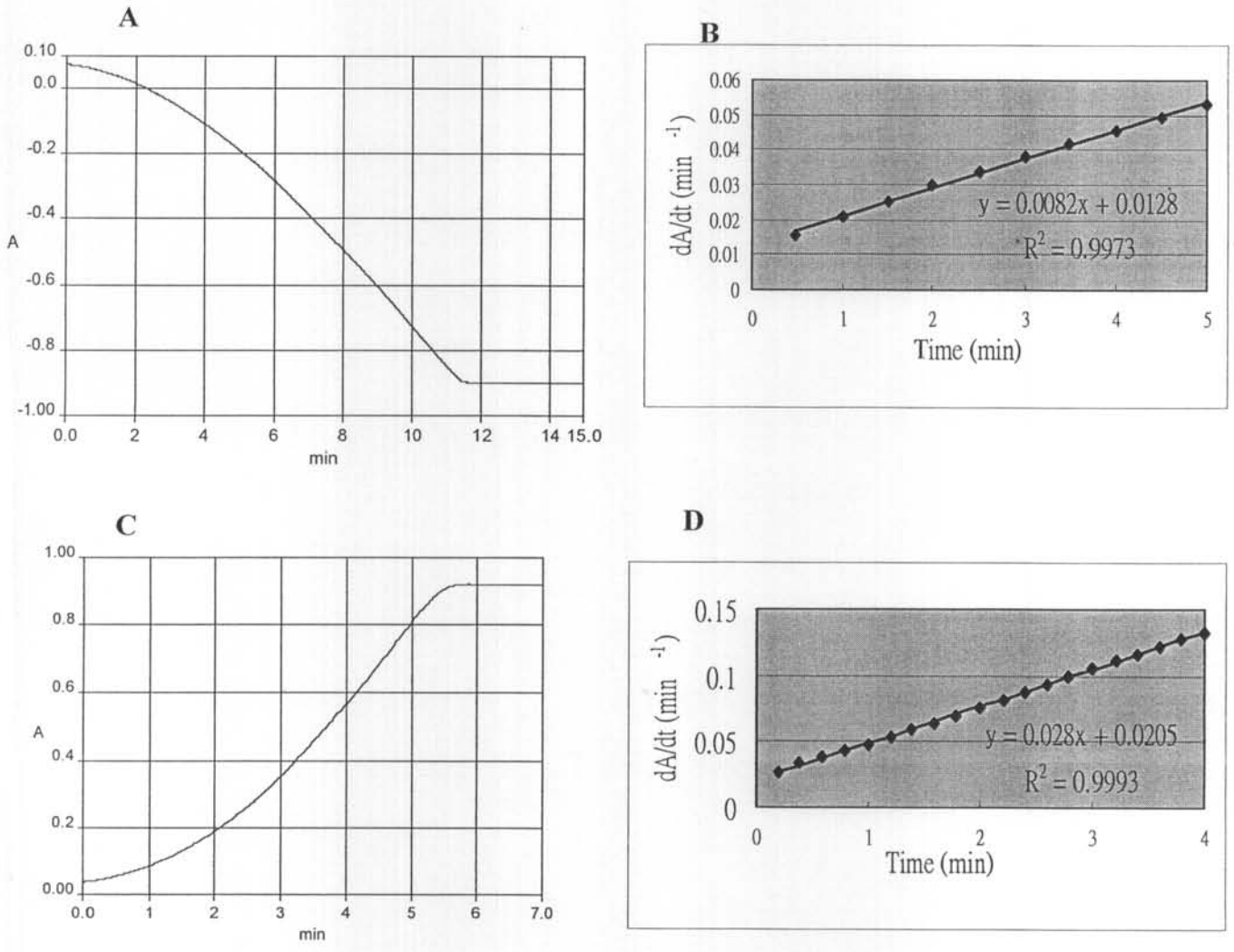


Figure 6.11 Hysteretic kinetics of MBP-Hybrid5. (A) Progress curve of penicillin G hydrolysis. (B) Linear increase of enzyme activity with time during reaction. (C) Progress curve of nitrocefin hydrolysis. (D) Linear increase of enzyme activity with time during reaction.

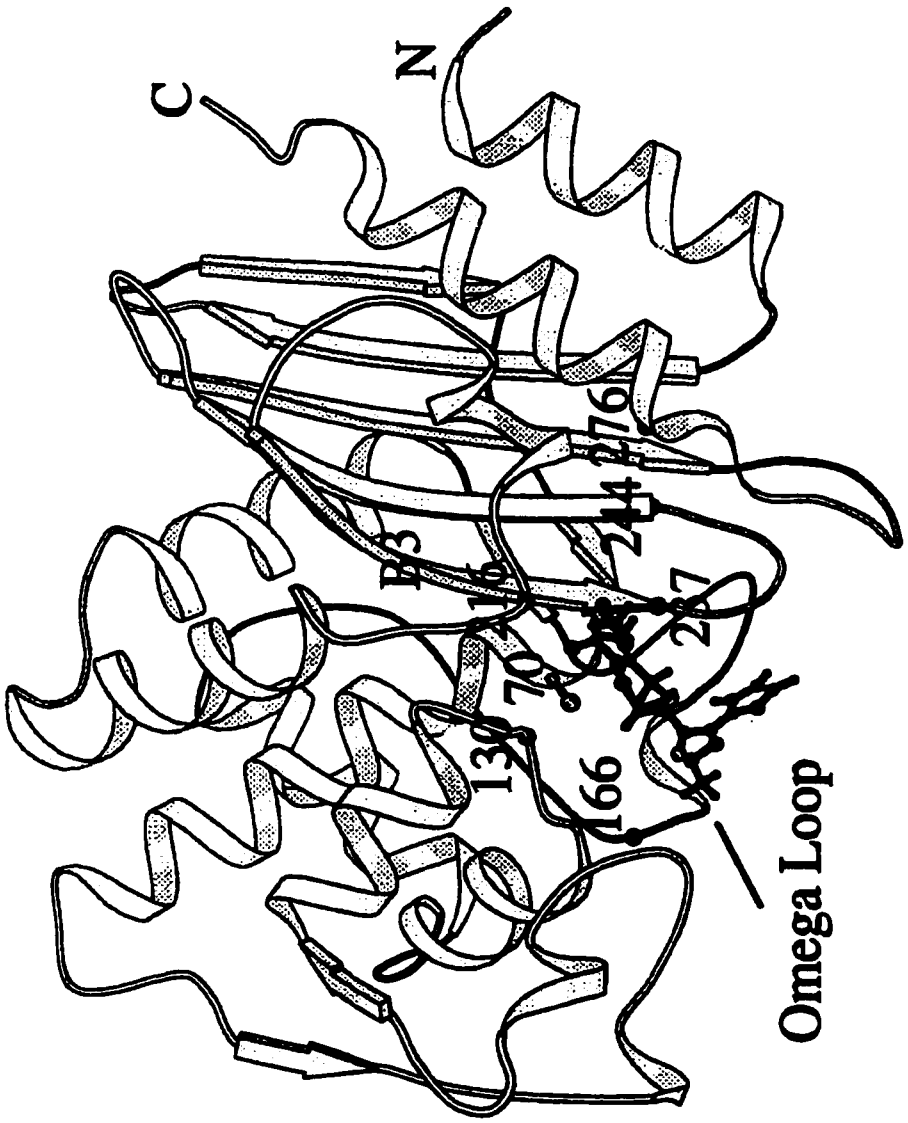


Figure 6.12 Folding of the *P. vulgaris* K1 β -lactamase. Cefuroxime is positioned to show the location of the β-lactam binding site at the edge of the β-sheet (after Nukaga *et al.*, 2002).

The cephaloridine hydrolysis by β-lactamases exhibits two distinctive kinetic schemes. The first (Scheme 1) follows the simple acylation and deacylation steps as mentioned before. As shown in Figure 6.13 A, the progress curve of cephaloridine hydrolysis by MBP-PenPC is very similar to that of penicillin G hydrolysis. When the absorbance stopped to change, the curve can be restored by the addition of the same amount of cephaloridine (the 2nd assay) but not the same amount of enzyme (the 3rd assay), which indicates that there is no product inhibition and enzyme inactivation during reaction. The second mechanism (Scheme 2) is termed the branched pathway (Chen and Herzberg, 1999) and is consistent with substrate-induced progress inactivation or burst kinetics (Waley, 1991). Progress inactivation is well documented in β-lactamases (Carrey *et al.*, 1984; Citri *et al.*, 1976; Faraci and Pratt, 1985)

$$E + S \overset{K_S}{\rightleftharpoons} ES \overset{k_2}{\rightarrow} EC \overset{k_3}{\rightarrow} E + P$$
$$\qquad\qquad\qquad\quad k_{-4} \downarrow\uparrow k_{+4}$$
$$\qquad\qquad\qquad\quad EC^*$$

Scheme 2

where, the initial acyl enzyme, EC, is converted to a second from of an acyl enzyme, EC*, which does not turn over (or turns over more slowly). For this mechanism, the progress curve exhibits an initial exponential phase followed by a slower steady-state phase, since a less active form of the enzyme accumulates. Branching at ES may also result in the accumulation of the less active enzyme. Usually, the initial bust of product

is much greater than the concentration of the enzyme. The product formation can be described by the equation (Zawadzke *et al.*, 1996)

$$P = v_s t-(v_s-v_i)(1-e^{-kt})/k$$

where P is the concentration of the product at time t, v_i is the initial velocity, v_s is the steady-state velocity, and k is the rate constant characterizing the change. The branched pathway may be contrasted with a progress curve that follows Scheme 1, in the case where $k_2 \gg k_3$. A fast burst with the amplitude that equals to 1 mol of product/mol of enzyme is characteristic of this mechanism, if the acyl enzyme and the product absorb light similarly. The apparent steady-state rate, k_{cat}, is then equivalent to k_3, the rate of deacylation. In addition, Escobar *et al.* (1994) discussed the case in which $k_3 < k_4$ in a branched pathway, a condition that may also lead to a burst with amplitude of 1.

To attempt to distinguish between a simple linear mechanism (Scheme 1) and the branched pathway (Scheme 2), the reaction may be monitored in the presence of 0.5 M ammonium sulfate. Mitchinson and Pain (1985) have shown that sulfate stabilizes the native form of β-lactamase from *S. aureus*; hence it was expected that for a branched pathway the burst stoichiometry would increase. A similar experiment has been done with the E166C β-lactamase from *B. licheniformis*, for which a doubling of the burst

stoichiometry was observed in the presence of 0.5 M ammonium sulfate (Escobar *et al.*, 1994). This was interpreted as indicative of a branched pathway.

The progress curve of cephaloridine hydrolysis by MBP-Hybrid4 (Figure 6.13B and Figure 6.13C) or MBP-PC1 (Figure 6.13F) was found to exhibit an initial exponential phase followed by a slower steady-state phase, the inset of Figure 6.13F clearly indicates that the reaction exhibits exponential decay of enzymes activity during assay. When the reaction reached the slow linear phase, the exponential phase may be restored by the addition of the same amount of enzyme (Figure 6.13D) but not the same amount of cephaloridine (Figure 6.13E), which suggests that exponential decay of enzyme activity is due to the enzyme inactivation but not substrate exhaustion.

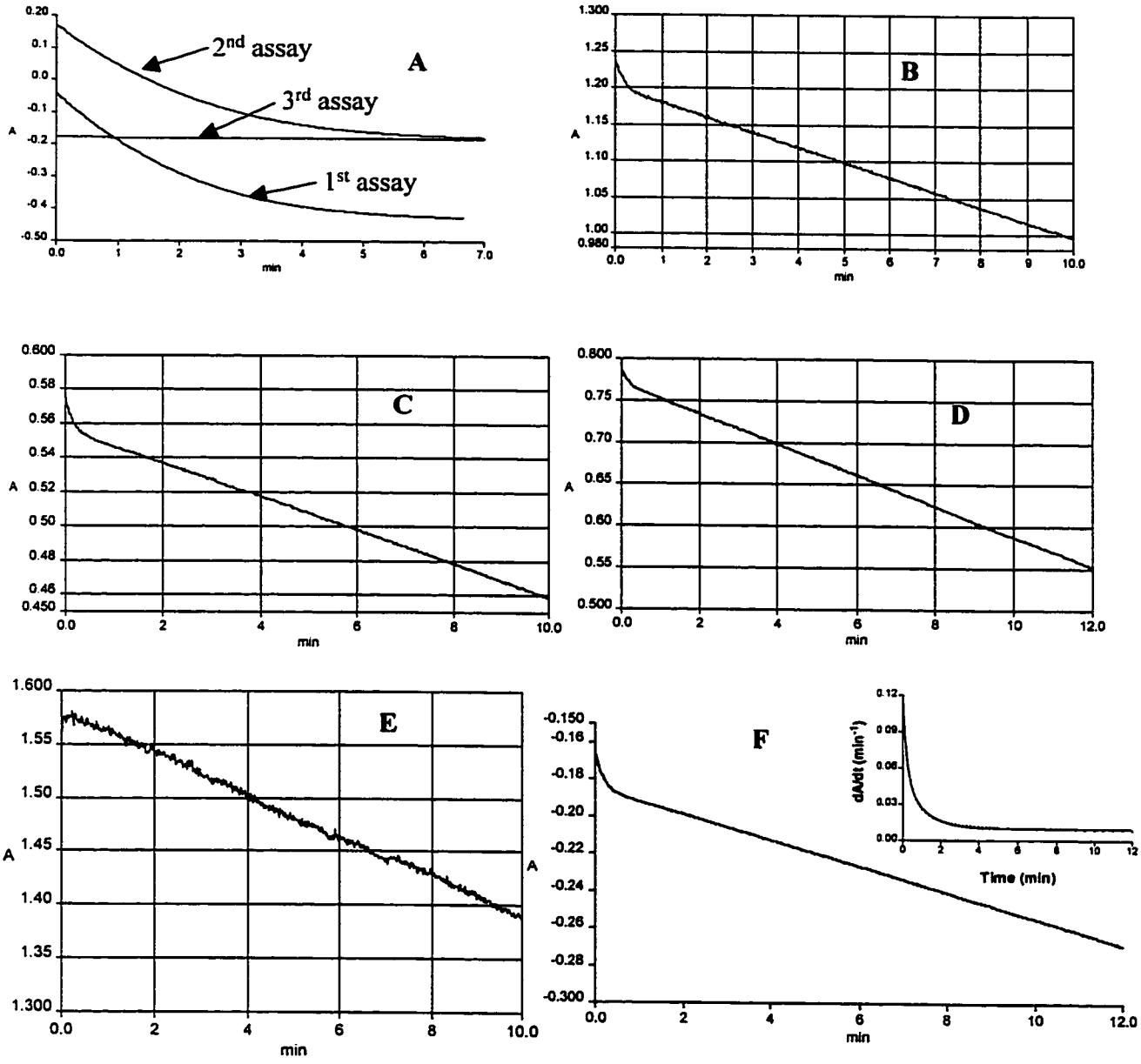


Figure 6.13 Progress curves of cephaloridine hydrolysis by β-lactamases. The 1st assay in (A) was performed by the addition of 5 μl of 15.5 μM MBP-PenPC into 995 μl assay buffer with 50 μM cephaloridine; the 2nd assay was performed by the addition of 50 μl of 1 mM cephaloridine to the mixture when the reaction in the 1st assay reached the end; When the reaction in the 2nd assay finished, 5 μl of 15.5 μM MBP-PenPC was added to the mixture and the 3rd assay was started. The curve (B) or (C) indicates the reaction by the mixture of 100 μl or 200 μl of 10.6 μM MBP-Hybrid 4 with 50 μM cephaloridine. When curve (C) reached the slower steady-state linear phase, another 100 μl of 10.6 μM MBP-hybrid 4 was added into the mixture, the reaction was recorded in curve (D), which is very similar to curve (C). However, when curve (D) went to linear phase, the addition of 50 μl of 1 mM cephaloridine did not restore the exponential phase (E). Curve (F) is the hydrolysis process of cephaloridine by MBP-PC1; the inset clearly indicates that the reaction exhibits exponential decay of enzyme activity during assay.

6.8 Discussion

From the thermal inactivation study, PenP shows much higher thermal stability than PenPC, which is consistent with the published data (Matagne *et al.*, 1990). Most interestingly, for the hybrid enzymes of PenP and PenPC, the more protein sequence is derived from PenP, the higher is the thermal stability of the protein. It seems that thermostability in different enzymes is achieved by a combination of various interactions and there are more interactions inside the PenP enzyme to stabilize the protein conformation. To date, no general rules as to which factors lead to increased protein stability has emerged, except that cumulative effects of hydrogen bonding, electrostatic interactions, better hydrophobic internal packing, helix-dipole stabilization, enhanced stability of secondary structure elements, etc., all come into play (Dangi *et al.*, 2002). The very high thermal stability of PenP may be due to its larger cumulative effects of all these kinds of interactions. For some unknown reasons, many enzymes produced by mesophilic *Bacillus licheniformis*, such as α-amylase, xylose isomerase, and phytase, are thermostable (Machius *et al.*, 1995; Vieille *et al.*, 2001; Tye *et al.*, 2002)

Thermal denaturation of MBP fusion enzymes of PC1, PenPC and their hybrids, which was monitored by measuring the far-UV protein ellipticity at 222 nm, suggests that the overall conformational changes of all the MBP-β-lactamases to elevated

temperatures are slightly different, as well as the steepness of the unfolding curves. However, for thermal inactivation, the difference is very significant. From our preliminary study, all MBP-β-lactamases, except for MBP-PenPC, lost almost all the activity after incubated in the MBP column buffer (0.05 M sodium phosphate, 0.5 M NaCl, 0.1 mM EDTA, pH 7.0) for 5 min at 65°C (data not shown). It seems that the change in activity is much more sensitive than the change in overall conformation. This indicates that the structure in the actives site is more flexible than other structures since the change in activity is the consequence of the change of the active site structure. PenPC shows much higher stability than the other enzymes for its performance in β-lactam hydrolysis. We obtained similar result from the GdnHCl inactivation experiments. As shown in Figure 6.3, MBP-PenPC shows much higher Cm value than the other enzymes. Moreover, while the Cm values of MBP-Hybrid 1 and MBP-Hybrid 4 are similar to that of MBP-PC1, MBP-Hybrid11 is very sensitive to GdnHCl, which reveals a reduced stability of the hybrid enzyme. For MBP-Hybrid6, MBP-Hybrid7 and MBP-Hybrid8, obvious activity decrease was observed when these purified samples were stored at 4 °C, which indicates the more reduced stability of these fusion enzymes. Therefore, for MBP fusion enzymes of PC1, PenPC and their hybrids, compared with their parents, hybrid enzymes lose their stability to different extents, especially those with crossover events occurred near the middle of the two

parental β-lactamases, such as Hybrid6, Hybrid7 and Hybrid8. These hybrid enzymes are more sensitive to the environmental conditions (such as elevated temperatures and GdnHCl) than their parents, which may be due to the structural perturbations in the active sites of these hybrid enzymes, especially the Ω-loop, which forms part of the active site depression. The conformational integrity of the Ω–loop in class A β-lactamases is crucial for hydrolysis and determining substrate binding affinity (Banerjee *et al.*, 1998). By comparison and alignment of amino acid sequences (Figure 6.14), the Ω–loop of MBP-Hybrid8 is found to be a "hybrid Ω–loop" (half from PenPC, and half from PC1). This new Ω–loop may cause the dramatic loss of activity. The packing of the Ω–loop against the rest of the molecule is imperfect in PC1 and the peptide bond between the catalytic residue, Glu 166, and the following residue, Ile 167, is a *cis* bond, an energetically unfavorable conformation. Non-proline *cis* peptide bonds are rare in proteins of known structure. A scrutiny of the amino acid sequences of various class A β-lactamases documented in the literature (Ambler *et al.*, 1991) revealed that position 167 is often (14 out of 20 instances) occupied by a proline residue, a feature in conformity with the expected preference for this residue in peptide bonds of *cis* conformation. Threonine appears to be the next preferred residue in this location since it has been found in four class A β-lactamases, one of which is PenPC. The hybrid Ω-loop of MBP-hybrid8 may not be able to form the correct

conformation, and therefore causes the dramatic lost of catalytic activity of the enzyme. Again, we still need the data from the study of protein crystal structures to prove this speculation.

The data presented above unequivocally indicate that the stabilities of the enzymes studied here decrease as follow: PenP > PenPC > PC1, which is consistent to the study of Vanhove *et al.* (1995). The structural feature of Ω-loop could, at least in part, explains the different stability of β-lactamases. In PenP, residue 167 is a proline but it is a threonine and an isoleucine in PnePC and PC1 respectively. Since the *cis* conformation is strongly unfovourable for a peptide bonds that do not involve proline residues. One can assume that the lowest stability of PC1 is, in part, due to the presence of this *cis* peptide bond in the structure.

PenPC	163	**N R F E T E L N E A I P G D I R** 178
Hybrid 8	163	**N R F E T** E L N Y Y S P K S K K 178
PC1	163	V R Y E I E L N Y Y S P K S K K 178

Figure 6.14 Comparison of the Ω-loop sequences of PenPC, PC1 and Hybrid8. The junction region of the hybrid Ω-loop of Hybrid8 is underlined.

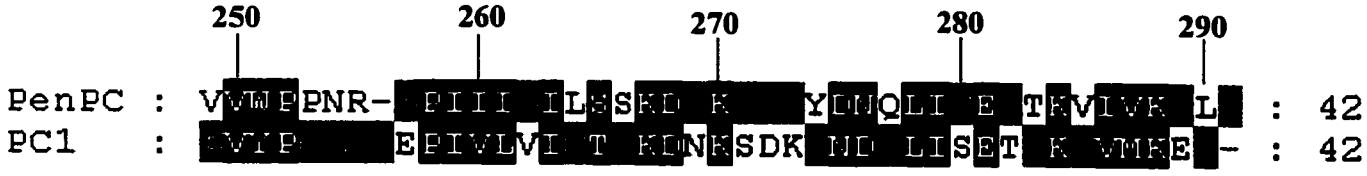


Figure 6.15 Alignment of the primary structures of the C-terminal 42 amino acids of PenPC and PC1.

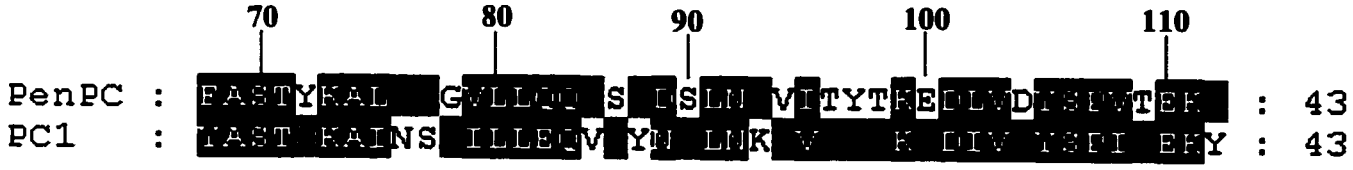


Figure 6.16 Alignment of the 43 amino acids region in between the crossover points of Hybrid5 and Hybrid6.

By the comparison of primary protein structures and enzymatic properties between PenPC and Hybrid11, we were surprised to find that the C-terminus of PenPC is essential to its stability and effective catalysis. The replacement of it (42 amino acids, from V 249 to R 291) with the corresponding sequence from PC1 is of disastrous consequence. The identity and similarity of this C-terminal region between PenPC and PC1 are 30% and 62%, respectively, which are quite similar to the identity (34%) and similarity (57%) between the two entire polypeptides. As shown in Figure 6.15, some residues of PenPC in this region, especially those different from PC1, may be very critical for its stability and effective catalysis. One of such residues may be Ala 272. According to the studies from the TEM-1 β-lactamase, Met 272 was found to be one of the essential residues that are not directly located in the active pocket and yet may influence substrate binding and catalysis (Huang *et al.*, 1996). The Met 272 side-chain is 47% solvent exposed and within van der Waals distance of the Arg 244 side chain which has been shown to participate in substrate binding (Delaire *et al.*, 1992; Zafaralla *et al.*, 1992). Thus, Met 272 may not tolerant of amino acid residue substitutions because such substitutions would alter the position of Arg 244 and thereby reduce the catalytic activity of the enzyme. In the *B. cereus* (and *B. licheniformis*) or *S. aureus* enzymes, an alanine or aspartic acid residue, respectively, occurs at this position. Therefore, residue 272 makes different interactions in the

different enzymes and the replacement of Ala 272 by Asp in PenPC may be one of the reasons why Hybrid11 dramatically loses its stability and catalytic activity.

As shown in Table 6.4 and Table 6.5, the K_m values of MBP-PC1, MBP-Hybrid1, MBP-Hybrid4, and MBP-Hybrid5 towards penicillin G, ampicillin, nitrocefin and cephaloridine are similarly very low, indicates that they show high affinity to these β-lactams. On the contrary, MBP-PenPC, MBP-Hybrid6, MBP-Hybrid7, MBP-Hybrid8 and MBP-Hybrid11 similarly show relatively low affinity (higher K_m) towards these β-lactams. Therefore, the region in between the crossover points in Hybrid5 and Hybrid6 may be very important for the determination of substrate specificity of Class A β-lactamases to these substrates, since the K_m values of MBP-Hybrid5 (similar to PC1) for many β-lactams are significantly different from those of MBP-Hybrid6 (similar to PenPC). By alignment of protein sequences alignment, the two hybrids differ at a 43-amino-acid region (for PenPC, from Phe 68 to His 112; for PC1, from Tyr 68 to Tyr 112) , covering Ser 70 and Lys 73, which are crucial active site residues for Class A β-lactamases. The identity and similarity of this region between PenPC and PC1 are 41% and 69%, respectively, which are much higher than those of the two entire polypeptides (Figure 6.16). Some residues in this region may be very critical for determining substrate specificity of β-lactamases.

The Ω-Loop was considered to be implicated in substrate specificity both in class A β-lactamases and class C β-lactamases (Banerjee *et al.*, 1998; Nukaga *et al.*, 1998; Palzkill *et al.*, 1994). However, there are some exceptions. The class A PSE-4 β-lactamase from *Pseudomonas aeruginosa* strain Dalgleish is capable of hydrolyzing carbenicillin at a high rate (Boissinot and Levesque, 1990). The roles of amino acids 161-179 in modulating ceftazidime resistance and hydrolysis in the PSE-4 Ω-loop are different from those in TEM-1 (Therrien *et al.*, 1998). By cassette mutagenesis, amino acids 163-179 (Ω-loop) was replaced with TEM-1, SHV-1 and *Streptomyces albus* G β-lactamase Ω-loops. Phenotypic analysis of *E. coli* recombinants expressing the Ω-loop PSE-4 mutant enzymes gave MICs and kinetic data similar to those of the wild-type PSE-4 (Sanschagrin *et al.*, 2000). Our results also suggest that the protein sequence located at 68-112 in class A β-lactamases (according to ABL scheme) (Ambler *et al.*, 1991) may play a more important role in substrate binding than the Ω-loop does, since the K_m values of MBP-Hybrid6 and MBP-Hybrid7, whose Ω-loops are from PC1, are similar to those of MBP-PenPC. It is quite reasonable because this region includes two important residues Ser 70, Lys 73 and is very close to the Glu-166 and the Ω-loop (Figure 6.17).

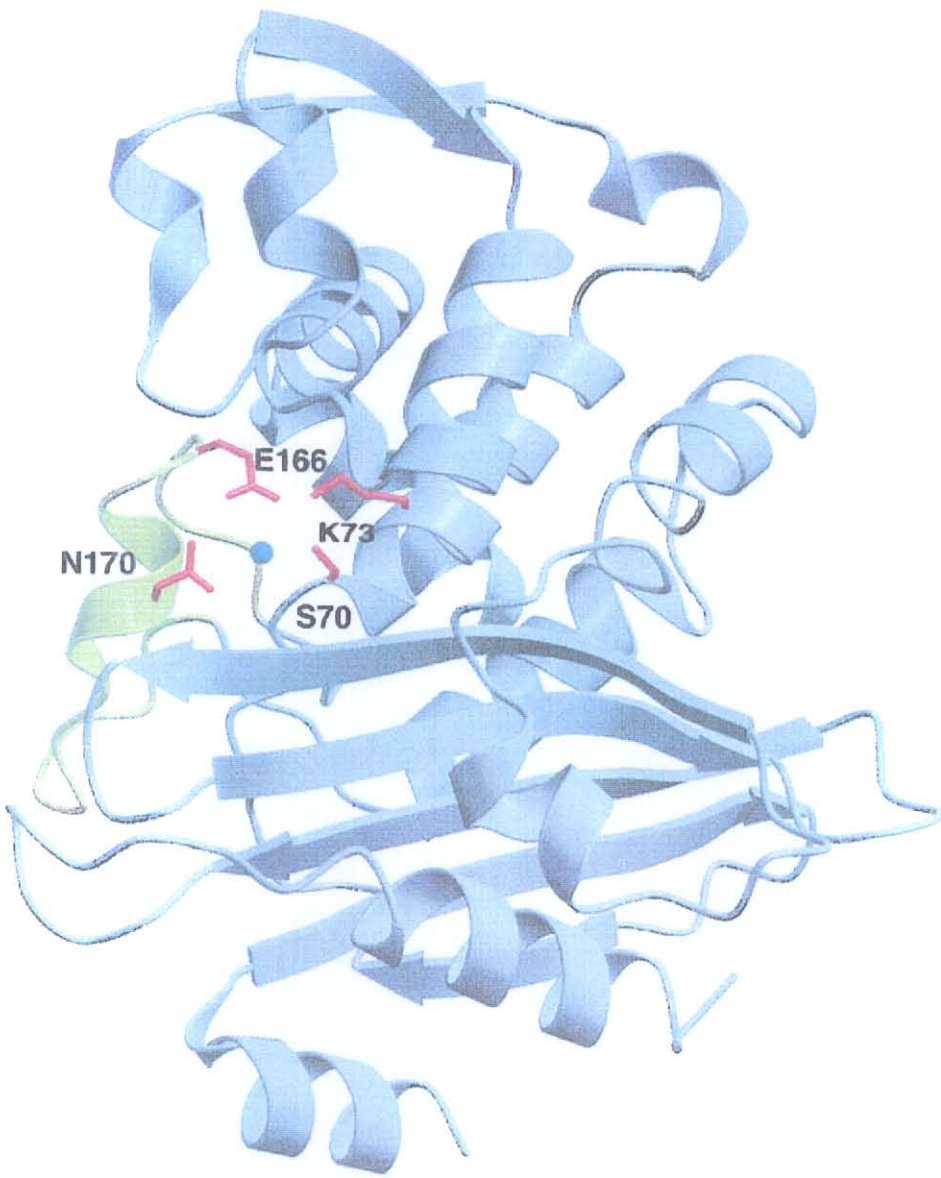


Figure 6.17 Overall fold of β-lactamase, highlighting the Ω-loop (gold) and key active site residues, Ser 70, Lys 73, Glu 166, and Asn 170 (red), and the hydrolytic water molecule (blue) (after Chen & Herzberg, 2001).

From the crystal structure studies of the PC1 enzyme (Herzberg and Moult, 1987; Herzberg, 1991), the region from Tyr 68 to Tyr 112 covers helix α2 (71-82), helix α3 (107-113) and the loops connecting them (Figure 1.7B). The internal cavities in the enzyme are larger in volume than a water molecular. This shows that the packing of this enzyme is strikingly imperfect. Two cavities are adjacent to the central helix α2, and are very large. One cavity is rather long and narrow. The second is an irregularly shaped cave and its limits are defined by helix α2, by Ω-loop and by residues on helices α5 and α6. Such a loose packing suggests a possible conformational flexibility of the molecule between the Ω-loop and helix α2, which would affect the shape of the substrate binding site. Ser 70 is situated at the amino terminus of the buried central helix α2 of the helical domain, suggesting a role for the dipole of this helix in the catalytic mechanism (Hol, 1985). In addition, analysis of the high-resolution three-dimensional structure of the TEM-1 enzyme (Jelsch *et al.*, 1993) shows that residue 104 is within a large loop encompassing residues 101 to 111. The 101-111 loop is strongly conserved among all β-lactamases except the residue 104, which is highly variable (Figure 6.18). This loop is also facing the Ω-loop. Even no evidence indicates a direct role for residue 104 in the substrate specificity of the class A β-lactamases, this residue may contribute to the precise positioning of residues 130-132 which are involved in substrate binding and catalysis (Petit *et al.*, 1995; Sowek *et al.*, 1991).

Changing residue 104 could also modify slightly the local electrostatic potential in this part of the active site (Petit *et al.*, 1995). Although position 104 alone seems insufficient to confer substrate binding, the K_m values of the E104K/R164S mutant of TEM-1 toward cephaloridine, cefotaxime, aztreonam, and penicillin G are significantly lower than those of TEM-1 (Table 6.6) (Sowek *et al.*, 1991). For class A β-lactamases, another non-conserved residue within this region (from 68-112) is position 76. The TEM-1 Leu 76 is completely buried on helix α2 of the enzyme (Jelsch *et al*, 1993). Leu 76 is part of a buried, hydrophobic cluster of residues including Phe 72, Ala 126, Ala 135, Leu 139, Pro 145, Leu 148 and Leu 162. The *B. cereus* and *S .aureus* enzymes have a threonine and a glutamine residue, respectively, at position 76. In the *B. cereus* and *S. aureus* enzymes, the residues surrounding residue 76 are thus predominantly hydrophilic; residue 76 interacts with its neighbors by means of hydrogen bonds rather than hydrophobic interactions, as in TEM-1. It seems that this structural region is stabilized by different residue-residue interactions among class A β-lactamases. The changes of some residues may cause the structural perturbations and have effect on substrate binding. However, the exact reason why the residues in this region (68-112) are so critical for determining substrate specificity is still unclear. Further studies to dissect and understand the exact structural property and

functions of this region may provide new insights into the mechanism of β-lactamase action.

Table 6.6 Binding parameters (K_m, μM) for mutant β-lactamases (after Sowek *et al.*, 1991)

Enzymes	Cephaloridine	Cefotaxime	Ceftazidime	Aztreonam	Penicllin G
TEM-1	660	450	80	1500	26
E104K	660	470	150	1400	19
R164S	100	230	260	1400	16
E104K/R164S	90	84	150	64	5.8

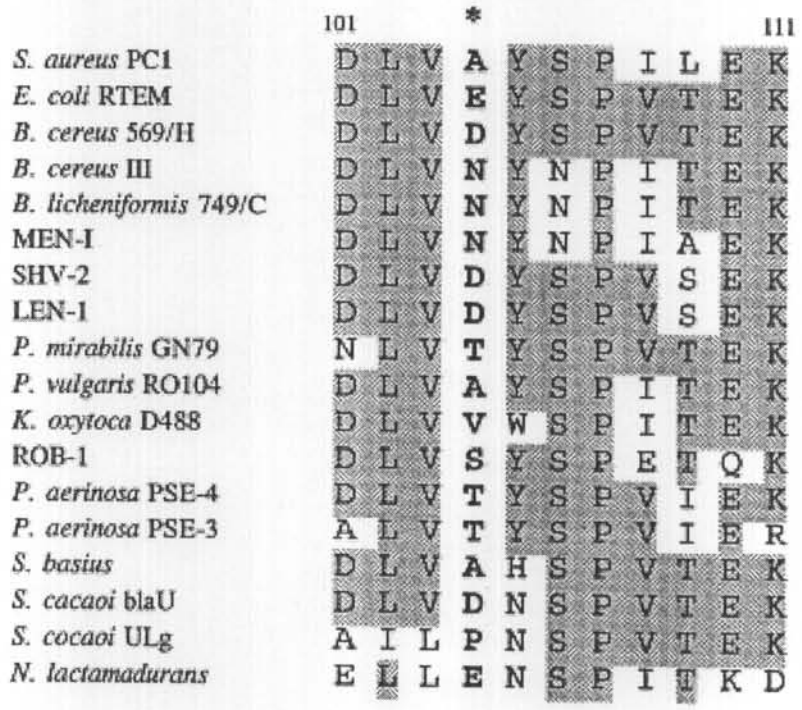


Figure 6.18 Amino acid sequence alignment of class A β-lactamases in the region of the highly conserved region around position 104. Hatched residues denote complete identity among the 18 aligned sequences, while residue 104 is in bold type and denoted by the asterisk. The figure is after Guo *et al.* (1999).

The kinetic studies of MBP-fusion enzymes of PC1, PenPC and their hybrids (Table 6.4 and Table 6.5) also suggest that some hybrids, especially those with crossover events occurred near the middle of the two β-lactamases, such as Hybrid6, Hybrid7 and Hybrid8, dramatically lost their catalytic efficiency in hydrolyzing penicillin G, ampicillin, nitrocefin and cephaloridine. In contrary, they have high apparent substrate affinity (much lower K_m) to other β-lactams with bulky side-chains such as methicillin and oxacillin. This suggests that the generation of hybrid enzymes is similar to the evolution process. In carving out a larger active site to accommodate the β-lactams with bulky side-chains, these new enzymes lost their power to hydrolyze β-lactams with simple side-chains. Also, they lost their internal actions that formerly contributed to their internal integrity, lowering their stability.

From the kinetic studies of PenP, PenPC and their hybrid β-lactamases, the overall kinetic property of Hybrid A is very similar to that of PenP, whereas the overall kinetic property of HybridB, HybridC, HybridD or HybridE is very close to that of PenPC. That is reasonable because most of the protein sequence of HybridA is derived form PenP (Figure 6.6) and most of the protein sequence of HybridB, HybridC, HybridD or HybridE is derived from PenPC (Figure 6.6). For HybridA, the K_m and k_{cat} values towards 6-APA and the K_m value towards methicillin are in the middle of those of the two parents. It is also reasonable considering that the protein sequence of

HybridF is half from PenP and half from PenPC (Figure 6.6). More interestingly, HybridF and HybridB show a much higher apparent binding affinity (lower K_m) towards cefuroxime (oxyimino β-lactam) than their parents. We also obtained a similar result for MBP-Hybrid5, which show a higher apparent binding affinity (lower K_m) towards cefuroxime than their parents.

Structural perturbations leading to extended activity in the class A β-lactamases have been discussed (Banerjee *et al.*, 1998; Huletsky *et al.*, 1993; Knox, 1995; Nukaga *et al.*, 2002; Raquet *et al.*, 1994). Most third-generation β-lactams which are resistant to class A and class C β-lactamases have a bulky and plane group such as an aminothiazolemethoxyimino or aminothiazolecarboxypropylozyimino side chain at position 7 of their cephalosporin nucleus or at position 2 of their monobactam nucleus, and they are named oxyimino β-lactams. The structural factors that result in the extended-spectrum phenotypes are quite diverse. For example, the three-dimensional structure of the SHV-1 β-lactamase, which possesses a somehow broader substrate profile than the TEM-1 enzyme. However, there are a few of subtle differences that differentiate the active sites of the two enzymes. The Ser 130 to Asn 132 loop and the neighboring Asp 104/ Tyr 105 loop in the SHV-1 enzyme have shifted away from the active site by about 0.7-1.2 Å, in comparison to the TEM-1 enzyme, thus widening the active site. A similar shift of Asn 132, seen in the NMC-A β-lactamase (Swaren *et al.*,

1998), was observed for the SHV-1 enzyme as well. As shown by the x-ray structure of another ESBL, Toho-1 (Ibuka *et al.*, 1999), and the role for Arg 244 in other class A β-lactamases is fulfilled by residue Arg 276 in the Toho-1 β-lactamase. The structure of the Per-1 β-lactamase shows significant differences in the Ω-loop, β3-strand and α2-helix regions compared to the corresponding regions in the TEM-1 β-lactamase. The collective effect of these differences is an enlargement of the active site pocket in the Per-1 enzyme. Therefore, it would appear that a theme for evolution of the extended –spectrum β-lactamases is enlargement of the active site in order to alleviate the unfavorable steric interaction with certain substrates that have been relatively immune to the deleterious action of β-lactamase (Kotra *et al.*, 2002). We believe that the active sites of MBP-Hybrid5, HybridF and HybridB are also enlarged due to the lack of some interactions and consequently, their binding affinity to oxyimino β-lactams is improved. It seems that homologous recombination among β-lactamases may be one of the approaches for the generation of extended-spectrum β-lactamases during evolution. However, the structural rationale for the kinetic properties of these hybrid enzymes still remains speculative in the absence of a crystal structure.

Chapter Seven

Expression of β-lactamase inhibitory protein (BLIP) in *Bacillus subtilis*

7.1 Introduction

As introduced in Chapter 1, β-lactam antibiotics such as penicillins and cephalosporins are among the most often used antimicrobial agents. Due to the widespread use of β-lactam antibiotics, bacterial resistance has been increasing at an alarming rate and represents a serious threat to the continued use of antibiotic therapy. The most common mechanism of bacterial resistance to β-lactam antibiotics is the synthesis of β-lactamases that cleave the amide bond in the β-lactam ring to generate ineffective products. TEM-1 β-lactamase is a class A enzyme encoded by the bla_{TEM-1} gene. Epidemiological studies have shown that TEM-1 is the most common plasmid-encoded β-lactamase in gram-negative bacteria (Wiedemann *et al.*, 1989). It is able to efficiently hydrolyze penicillins and many cephalosporins; therefore, it is an important source of bacterial resistance to β-lactam antibiotics. The SHV-1 β-lactamase is 68% identical to the TEM-1 β-lactamase and also occurs frequently in gram-negative bacteria (Barthelemy *et al.*, 1988; Bush, 1998). The SHV-1 β-lactamase exhibits a substrate hydrolysis profile similar to that of TEM-1. To overcome the drug resistance mediated by TEM-1 and SHV-1 β-lactamases, extended-spectrum cephalosporins such as aztreonam, cefotaxime, and ceftazidime were developed, in part, because the TEM-1 and SHV-1 β-lactamases are not able to hydrolyze these antibiotics. An additional strategy that has been employed to combat

antimicrobial resistance is the use of β-lactamase inhibitors such as the clavulanic acid, sulbactam, and tazobactam. Although not capable of antimicrobial activity themselves, these suicide inhibitors are used in conjunction with various β-lactam antibiotics to bind β-lactamase and prevent the hydrolysis of the antibiotics, thereby restoring the therapeutic value to the antimicrobial agent. However, both of these approaches apply selective pressure for mutations that result in a β-lactamase that either cleaves extended-spectrum cephalosporins or is no longer sensitive to β-lactamase inhibitors. Both types of mutations have been found in the genes encoding for TEM-1 and SHV-1 β-lactamases from clinical isolates which are resistant to these therapies. This has led to an increasing problem of resistance to antibiotics and a corresponding decrease of effective therapies for some bacterial infections. The emergence of resistance to β-lactamase inhibitors and the relatively rapid emergence of resistance to new antibiotics imply that the design of new antibiotics must keep pace with the evolution of bacterial resistance.

Clavulanic acid was initially purified from the soil bacterium *Streptomyces clavuligerus*, which also produces a protein inhibitor of β-lactamases called β-lactamase inhibitory protein (BLIP) (Doran *et al.*, 1990). BLIP is a 165 amino acid protein composed of two-domains of ~78 residues each and has been shown to bind to and inhibit the TEM-1 β-lactamase with a K_i of 0.1 to 0.6 nM (Petrosino *et al.*, 1999;

Rudgers and Palzkill, 1999; Strynadka *et al.*, 1994). In addition, BLIP inhibits β-lactamases from both Gram-positive and Gram-negative bacteria to varying degrees and also inhibits the cell-wall transpeptidase PBP5 from *Enterococcus faecalis*. However, BLIP does not efficiently bind to class B, C, or D β-lactamases (Strynadka *et al.*, 1994).

In terms of known protein-inhibitor complexes, TEM-1-BLIP is unique in that the inhibitor provides the concave surface and the enzyme presents a convex face for the interaction. The co-crystal structure of TEM-1 β-lactamase and BLIP reveals that BLIP binds just outside the active site pocket of β-lactamase and inserts two loops, one from each domain, into the active site of the enzyme (Figure 7.1) (Strynadka *et al.*, 1996). The structure of the complex also indicates that a β-hairpin including residues 46-51 of BLIP inserts into the active site of β-lactamase. An aspartic acid residue at position 49 of the hairpin is positioned in the active site to form hydrogen bonds with four catalytic residues of β-lactamase: Ser 130, Lys 234, Ser 235, and Arg 244 (ABL numbering). In addition, a phenylalanine at position 142 on the other loop occupies a position in the active site similar to the position that the benzyl group of β-lactam antibiotic penicillin G occupies during substrate binding and catalysis (Strynadka *et al.*, 1996). Replacement of Asp 49 with Ala lowers the binding affinity approximately 80-fold, whereas substitution of Phe 142 with Ala results in a 300-fold decrease in

binding affinity. Thus, Asp 49 and Phe1 42 make important contributions to the stability of the inhibitory complex. However, since the D49A and F142A variants still bind TEM-1 β-lactamase with nanomolar affinity, other interactions must also contribute to the strong levels of inhibition observed. The identification of the epitopes responsible for the remaining binding energy will facilitate the engineering of tighter, smaller inhibitors for these β-lactamases (Petrosino *et al.*, 1999). Moreover, wild-type BLIP has a higher K_i for the E104K β-lactamase mutant, suggesting that interactions between BLIP and β-lactamase residue Glu 104 are important for wild-type levels of BLIP inhibition.

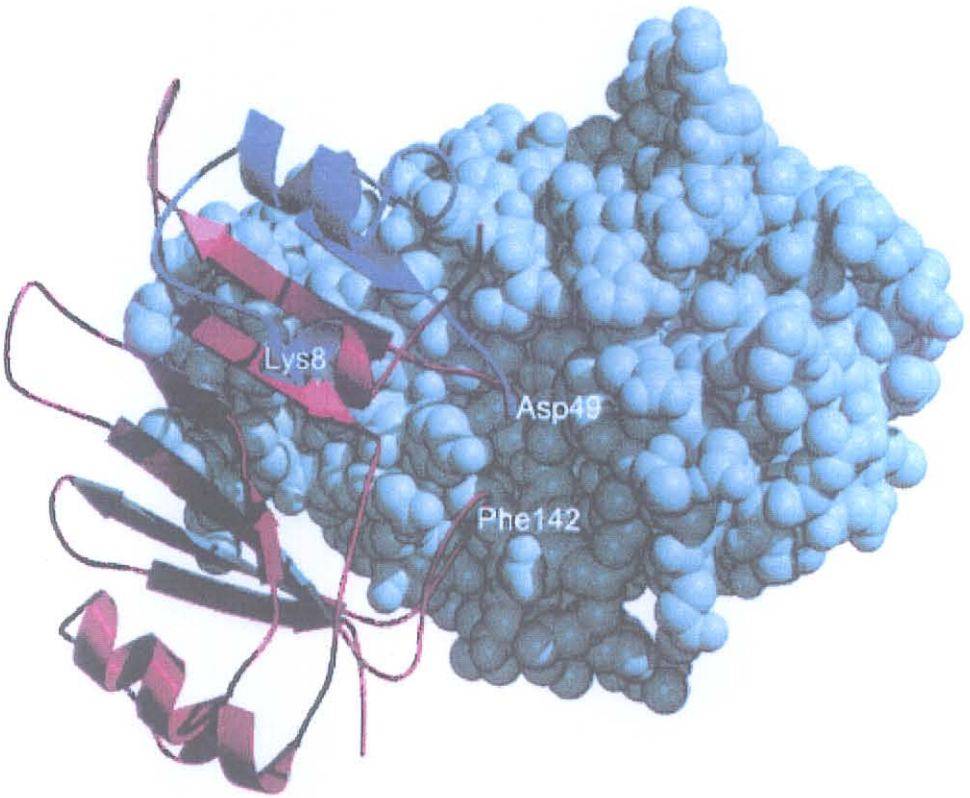


Figure 7.1 Structure of the complex between BLIP and TEM-1 ß-lactamase (after Rudgers and Palzkill, 2001). TEM-1 β-lactamase is shown in space-fill and is colored gray. The BLIP structure is presented in ribbon format and colored red. The K8–D49 peptide region of BLIP is indicated in blue. The position of the BLIP Asp 49 and Phe 142 turn regions that insert into the active site of TEM-1 β-lactamase are labeled.

Very interestingly, even though TEM-1 and SHV-1 enzymes hydrolyze a similar profile of penicillins and cephalosporins and their amino acid sequences show 68 % identity, the K_i of BLIP for SHV-1 was found to be 1.0 μM, which is 9,000-fold higher than what was found for TEM-1 (Petrosino *et al.*, 1999). It suggests that the interactions that make BLIP a tight inhibitor of TEM-1 are not conserved with SHV-1 and that small structural differences between the β-lactamases are significant with respect to BLIP binding. It is of great interest to delineate the important amino acids in β-lactamases, which are critical for interaction with BLIP. A better understanding of the interactions between BLIP and β-lactamases could serve as a stating point for the design of novel proteins that can much more effectively inhibit β-lactamases. Considering the extensive amino acid sequence homology among class A β-lactamases, the creation of β-lactamase hybrids may be an ideal approach in this aspect. BLIP inhibits a large variety of β-lactamases from different bacterial species to varying degrees (Strynadka *et al.*, 1994). Whereas BLIP has been shown to be able to effectively inhibit the *B. licheniformis* 749/C β-lactamase with a Ki of lower than 3 μM, commercially available (Sigma) β-lactamases derived from *B. cereus* (including PenPC) and *S. aureus* PC1 β-lactamase were not inhibited by BLIP (Doran *et al.*, 1990; Strynadka *et al.*, 1996; Strynadka *et al.*, 1994). Therefore, we have good grounds for believing that the available hybrid β-lactamases are very useful for the identification

of amino acids involved in BLIP binding. However, the prerequisite to this analysis is to get sufficient amount of soluble BLIP.

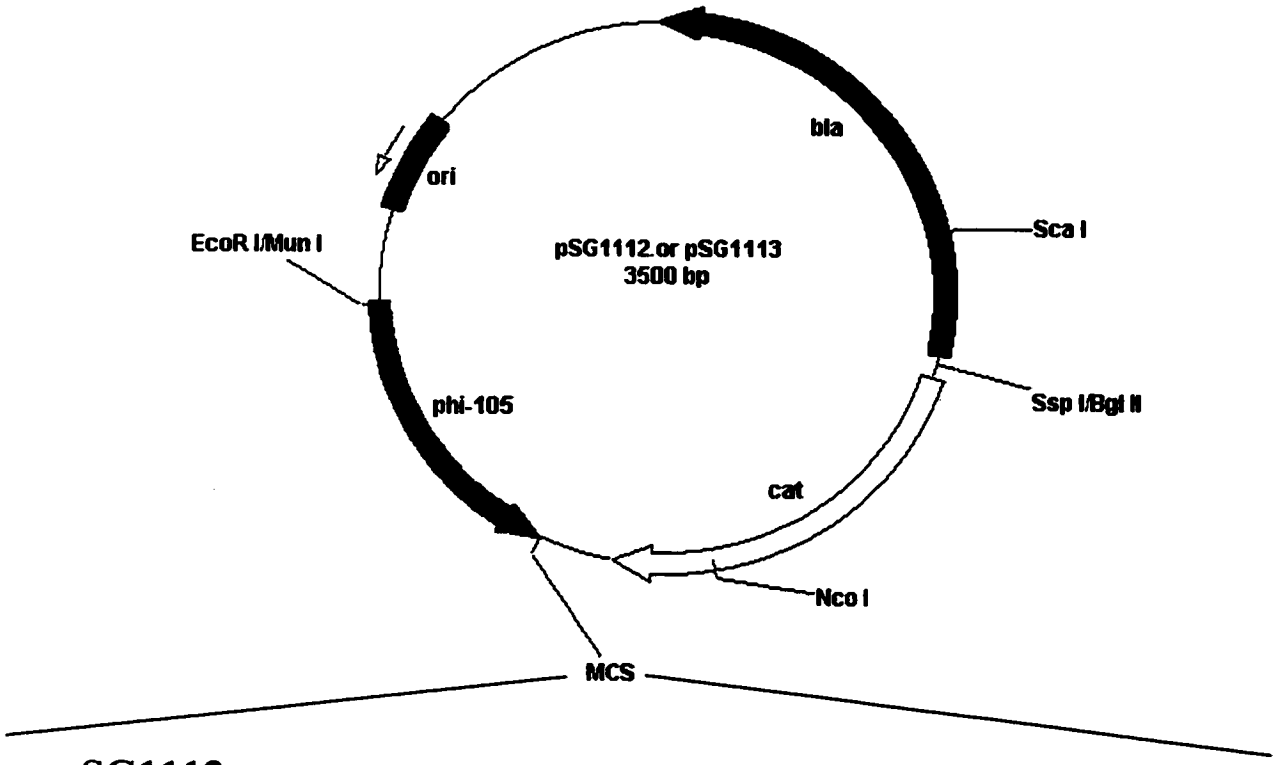
BLIP expressed in its native *S. clavuligereus* produces large quantities of protein, whereas expression in another Streptomyces species, *Streptomyces lividans*, produces limited quantities of BLIP (in amounts approximate 12-fold lower than that produced by *S. clavuligerus)* (Doran *et al.*, 1990; Paradkar *et al.*, 1994). In order to facilitate the study of BLIP mutants and to allow protein-engineering techniques to be performed, functional BLIP had been expressed in *E. coli* (Albeck and Schreiber, 1999; Petrosino *et al.*, 1999; Rudgers and Palzkill, 1999). When expressed as a His-tagged protein, about 0.25 mg of >90% pure BLIP was isolated for every one liter of culture. The His-tagged BLIP interacts with 1 nM TEM-1 β-lactamase and resulted in a K_i of 0.11 nM, which is slightly lower than the previously reported value of 0.6 nM for BLIP purified from *S. clavuligereus* (Petrosino *et al.*, 1999). BLIP was also reported to have been expressed and purified as a fusion to the C-terminus of maltose binding protein (MBP). Binding experiments demonstrated the MBP-BLIP fusion protein has the same K_i for β-lactamase inhibition as the wild-type BLIP. However, the yield and the purity of the MBP-BLIP was not mentioned in the paper (Rudgers and Palzkill, 1999). When the *blip* gene (498 bp) was subcloned into a pET-9a vector (Promega), high BLIP expression was achieved by growing cells in a fermentor. Even though about 2

mg pure protein was isolated from a 1-liter culture, the protein has to be refolded from inclusion body which is time-consuming and of very low recovery (Albeck and Schreiber, 1999). Therefore, all these heterologous intracellular expressions of protein in *E. coli* are not good enough for the production of BLIP. As mentioned in Chapter 3, *B. subtilis* 1A304(ϕ105MU331) has been successfully used to facilitate the expression and purification of a number of heterologous proteins including PenPC, PenP β-lactamase and their hybrid derivatives in our laboratory. Since both *B. subtilis* and *S. clavuligerus* are gram-positive, it is very likely that BLIP may be properly processed and secreted into culture medium when expressed in *B. subtilis*.

7.2 Subcloning the *blip* gene into the plasmids pSG1112 and pSG1113

To facilitate the introduction of heterologous gene for expression in *B. subtilis* strain 1A304(ϕ105MU331), vectors pSG1112 and pSG1113 were employed. These vectors are derivatives of pSG703 (Thornewell *et al.*, 1993) and their maps are shown in Figure 7.2. These vectors carried a pBR322 replication origin for *E. coli*, a *cat* gene for chloramphenicol resistance, a *bla* gene for ampicillin resistance, and a fragment containing the promoter and the Shine-Dalgarno-type ribosome-binding site (SD) from prophage ϕ105MU331, followed by a MCS containing several unique restriction sites. The two integration vectors are identical except that pSG1113 includes the sequence encoding for six-histidine amino acids and the distance between SD and start

codon ATG in pSG1113 is two bases longer than that in pSG1112. Chromosomal DNA of *Stretomyces clavuligerus* (ATCC 27064) was purified by Wizard® Genomic DNA Purification Kit (Promega) and used as template of PCR reaction. Primers LHB31 (5'-gtatat**CATATG**aggacagtggggatcggcgcg-3') and LHB33 (5'-gatata**TC TAGA**ggtcgactccttcggcgacg-3') were designed to amplify the *blip* gene, covering the sequence encoding the signal peptide and the sequence of transcriptional terminator (designated *blip F*). Primers LHB32 (5'-gatata**CATATG**gcgggggtgatgaccggggcg –3') and LHB33 were used to amplify *blip* without the sequence encoding signal peptide (designated *blip M*). Considering the high GC content of the *blip* gene (66%), which was within the range of 62 to 74 % G+C characteristic of *Streptomyces* genes (Doran *et al.*, 1990), 5% DMSO was included in the PCR reaction to prevent the formation of secondary structures. For PCR reaction, thirty-five cycles of amplification were performed with the following temperatures: 30 s at 94°C, 30 s at 50°C, 30 s at 72°C. The two PCR products were digested by *Nde*I and *Xba*I, and DNA fragments were recovered from argarose gel and ligated with similarly digested pSG1112 and pSG1113. The correct recombinant plasmids were screened by *Nde*I and *Xba*I digestion. All recombinant plasmids (pSG1112*blipF*, pSG1113*blipF*, pSG1112*blipM*, pSG1113*blipM*) were confirmed by DNA sequencing.



pSG1112

fmet

AATGGAGGGATTCATATGAAAAGCGGGGATCCTCTAGAAGTCGACCCT

SD Nde I BamH I Xba I Sal I

pSG1113

His His His His His His

ATTGGAGGGATTCATAGATGCATCACCATCACCATCATATGAAAAGCGGGGATCCTCTAGA

SD fmet Nde I BamH I Xba I

Figure 7.2 Plasmid maps of pSG1112 and pSG1113. Heterologous genes were inserted into the multicloning sites (MCS) of the vectors. The vectors carried a pBR322 replication origin for *E. coli*, the promoter and the Shine-Dalgarno-type ribosome binding site (SD) of prophage ϕ105MU331, a *cat* gene for chloramphenicol resistance, and a *bla* gene for ampicillin resistance. The two integration vectors are identical except that pSG1113 includes the sequence encoding for six-histidine amino acids the distance between SD and start codon ATG in pSG1113 is two bases longer than that in pSG1112 .

7.3 Transformation of the recombinant plasmids into *B. subtilis* strain 1A304(ϕ105MU331)

For protein expression, the constructed integration recombinant plasmids were transformed into *B. subtilis* 1A304 (ϕ105 MU331). Similar to the transformation with undigested pSG703 derivatives, the transformation of 1A304(ϕ105MU331) with undigested pSG1112 or pSG1113 derivatives gave both Cm^REr^R and Cm^REr^S colonies (Thornewell *et al.*, 1993). There are two crossover sites between plasmid and prophage, one is at the ϕ105 sequences of plasmid and prophage, and the other is at the complete *cat* gene of the plasmid and the truncated *cat* gene of the prophage. Whereas the Cm^REr^R colonies were resulted from Campbell-type single crossover between plasmid and prophage, the Cm^REr^S colonies were resulted from double crossover homologous recombination between plasmid and prophage. As mentioned in Chapter 4, the protein expression level of the Cm^REr^S transformants was about 10-fold higher than that of the Cm^REr^R transformants when PenPC and some of its mutants were expressed in the system, therefore, Cm^REr^S transformants were chosen for the expression of BLIP.

7.4 Small scale expression of BLIP from *B. subtilis*

For protein expression, as mentioned in Chapter 2, the transformants obtained from different pSG1112 or pSG1113 derivatives were separately picked and grown to generate a seed culture (5 ml) first and 1 ml of it was inoculated into 15 ml culture.

Prophage induction was done at 50°C for 4 min, and then growth was continued at 37°C. Strain 1A304(ϕ105MU331) was similarly treated as a control. Samples of the culture were taken hourly after induction and stored in a -20°C freezer for further analysis. Both the supernatants and cell pellets of culture samples collected at different time points after thermo-induction were studied by SDS/PAGE analysis (data not shown), but no obvious protein band corresponding to the size of BLIP (17 kDa) was observed. A high sensitivity β-lactamase inhibition assay was then performed for the detection of the expression of BLIP. The inhibition assays suggested that only the culture supernatant from the strain transformed with the construct pSG1112*blip*F was found to inhibit the Penase activity. It is very surprising, because the construct pSG1113*blip*F is very similar to pSG1112*blip*F, except that pSG1113 includes the sequence encoding for six-histidine amino acids exactly before the start codon of the *blip* gene. One possibility is that the additional six-histidine amino acids may disable the function of the signal peptide. Further study is worth to clarify this issue. The expression of intracellular BLIP with or without the His-tag is found to be unsuccessful, perhaps due to the rapid degradation of the expressed BLIP by the proteases in the cytosol.

7.5 Optimizing conditions for BLIP expression

Since the expression of BLIP in the *B. subtilis* system was not as high as that of

PenPC, several parameters were optimized for the expression.

The effect of oxygen supply on the bacterial cell growth and BLIP expression was studied using three different types of 1-liter flasks: (i) without baffles, (ii) with baffles, (iii) with baffles and the addition of antifoam in the culture. All these 1-liter flasks were used to culture only 100 ml of cells to make sure that there was sufficient oxygen supply for cell growth. While the cells grew faster in the flasks with baffles than those in the flasks without baffles (data not shown), the BLIP expression in the flasks without baffles was slightly higher that that in the flasks with baffles (Figure 7.3). It seems that the relatively lower oxygen supply was beneficial for BLIP production, even though oxygen is essential for aerobic growth. Similar results were reported in the production of β-lactamase both in *E. coli* and *B. subtilis* as well as the production of other recombinant proteins in *E. coli*. (Li *et al.*, 1992; Sargantanis and Karim, 1998).

The density of cells during heat shock is also very critical for heterologous protein expression. From our study, as shown in Figure 7.4, an OD_{600} value close to 3.4 is an ideal point for heat shock.

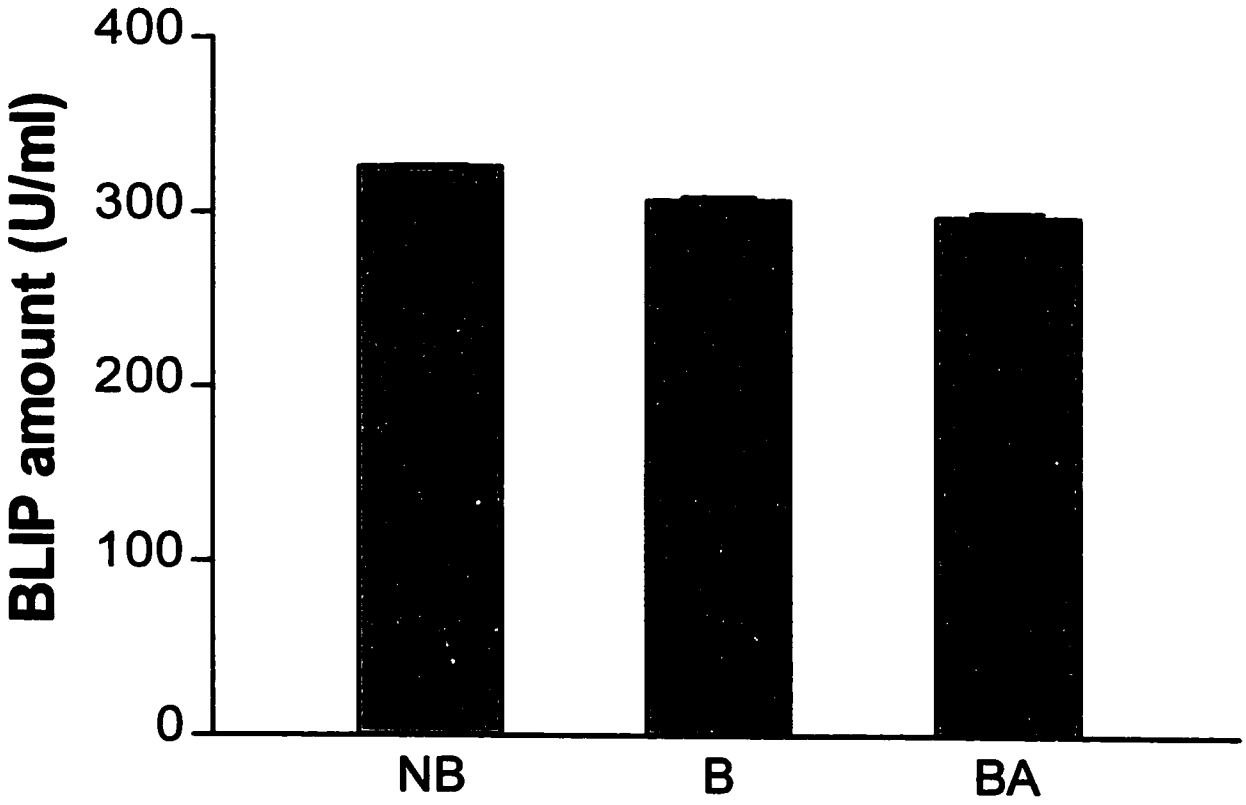


Figure 7.3 Comparison of BLIP expression in different types of culture flasks. Cells in flasks without baffles (NB), with baffles (B), or with baffles and addition of antifoam in the medium (BA) grew at 37°C at 300 rpm. Thermo-induction was carried out at 50°C for 4 min when the OD_{600} of culture reached about 3.4, and then cells were allowed to grow at 37°C for protein expression. Samples were harvested 5 h after thermo-induction and their supernatants were used for the Penase inhibition assay. The error bars were calculated from the deviation of two measurements. No BLIP expression was detected from the cells without heat shock.

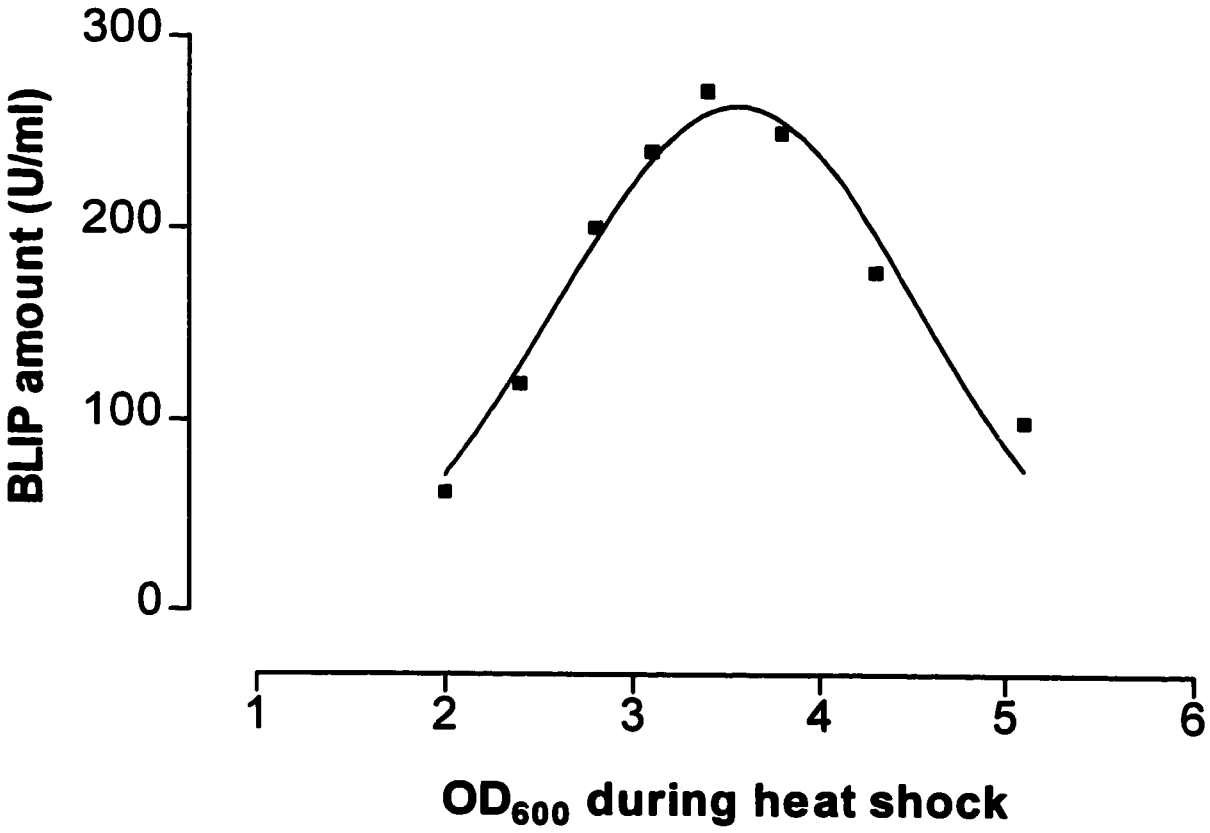


Figure 7.4 Effect of OD_{600} values for heat shock on BLIP production

Different flasks of bacterial cells were cultured at 37°C and subjected to heat shock 50°C for 4 min) at different cell densities (indicated by OD_{600}, from 2.0 to 5.1). Samples were taken at 5.5 h after thermo-induction and their supernatants were used for the Penase inhibition assay. Each single point represents result from a single measurement.

The duration of heat shock may greatly affect expression. Whereas heat shock at 50°C for too long time may severely cause cell death, too little heat shock may not effectively turn on the strong phage promoter to induce the protein expression. Compromised by cell death and induction of protein expression, it seems that there is an optimal duration for thermo-induction. Compared with the cells which were heat-shocked for 2 min at 50 °C, the cells which were heat-shocked for 4 or 6 min grew slightly slower but protein expression was increased by about 2-fold (Figure 7.5 and Figure 7.6). It is also found that there is no significant difference between the cells heat-shocked for 4 min and the cells heat-shocked for 6 min. Therefore, heat shock for 4 min at 50°C is used for the induction of BLIP expression.

In order to determine the time to harvest the cells after thermo-induction, cells were cultured at 37°C and heat shock was performed at 50°C for 4 min when the OD_{600} of culture was 3.4. Samples at different time points after heat shock were taken and their supernatants were subjected to Penase inhibition assay and SDS/PAGE analysis. As shown in Figure 7.7, it seems that the expression of BLIP was maximum around 5-6 h after thermo-induction and protein degradation became serious at 6 h after thermo-induction. Therefore, cells were harvested at the fifth hour after thermo-induction.

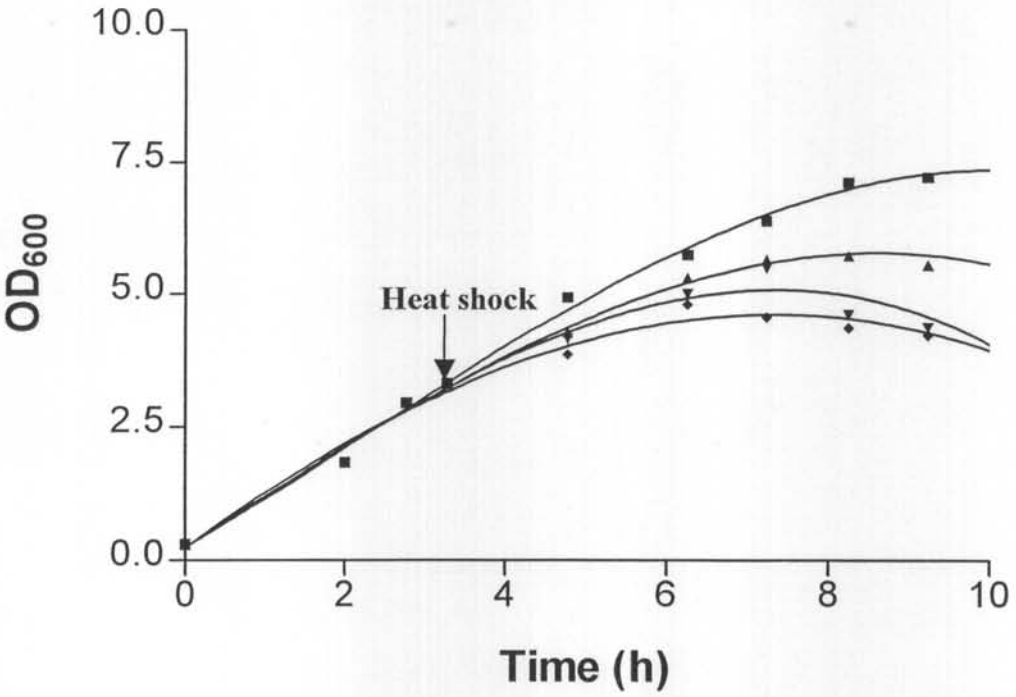


Figure 7.5 Effect of heat shock duration on cell growth. Cells were cultured at 37 °C at 300 rpm and heat shock was performed at 50 °C for 0 (■), 2 (▲) , 4 (▼) or 6 (◆) min when OD_{600} of culture reached 3.3. The cell growth was continually monitored after heat shock.

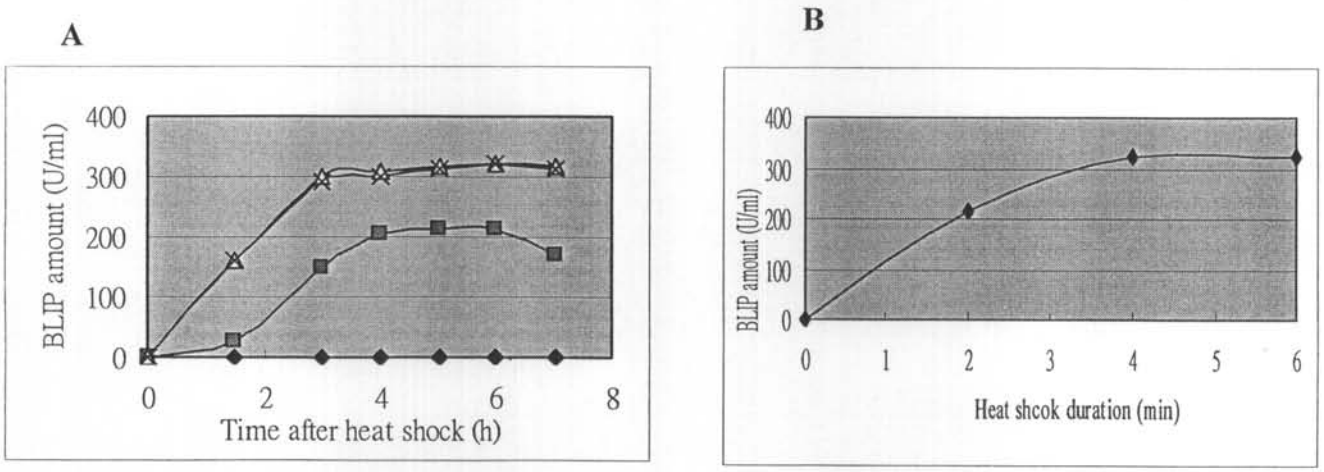


Figure 7.6 Effect of heat shock duration on BLIP production. Cells were cultured at 37 °C at 300 rpm and heat shock was performed at 50 °C for 0 (◆), 2 (■), 4(Δ) or 6 (×) min when OD_{600} of culture reached 3.3. Samples were taken at different time points after heat shock and their supernatants were used for Penase inhibition assay (A).By the comparison of samples at 6 h after heat shock, the effect of heat shock duration on BLIP production is clearly indicated (B).

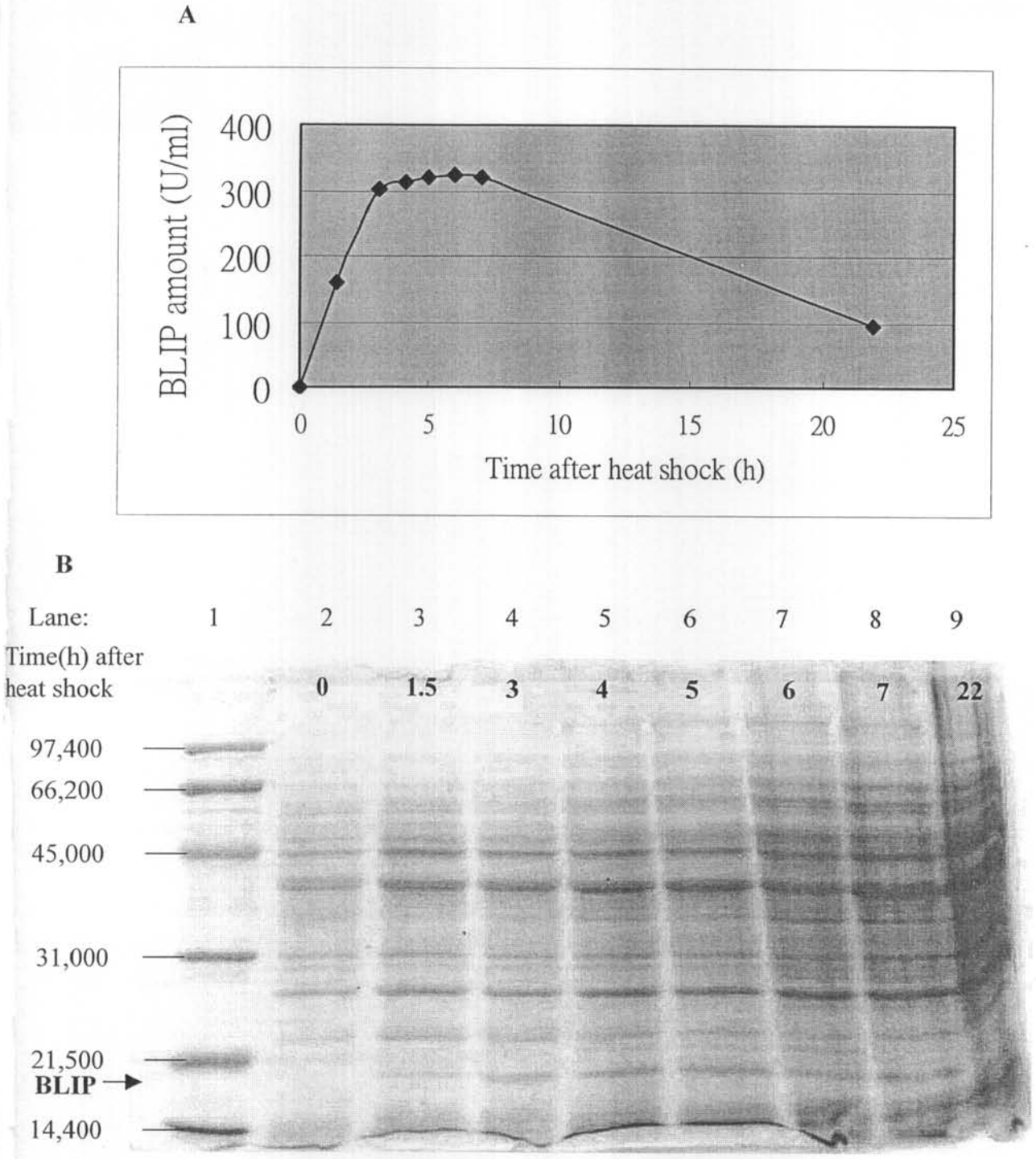


Figure 7.7 Analysis of BLIP expression at different time points after thermo-induction by (A) Penase inhibition assay and (B) SDS/PAGE. Lane 1 is the MW marker; lanes 2-9 contain 100 µl (concentrated down to 10 µl) of culture supernatant collected at 0, 1.5, 3, 4, 5,6,7 and 22 h after thermo-induction.

7.5 Large-scale expression and purification of BLIP

According to the above pilot experiments, for a larger scale protein expression, cells were cultured in 1-liter flasks without baffles. Heat shock was performed at 50°C for 4 min when OD_{600} of culture was around 3.4 and the supernatants of the culture 5 h after thermo-induction were harvested for BLIP purification.

For protein purification, the harvested supernatants of the culture were subjected to ammonium sulfate precipitation. Ammonium sulfate (51.6 g) was gradually added with stirring to 100 ml of culture supernatant at 4°C to give 80 % saturation (Doran *et al.*, 1990). The precipitated protein was collected by centrifugation for 20 min at 18,000 g and dissolved in 25 mM Tris-HCl buffer (pH 8.4) and then desalted by 3 times of dialysis against the same buffer (Albeck and Schreiber, 1999) or by passing throught a desalting column (Hi Prep 26/10 Desalting, Pharmacia).

Desalted protein solution was applied to a Q Sepharose column (Hi Prep 16/10 XL, Pharmacia), which was pre-equilibrated with 25 mM Tris-HCl buffer (pH 8.4). A gradient of two segments (1 - 0.2 M NaCl and 0.2 – 1 M NaCL) was established for the protein elution and BLIP was eluted out from the column in the first segment (Figure 7.8). The fractions were analyzed by Penase inhibition assay, Bradford test and SDS/PAGE analysis to find the fractions containing BLIP and to estimate the yield and purity of BLIP in these fractions.

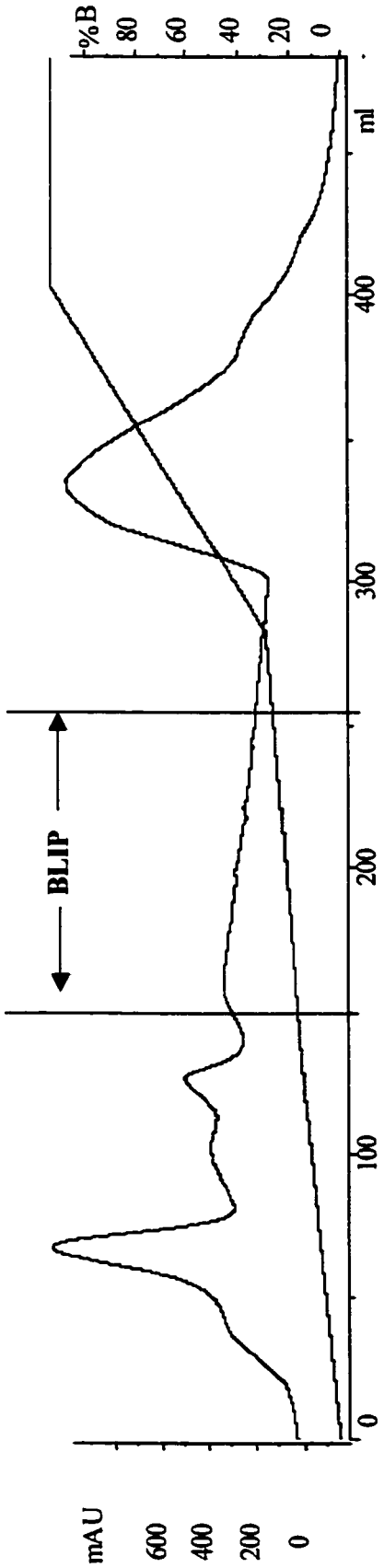


Figure 7.8 Elution profile of BLIP from a Q Sepharose column. Desalted protein solution was applied to a Q Sepharose column (Hi Prep 16/10 XL, Pharmacia), which was pre-equilibrated with 25 mM Tris-HCl buffer (pH 8.4). After washed with the starting buffer, the column was eluted with a gradient of two segments (0 - 0.2 M NaCl and 0.2 – 1 M NaCl) and BLIP was eluted from the column in the first segment. Fractions with a total elution volume of 150 – 262 ml were collected. Where 100% B is equal to 1 M NaCl, and 1 mAU is approximately equal to protein concentration of 1 mg/ml.

To get further purification, the selected fractions containing BLIP were pooled together and concentrated by ultrafiltration using a YM1 filter (MW cut-off: 1,000 Da). The buffer of the protein solution was also changed to 0.1 M sodium phosphate buffer (pH 7.0) (Doran *et al.*, 1990) by ultrafiltration. Then the protein solution was loaded onto a DEAE column (HiTrap DEAE Sepharose FF, 1 ml, Phamacia), which was pre-equilibrated with 0.1 M sodium phosphate buffer (pH 7.0). Proteins were eluted by a linear gradient (0 – 1 M NaCl). Similarly, the fractions were analyzed by Penase inhibition assay, Bradford test and SDS/PAGE analysis to find the fractions containing BLIP and to estimate the yield and purity of BLIP in these fractions.

The purification of BLIP was summarized in Table 7.1 and the samples of different purification steps were analyzed by SDS/PAGE (Figure 7.9). The sample after DEAE ion exchange chromatography was not included in Figure 7.9, which was much more pure than the BLIP sample eluted from Q Sepharose.

Table 7.1 Purification of BLIP

	Total BLIP (U)	Total protein (mg)	Specific activity (U/mg of protein)	Recovery (%)	Fold of purification
Culture supernatant	937872	581	1614	100	1
Ammonium sulfate concentrate	1396358	423.4	3298	149*	2
Desalted sample	1678018	279.6	6001	179*	3.7
After Q Sepharose	813660	13.7	59304	86	36.7
After DEAE column	485270	5.8	84102	52	52

* The recovery of more than 100% may be due to the existence of some components in the crude culture supernatant, which may interfere the interaction between Panase and BLIP.

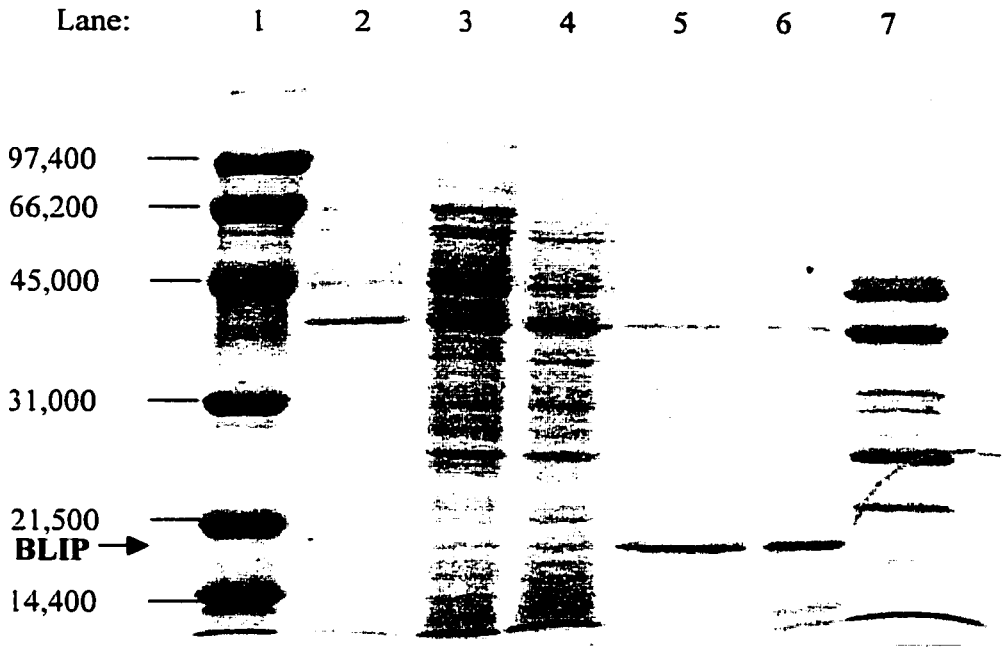


Figure 7.9 SDS/PAGE analysis of protein samples at different stages during BLIP purification. 0.5 µl of protein MW marker (Lane 1), 10 µl of culture supernatant (Lane 2), 5 µl of ammonium sulfate concentrate (Lane 3), 10 µl of desalted protein sample (Lane 4), 100 µl of one of the fractions eluted from Q Sepharose column (Lane 5), 5 µl of concentrated sample of pooled fractions (from Q Sepharose) containing BLIP (Lane 6), and 100 µl of flow-through from Q Sepharose column (Lane 7) were loaded onto a 12% polyacrylamide gel for analysis .

7.6 Discussion

To our knowledge, this is the first time that BLIP was heterologously expressed as a secreted form in *B. subtilis*. The secreted BLIP is believed to be more identical to its native form in terms of its structure and function. After optimization, about 3.8 mg of pure (>95%) BLIP was expressed and purified in *B. subtilis* using strong promoter of the prophage ϕ105MU331. As far as we know, this system is much better than all the reported methods so far. To further improve the yield, we are now constructing another vector to facilitate the higher expression and secretion of BLIP in *B. subtilis* strain 1A304(ϕ105MU331), by the use of the powerful promoter and signal sequence from the *B. amyloliquefaciens* α-amylase, as well as the strong promoter of the prophage ϕ105MU331. We are now also trying to produce BLIP in a fermenter and hopefully, much higher expression level will be achieved. Moreover, *B. subtilis* strain 1A304(ϕ105MU331) and its derivatives are found to be able to grow well in defined Spizizen minimal medium (SMM) (Anagnostopoulos and Spizizen, 1961) with the supplements of glucose, amino acids and other nutrients. Some proteins had also been successfully expressed in the defined pre-transfromation medium (PTM) (for detail, see Chapter 2) from *B. subtilis* 1A304(ϕ105MU331) derivatives. It is likely that large amount of BLIP with ^{13}C and $^{N}15$ labeling could be obtained by our method, which may be very useful for the NMR studies of BLIP structure and function in solution.

Without doubt, the production of high levels of BLIP with high purity by our method will certainly facilitate the many important studies of BLIP, especially the interaction between various β-lactamases and BLIP. The better understanding of the interaction between β-lactamases and BLIP could serve as a starting point for the design of novel inhibitory proteins to effectively counter the deleterious effects of β-lactamases.

Conclusions

(1) Eighteen chimeric β-lactamase genes (six from *penPC* and *penP*, 12 from *penPC* and *pc1*) were successfully generated in *E. coli* RR1 by *in vivo* intramolecular recombination within the homologous regions between two class A β-lactamase genes [the β-lactamase I gene from *Bacillus cereus* (*penPC*) and the β-lactamase gene from *Bacillus licheniformis* (*penP*); or *penPC* and the β-lactamase gene from *Staphylococcus aureus* (*pc1*)]. These hybrid genes encode novel β-lactamases with their N-terminal moiety derived from PenPC and C-terminal moiety derived from either PenP or PC1. It is concluded from our studies that the frequency of homologous recombination is a function of the sequence identity between the genes concerned and 53% identity may be marginal for performing intramolecular homologous recombination between the genes interested. The double-strand breaks inside a homologous region was also found to stimulate recombination in our study, but the gradient of recombination frequencies related to the location of the break was not observed. For unknown reasons, the crossover site (5' –AACTTGAG- 3') for the generation of *hybrid A* was found to be a particular hot spot of recombination, which is different for the octameric Chi (χ) sequence. Even though the molecular details of the recombination processes are still far from being completely deciphered, homologous

recombination is proved to be a very powerful approach to generate hybrid genes between partially homologous genes.

(2) While PenPC, PenP and all their hybrid β-lactamases were successfully expressed in *B. subtilis*, *B. subtilis* may not be an ideal host for the protein expression of PC1 and its hybrid derivatives, even though the S. *aureus pc1* promoter is active *in vivo* in *B. subtilis*. The failure of the expression of PC1 and its hybrid derivatives might result from proteolytic degradation of secreted enzymes by host proteases. It was found that too much oxygen supply (with higher ratio of flask volume to culture volume and the use of baffled flasks) would decrease the yield of expressed proteins, possibly, because the phage DNA content is expected to increase with a decrease in cell growth rate under low DO supply. Compared with PenPC and HybridB-E, HybridF β-lactamase was found to bind to Celite with lower affinity, possibly, because half of its protein sequence is derived from PenP and effective binding to Celite is one of the properties of PenPC structure. The expressed PenPC, HybridB, HybridC, HybridD, HybridE and HybridF were purified to high purity by affinity binding to Celite, but PenP and Hybrid A were purified by cation–exchange chromatography. The powerful *B. subtilis* expression system was also used for the successful expression of the

β-lactamase inhibitory protein (BLIP) from *Streptomyces clavuligerus*. After optimization, about 3.8 mg of pure (>95%) BLIP was expressed and purified from 1-liter culture.

(3) PC1 and its hybrid β-lactamases were successfully expressed in *E. coli* as GST fusion proteins. The expressed GST fusion proteins with PC1, PenPC, Hybrid1, Hybrid2, Hybrid3, and Hybrid4 were highly soluble. However, the expressed GST-Hybrid5 was only partially soluble, and the other GST fusion proteins were found to be expressed as inclusion bodies. All efforts made failed to improve the solubility of these miss-folded fusion proteins. The formation of inclusion body might result from the incorrect protein folding when these hybrid β-lactamases were expressed in the cytosol. Nevertheless, when all these hybrids were expressed as maltose binding protein (MBP) fusions in *E. coli*, all the expressed fusion proteins were found to be highly soluble. One possibility, which seems to be consistent with all the available experimental evidences, is that MBP may function as an internal chaperone. All MBP-β-lactamses were successfully purified by a one-step amylose affinity column. We also found that the small amount of contaminants of the specific signal peptidases from the host cells may cause the cleavage of purified GST-PC1 and other GST-β-lactamases during storage. More

powerful centrifugation or the addition of efficient signal peptidase inhibitor might be helpful to diminish the cleavage of fusion proteins during protein purification. Moreover, the addition of IPTG was found to cause higher expression of TEM-1 in the cells transformed with the pGEX-6P-1 vector than in the cells transformed with the pMAL-c2 vector, as a result of transcriptional read-through from the inducible GST or MBP gene into the downstream TEM-1 β-lactamase coding region. The expressed TEM-1 is not a problem for futher studies since it can be successfully separated from MBP-β-lactamases by chromatography.

(4) From our thermal inactivation study, PenP shows much higher thermal stability than PenPC. Most interestingly, for the hybrid enzymes of PenP and PenPC, the more protein sequence is derived from PenP, the higher is the thermal stability of the hybrid protein. It seems that thermostability in different enzymes is achieved by a combination of various interactions. Thermal denaturation of MBP fusion enzymes of PC1, PenPC and their hybrids suggests that the overall conformational changes of all MBP-β-lactamases to elevated temperatures are very similar. However, for thermal inactivation, all MBP-β-lactamases except for MBP-PenPC lost almost all the activity after incubated at 65°C for 5 min. It seems that the

change in activity is much more sensitive than the change in overall conformation. For MBP fusion enzymes of PC1, PenPC and their hybrids, compared with their parents, hybrid enzymes lost their stability to different extents, especially those formed by crossover events occurred near the middle of the two β-lactamases, which may be due to the structural perturbation near the active site of these hybrid enzymes, especially the Ω-loop, which forms part of the active site depression.

(5) The C-terminus of PenPC (from V 249 to R291) was found to be essential to its stability and effective catalysis. The replacement of these 42 amino acids with the corresponding sequence from PC1 is of disastrous consequence. The identity and similarity of this C-terminal region between PenPC and PC1 are 30 % and 62%, respectively, which are quite similar to the identity (34%) and similarity (57%) between the two entire polypeptides. Some residues of PenPC in this region, especially those different from PC1, may be very critical for their stability and effective catalysis. One of the residues may be Ala 272. This is the first time that the C-terminal of PenPC is reported to play such an important role in protein structure and function.

(6) The region in between the crossover points (68-112, ABL Scheme) in Hybrid5 and Hybrid6 may be very important for the determination of

substrate specificity of Class A β-lactamases, since the K_m values of MBP-Hybrid5 (similar to PC1) for many β-lactams are significantly different from those of MBP-Hybrid6 (similar to PenPC). By protein sequences alignment, the two hybrids differ at a 43-amino-acid region, covering Ser 70 and Lys 73, which are crucial active site residues for Class A β-lactamases. The identity and similarity of this region between PenPC and PC1 are 41% and 69%, respectively, which are much higher than those of the two entire polypeptides. Some residues in this region may play an important role in determining substrate specificity. Residues at position 76 and 104 may be two of them. It is quite reasonable because this region includes two important residues Ser 70, Lys 73 and is very close to Glu 166 and the Ω-loop. Further studies to dissect and understand the exact structural property and functions of this region may provide new insights into the mechanism of β-lactamase action.

(7) The kinetic studies of MBP-fusion enzymes of PC1, PenPC and their hybrids (Table 6.4 and Table 6.5) also suggest that some hybrids, especially those with crossover events occurred near the middle of the two β-lactamases, such as Hybrid6, Hybrid7 and Hybrid8, dramatically lost their catalytic efficiency in hydrolyzing penicillin G, ampicillin, nitrocefin and cephaloridine. In

contrary, they have high apparent substrate affinity (much lower Km) to other β-lactams with bulky side-chains such as methicillin and oxacillin. This suggests that the generation of hybrid enzymes is similar to the evolution process. In carving out a larger active site to accommodate the β-lactams with bulky side-chains, these new enzymes lost their power to hydrolyze β-lactams with simple side-chains. Also, they lost their internal actions that formerly contributed to their internal integrity, lowering their stability.

(8) MBP-Hybrid5, HybridF and HybridB β-lactamases were found to show higher binding affinity to oxyimino β-lactams (such as cefuroxime) than their parents, possibly due to the enlargement of the active site pocket. It seems that homologous recombination among β-lactamases may be one of the approaches for the generation of extended-spectrum β-lactamases during evolution. However, the structural rationale for the kinetic properties of these hybrid enzymes still remains speculative in the absence of crystal structures.

(9) Based on our studies of the properties of generated hybrid enzymes, we observd two kinds of correlation among hybrids and their parents: (i) linear correlation and (ii) non-linear correlation. For linear correlation, the properties of the hybrids are similar to one of their parents or intermediate between those of the two parent enzymes. In this case, if any two enzymes

(two hybrids, or hybrid and one of its parent) behave differently, it is likely that the consequence is resulted from the residues located in the non-identical region between the two partially homologous enzymes. For non-linear correlation, the hybrids have some novel properties that their parents do not have. We attribute these novel properties to the structural perturbations of the hybrid enzymes.

References

Abastado, J. P., Darche, S., Godeau, F., Cami, B., and Kourilsky, P. (1987). Intramolecular recombination between partially homologous sequences in *Escherichia coli* and *Xenopus laevis* oocytes. Proc Natl Acad Sci U S A *84*, 6496-500.

Abraham, E.P., Chain, E. (1940) An enzyme from bacteria able to destroy penicillin. Nature 146, 837

Albeck, S., and Schreiber, G. (1999). Biophysical characterization of the interaction of the β-lactamase TEM-1 with its protein inhibitor BLIP. Biochemistry *38*, 11-21.

Ambler, R. P. (1980). The structure of β-lactamases. Philos Trans R Soc Lond B Biol Sci *289*, 321-31.

Ambler, R. P., Coulson, A. F., Frere, J. M., Ghuysen, J. M., Joris, B., Forsman, M., Levesque, R. C., Tiraby, G., and Waley, S. G. (1991). A standard numbering scheme for the class A β-lactamases. Biochem J *276*, 269-70.

Anagnostopoulos, C., and Spizizen, J. (1961). Requirements for transformation in *Bacillus subtilis*. Journal of Bacteriology *81*, 741-746.

Armstrong, R. N. (1990). Structure-function relationships in enzymatic catalysis. Can chimeric enzymes contribute? Chem. Rev. *90*, 1309-1325.

Arnold, L. D., and Viswanatha, T. (1983). Thermal denaturation of native and cross-linked *Bacillus cereus* 569/H β-lactamase I. Biochim Biophys Acta *749*, 192-7.

Arriaga, P., Menendez, M., Villacorta, J. M., and Laynez, J. (1992). Differential scanning calorimetric study of the thermal unfolding of β-lactamase I from *Bacillus cereus*. Biochemistry *31*, 6603-7.

Babbitt, P. C., and Gerlt, J. A. (1997). Understanding enzyme super families. Chemistry as the fundamental determinant in the evolution of new catalytic activities. J Biol Chem *272*, 30591-4.

Baillie, L. W., Moore, P., and McBride, B. W. (1998). A heat-inducible *Bacillus*

subtilis bacteriophage ϕ105 expression system for the production of the protective antigen of *Bacillus anthracis*. FEMS Microbiol Lett *163*, 43-7.

Baker, P. J., Waugh, M. L., Wang, X. G., Stillman, T. J., Turnbull, A. P., Engel, P. C., and Rice, D. W. (1997). Determinants of substrate specificity in the superfamily of amino acid dehydrogenases. Biochemistry *36*, 16109-15.

Banerjee, S., Pieper, U., Kapadia, G., Pannell, L. K., and Herzberg, O. (1998). Role of the Ω-loop in the activity, substrate specificity, and structure of class A β-lactamase. Biochemistry *37*, 3286-96.

Baneyx, F. (1999). Recombinant protein expression in *Escherichia coli*. Curr Opin Biotechnol *10*, 411-21.

Barthelemy, M., Peduzzi, J., Ben Yaghlane, H., and Labia, R. (1988). Single amino acid substitution between SHV-1 β-lactamase and cefotaxime-hydrolyzing SHV-2 enzyme. FEBS Lett *231*, 217-20.

Blazquez, J., Baquero, M. R., Canton, R., Alos, I., and Baquero, F. (1993). Characterization of a new TEM-type β-lactamase resistant to clavulanate, sulbactam, and tazobactam in a clinical isolate of *Escherichia coli*. Antimicrob Agents Chemother *37*, 2059-63.

Boissinot, M., and Levesque, R. C. (1990). Nucleotide sequence of the PSE-4 carbenicillinase gene and correlations with the *Staphylococcus aureus* PC1 β-lactamase crystal structure. J Biol Chem *265*, 1225-30.

Brown, R. P., Aplin, R. T., and Schofield, C. J. (1996). Inhibition of TEM-2 β-lactamase from *Escherichia coli* by clavulanic acid: observation of intermediates by electrospray ionization mass spectrometry. Biochemistry *35*, 12421-32.

Bush, K. (1998). Metallo-β-lactamases: a class apart. Clin Infect Dis *27 Suppl 1*, S48-53.

Bush, K. (2001). New β-lactamases in gram-negative bacteria: diversity and impact on the selection of antimicrobial therapy. Clin Infect Dis *32*, 1085-9.

Bush, K., Jacoby, G. A., and Medeiros, A. A. (1995). A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother *39*, 1211-33.

Canica, M. M., Barthelemy, M., Gilly, L., Labia, R., Krishnamoorthy, R., and Paul, G. (1997). Properties of IRT-14 (TEM-45), a newly characterized mutant of TEM-type β-lactamases. Antimicrob Agents Chemother *41*, 374-8.

Caramori, T., Albertini, A. M., and Galizzi, A. (1991). *In vivo* generation of hybrids between two *Bacillus thuringiensis* insect-toxin-encoding genes. Gene *98*, 37-44.

Carrey, E. A., and Pain, R. H. (1978). Conformation of a stable intermediate on the folding pathway of *Staphylococcus aureus* penicillinase. Biochim Biophys Acta *533*, 12-22.

Carrey, E. A., Virden, R., and Pain, R. H. (1984). The reversible deactivation of β-lactamase from *Staphylococcus aureus* by quinacillin and cephaloridine and its modification by antibodies. Biochim Biophys Acta *785*, 104-10.

Cartwright, S. J., and Coulson, A. F. (1979). A semi-synthetic penicillinase inactivator. Nature *278*, 360-1.

Chen, C. C., and Herzberg, O. (1999). Relocation of the catalytic carboxylate group in class A β-lactamase: the structure and function of the mutant enzyme Glu166→Gln:Asn170→Asp. Protein Eng *12*, 573-9.

Chen, C. C., Rahil, J., Pratt, R. F., and Herzberg, O. (1993). Structure of a phosphonate-inhibited β-lactamase. An analog of the tetrahedral transition state/intermediate of β-lactam hydrolysis. J Mol Biol *234*, 165-78.

Chen, C. C., Smith, T. J., Kapadia, G., Wasch, S., Zawadzke, L. E., Coulson, A., and Herzberg, O. (1996). Structure and kinetics of the β-lactamase mutants S70A and K73H from *Staphylococcus aureus* PC1. Biochemistry *35*, 12251-8.

Cheung, W. L. (2000). Properties of Novel Beta-lactamase Enzymes. MPhil thesis, In Applied Biology and Chemical Technology (Hong Kong: The Hong Kong Polytechnic University).

Citri, N., Samuni, A., and Zyk, N. (1976). Acquisition of substrate-specific parameters during the catalytic reaction of penicillinase. Proc Natl Acad Sci U S A *73*, 1048-52.

Citri, N., and Zyk, N. (1982). Interaction of the pBR 322-coded RTEM β-lactamase with substrates. Evidence for specific conformational transitions. Biochem J *201*, 425-7.

Clarke, A. J., Mezes, P. S., Vice, S. F., Dmitrienko, G. I., and Viswanatha, T. (1983). Inactivation of *Bacillus cereus* 569/H β-lactamase I by 6-β-(trifluoromethane sulfonyl)amidopenicillanic acid sulfone and its N- methyl derivative. Biochim Biophys Acta *748*, 389-97.

Conrad, B., Hoang, V., Polley, A., and Hofemeister, J. (1995). Hybrid *Bacillus amyloliquefaciens* X *Bacillus licheniformis* α-amylases. Construction, properties and sequence determinants. Eur J Biochem *230*, 481-90.

Dalbey, R. E., and Von Heijne, G. (1992). Signal peptidases in prokaryotes and eukaryotes-a new protease family. Trends Biochem Sci *17*, 474-8.

Damblon, C., Raquet, X., Lian, L. Y., Lamotte-Brasseur, J., Fonze, E., Charlier, P., Roberts, G. C., and Frere, J. M. (1996). The catalytic mechanism of β-lactamases: NMR titration of an active-site lysine residue of the TEM-1 enzyme. Proc Natl Acad Sci U S A *93*, 1747-52.

Damblon, C., Zhao, G. H., Jamin, M., Ledent, P., Dubus, A., Vanhove, M., Raquet, X., Christiaens, L., and Frere, J. M. (1995). Breakdown of the stereospecificity of DD-peptidases and β-lactamases with thiolester substrates. Biochem J *309*, 431-6.

Dangi, B., Dobrodumov, A. V., Louis, J. M., and Gronenborn, A. M. (2002). Solution structure and dynamics of the human-*Escherichia coli* thioredoxin chimera: insights into thermodynamic stability. Biochemistry *41*, 9376-88.

Davies, J. (1994). Inactivation of antibiotics and the dissemination of resistance genes. Science *264*, 375-82.

Davies, R. B., and Abraham, E. P. (1974a). Conformational changes in the extracellular β-lactamase I from *Bacillus cereus* 569/H/9. Biochem J *143*, 137-41.

Davies, R. B., and Abraham, E. P. (1974b). Metal cofactor requirements of β-lactamase II. Biochem J *143*, 129-35.

Davies, R. B., and Abraham, E. P. (1974c). Separation, purification and properties of β-lactamase I and β-lactamase II from *Bacillus cereus* 569/H/9. Biochem J *143*, 115-27.

De Meester, F., Joris, B., Lenzini, M. V., Dehottay, P., Erpicium, T., Dusart, J., Klein, D., Ghuysen, J. M., Frere, J. M., and Van Beeumen, J. (1987). The active sites of the β-lactamases of *Streptomyces cacaoi* and *Streptomyces albus* G. Biochem J *244*, 427-32.

De Meester, F., Joris, B., Reckinger, G., Bellefroid-Bourguignon, C., Frere, J. M., and Waley, S. G. (1987). Automated analysis of enzyme inactivation phenomena. Application to β-lactamases and DD-peptidases. Biochem Pharmacol *36*, 2393-403.

Delaire, M., Labia, R., Samama, J. P., and Masson, J. M. (1992). Site-directed mutagenesis at the active site of *Escherichia coli* TEM-1 β-lactamase. Suicide inhibitor-resistant mutants reveal the role of arginine 244 and methionine 69 in catalysis. J Biol Chem *267*, 20600-6.

di Guan, C., Li, P., Riggs, P. D., and Inouye, H. (1988). Vectors that facilitate the expression and purification of foreign peptides in *Escherichia coli* by fusion to maltose-binding protein. Gene *67*, 21-30.

Dideberg, O., Charlier, P., Wery, J. P., Dehottay, P., Dusart, J., Erpicum, T., Frere, J. M., and Ghuysen, J. M. (1987). The crystal structure of the β-lactamase of *Streptomyces albus* G at 0.3 nm resolution. Biochem J *245*, 911-3.

Doran, J. L., Leskiw, B. K., Aippersbach, S., and Jensen, S. E. (1990). Isolation and characterization of a β-lactamase-inhibitory protein from *Streptomyces clavuligerus* and cloning and analysis of the corresponding gene. J Bacteriol *172*, 4909-18.

Durkin, J. P., and Viswanatha, T. (1978). Clavulanic acid inhibition of β-lactamase I from *Bacillus cereus* 569/H. J Antibiot (Tokyo) *31*, 1162-9.

Errington, J. (1986). A general method for fusion of the *Escherichia coli lacZ* gene to chromosomal genes in *Bacillus subtilis*. J Gen Microbiol *132*, 2953-66.

Errington, J. (1993). Temperate phage vectors. In *Bacillus subtilis* and other Gram-positive Bacteria, H. J. A. a. L. R. Sonenshein A.L., ed. (Washington. D.C.: Americ Society for Microbiology), pp. 645-650.

Escobar, W. A., Miller, J., and Fink, A. L. (1994). Effects of site-specific mutagenesis of tyrosine 105 in a class A β-lactamase. Biochem J *303*, 555-8.

Escobar, W. A., Tan, A. K., Lewis, E. R., and Fink, A. L. (1994). Site-directed mutagenesis of glutamate-166 in β-lactamase leads to a branched path mechanism. Biochemistry *33*, 7619-26.

Faraci, W. S., and Pratt, R. F. (1985). Mechanism of inhibition of the PC1 β-lactamase of *Staphylococcus aureus* by cephalosporins: importance of the 3'-leaving group. Biochemistry *24*, 903-10.

Farzaneh, S., Chaibi, E. B., Peduzzi, J., Barthelemy, M., Labia, R., Blazquez, J., and Baquero, F. (1996). Implication of Ile-69 and Thr-182 residues in kinetic characteristics of IRT-3 (TEM-32) β-lactamase. Antimicrob Agents Chemother *40*, 2434-6.

Fisher, J., Belasco, J. G., Khosla, S., and Knowles, J. R. (1980). β-Lactamase proceeds via an acyl-enzyme intermediate. Interaction of the *Escherichia coli* RTEM enzyme with cefoxitin. Biochemistry *19*, 2895-901.

Fitter, J., Herrmann, R., Dencher, N. A., Blume, A., and Hauss, T. (2001). Activity and stability of a thermostable alpha-amylase compared to its mesophilic homologue: mechanisms of thermal adaptation. Biochemistry *40*, 10723-31.

Fleming, A. (1929). On the antibacterial action of cultures of *Penicillium*, with special reference to their use in the isolation of *B. influenzae*. British Journal of

Experimental Pathology *10*, 226-36.

Fox, J. D., Kapust, R. B., and Waugh, D. S. (2001). Single amino acid substitutions on the surface of *Escherichia coli* maltose-binding protein can have a profound impact on the solubility of fusion proteins. Protein Sci *10*, 622-30.

Frere, J. M., and Joris, B. (1985). Penicillin-sensitive enzymes in peptidoglycan biosynthesis. Crit Rev Microbiol *11*, 299-396.

Frere, J. M., Nguyen-Disteche, M., Coyette, A., and Joris, B. (1992). Mode of action: interaction with the penicillin binding proteins. In The Chemistry of β-Lactams, M. I. Page, ed. (Blackie, London: Bladkie Acadamic& Professional).

Frieden, C. (1970). Kinetic aspects of regulation of metabolic processes. The hysteretic enzyme concept. J Biol Chem *245*, 5788-99.

Garau, J. (1994). β-Lactamases: current situation and clinical importance. Intensive Care Med *20 Suppl 3*, S5-9.

Georgiou, G., and Valax, P. (1996). Expression of correctly folded proteins in *Escherichia coli*. Curr Opin Biotechnol *7*, 190-7.

Gerlt, J. A., and Babbitt, P. C. (1998). Mechanistically diverse enzyme superfamilies: the importance of chemistry in the evolution of catalysis. Curr Opin Chem Biol *2*, 607-12.

Ghuysen, J. M. (1991). Serine β-lactamases and penicillin-binding proteins. Annu Rev Microbiol *45*, 37-67.

Gibson, R. M., Christensen, H., and Waley, S. G. (1990). Site-directed mutagenesis of β-lactamase I. Single and double mutants of Glu-166 and Lys-73. Biochem J *272*, 613-9.

Gibson, R. M., and Errington, J. (1992). A novel *Bacillus subtilis* expression vector based on bacteriophage ϕ105. Gene *121*, 137-42.

Gorss, C. (1996). Function and regulation of the heat shock proteins. In

Escherichia coli and *Salmonella typhimurium*: cellular and molecular Biology, F. C. Neidhardt, Curtiss III, R., Ingrahem, K.B., et al., ed. (Washington, D.C.: American Society of Microbiology Press), pp. 1382-1399.

Greenfield, N. J. (1999). Applications of circular dichroism in protein and peptide analysis. Trends in analytical chemistry *18*, 236-244.

Guarneros, G., Montanez, C., Hernandez, T., and Court, D. (1982). Posttranscriptional control of bacteriophage λ gene expression from a site distal to the gene. Proc Natl Acad Sci U S A *79*, 238-42.

Guillaume, G., Vanhove, M., Lamotte-Brasseur, J., Ledent, P., Jamin, M., Joris, B., and Frere, J. M. (1997). Site-directed mutagenesis of glutamate 166 in two β-lactamases. Kinetic and molecular modeling studies. J Biol Chem *272*, 5438-44.

Guo, F., Huynh, J., Dmitrienko, G. I., Viswanatha, T., and Clarke, A. J. (1999). The role of the non-conserved residue at position 104 of class A β-lactamases in susceptibility to mechanism-based inhibitors. Biochim Biophys Acta *1431*, 132-47.

Hartl, F. U. (1996). Molecular chaperones in cellular protein folding. Nature *381*, 571-9.

Hasson, M. S., Schlichting, I., Moulai, J., Taylor, K., Barrett, W., Kenyon, G. L., Babbitt, P. C., Gerlt, J. A., Petsko, G. A., and Ringe, D. (1998). Evolution of an enzyme active site: the structure of a new crystal form of muconate lactonizing enzyme compared with mandelate racemase and enolase. Proc Natl Acad Sci U S A *95*, 10396-401.

Hemila, H., Pokkinen, M., and Palva, I. (1992). Improving the production of *E. coli* β-lactamase in *Bacillus subtilis:* the effect of glucose, pH and temperature on the production level. J Biotechnol *26*, 245-56.

Herzberg, O., and Moult, J. (1987). Bacterial resistance to beta-lactam antibiotics: crystal structure of β-lactamase from *Staphylococcus aureus* PC1 at 2.5 Å resolution. Science *236*, 694-701.

Herzberg, O. (1991). Refined crystal structure of β-lactamase from *Staphylococcus aureus* PC1 at 2.0 Å resolution. J Mol Biol *217*, 701-19.

Hess, G. F., and Graham, R. S. (1990). Efficiency of transcriptional terminators in *Bacillus subtilis*. Gene *95*, 137-41.

Hochuli, E. (1990). Purification of recombinant proteins with metal chelate adsorbent. Genet Eng *12*, 87-98.

Hockney, R. C. (1994). Recent developments in heterologous protein production in *Escherichia coli*. Trends Biotechnol *12*, 456-63.

Hokenson, M. J., Cope, G. A., Lewis, E. R., Oberg, K. A., and Fink, A. L. (2000). Enzyme-induced strain/distortion in the ground-state ES complex in β-lactamase catalysis revealed by FTIR. Biochemistry *39*, 6538-45.

Hol, W. G. (1985). The role of the α-helix dipole in protein function and structure. Prog Biophys Mol Biol *45*, 149-95.

Hopp, T. H., Price, K.S., Libby, R.T., March, C.J., Cerretti, D.P., Urdal, D.L. and Conlon, P.J. (1988). A short polypeptide marker sequence useful for recombinant protein identification and purification. Bio/Technology *6*, 1204-1210.

Hosseini-Mazinani, S. M., Nakajima, E., Ihara, Y., Kameyama, K. Z., and Sugimoto, K. (1996). Recovery of active β-lactamases from *Proteus vulgaris* and RTEM-1 hybrid by random mutagenesis by using a dnaQ strain of *Escherichia coli*. Antimicrob Agents Chemother *40*, 2152-9.

Huang, W., Petrosino, J., Hirsch, M., Shenkin, P. S., and Palzkill, T. (1996). Amino acid sequence determinants of β-lactamase structure and activity. J Mol Biol *258*, 688-703.

Huletsky, A., Knox, J. R., and Levesque, R. C. (1993). Role of Ser-238 and Lys-240 in the hydrolysis of third-generation cephalosporins by SHV-type β-lactamases probed by site-directed mutagenesis and three-dimensional modeling. J Biol Chem *268*, 3690-7.

Ibuka, A., Taguchi, A., Ishiguro, M., Fushinobu, S., Ishii, Y., Kamitori, S., Okuyama, K., Yamaguchi, K., Konno, M., and Matsuzawa, H. (1999). Crystal structure of the E166A mutant of extended-spectrum β-lactamase Toho-1 at 1.8 A resolution. J Mol Biol *285*, 2079-87.

Ishiguro, M., and Imajo, S. (1996). Modeling study on a hydrolytic mechanism of class Å β-lactamases. J Med Chem *39*, 2207-18.

Ishii, Y., Sonezaki, S., Iwasaki, Y., Tauchi, E., Shingu, Y., Okita, K., Ogawa, H. I., Kato, Y., and Kondo, A. (1998). Single-step purification and characterization of MBP (maltose binding protein)-DnaJ fusion protein and its utilization for structure-function analysis. J Biochem (Tokyo) *124*, 842-7.

Izui, K., Nielsen, J. B., Caulfield, M. P., and Lampen, J. O. (1980). Large exopenicillinase, initial extracellular form detected in cultures of *Bacillus licheniformis*. Biochemistry *19*, 1882-6.

Jacob, F., Joris, B., Dideberg, O., Dusart, J., Ghuysen, J. M., and Frere, J. M. (1990a). Engineering a novel β-lactamase by a single point mutation. Protein Eng *4*, 79-86.

Jacob, F., Joris, B., and Frere, J. M. (1991). Active-site serine mutants of the *Streptomyces albus* G β-lactamase. Biochem J *277*, 647-52.

Jacoby, G. A., and Medeiros, A. A. (1991). More extended-spectrum β-lactamases. Antimicrob Agents Chemother *35*, 1697-704.

Jacob, F., Joris, B., Lepage, S., Dusart, J., and Frere, J. M. (1990b). Role of the conserved amino acids of the 'SDN' loop (Ser130, Asp131 and Asn132) in a class A β-lactamase studied by site-directed mutagenesis. Biochem J *271*, 399-406.

Jamin, M., Wilkin, J. M., and Frere, J. M. (1995). Bacterial DD-transpeptidases and penicillin. Essays Biochem *29*, 1-24.

Jelsch, C., Mourey, L., Masson, J. M., and Samama, J. P. (1993). Crystal structure of *Escherichia coli* TEM1 β-lactamase at 1.8 Å resolution. Proteins *16*, 364-83.

Jenkinson, H. F., and Deadman, M. (1984). Construction and characterization of recombinant phage ϕ105d(Cmrmet) for cloning in *Bacillus subtilis*. J Gen Microbiol *130*, 2155-64.

Jones, D., and Errington, J. (1987). Construction of improved bacteriophage ϕ105 vectors for cloning by transfection in *Bacillus subtilis*. J Gen Microbiol *133*, 483-92.

Joris, B., Ghuysen, J. M., Dive, G., Renard, A., Dideberg, O., Charlier, P., Frere, J. M., Kelly, J. A., Boyington, J. C., Moews, P. C., and et al. (1988). The active-site-serine penicillin-recognizing enzymes as members of the *Streptomyces* R61 DD-peptidase family. Biochem J *250*, 313-24.

Joris, B., Ledent, P., Dideberg, O., Fonze, E., Lamotte-Brasseur, J., Kelly, J. A., Ghuysen, J. M., and Frere, J. M. (1991). Comparison of the sequences of class A β-lactamases and of the secondary structure elements of penicillin-recognizing proteins. Antimicrob Agents Chemother *35*, 2294-301.

Juteau, J. M., Billings, E., Knox, J. R., and Levesque, R. C. (1992). Site-saturation mutagenesis and three-dimensional modelling of ROB-1 define a substrate binding role of Ser130 in class A β-lactamases. Protein Eng *5*, 693-701.

Kapust, R. B., and Waugh, D. S. (1999). *Escherichia coli* maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. Protein Sci *8*, 1668-74.

Kelly, J. A., Dideberg, O., Charlier, P., Wery, J. P., Libert, M., Moews, P. C., Knox, J. R., Duez, C., Fraipont, C., Joris, B., and et al. (1986). On the origin of bacterial resistance to penicillin: comparison of a β-lactamase and a penicillin target. Science *231*, 1429-31.

Kimura, N., Nishi, A., Goto, M., and Furukawa, K. (1997). Functional analyses of a variety of chimeric dioxygenases constructed from two biphenyl dioxygenases that are similar structurally but different functionally. J Bacteriol *179*, 3936-43.

Kirby, W.M.M. (1944) Extraction of a highly potent penicillin inactivator from penicillin resistant staphylococci. Science 99, 452-3

Knox, J. R. (1995). Extended-spectrum and inhibitor-resistant TEM-type β-lactamases: mutations, specificity, and three-dimensional structure. Antimicrob Agents Chemother *39*, 2593-601.

Knox, J. R., and Moews, P. C. (1991). Beta-lactamase of *Bacillus licheniformis* 749/C. Refinement at 2 Å resolution and analysis of hydration. J Mol Biol *220*, 435-55.

Knox, J. R., Moews, P. C., Escobar, W. A., and Fink, A. L. (1993). A catalytically-impaired class A β-lactamase: 2 Å crystal structure and kinetics of the *Bacillus licheniformis* E166A mutant. Protein Eng *6*, 11-8.

Kohler, K., Ljungquist, C., Kondo, A., Veide, A., and Nilsson, B. (1991). Engineering proteins to enhance their partition coefficients in aqueous two-phase systems. Biotechnology (N Y) *9*, 642-6.

Korndorfer, I., Steipe, B., Huber, R., Tomschy, A., and Jaenicke, R. (1995). The crystal structure of holo-glyceraldehyde-3-phosphate dehydrogenase from the hyperthermophilic bacterium *Thermotoga maritima* at 2.5 Å resolution. J Mol Biol *246*, 511-21.

Kotra, l. P., Samama, J.-P., and Mobashery, S. (2002). β-Lactamases and resistance to β-lactam antibiotics. In Bacterial resistance to antimicrobials, K. Lewis, A. A. Salyers, H. W. Taber and r. G. Wax, eds. (New York. & Basel: Marcel Dekker, Inc), pp. 123-160.

Kwok, S. Y. (2000). Properties of Novel Pyridoxal Kinase Enzymes. MPhil thesis, In Applied Biology and Chemical Technology (Hong Kong: The Hong Kong Polytechnic University).

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-5.

Lamotte-Brasseur, J., Dive, G., Dideberg, O., Charlier, P., Frere, J. M., and Ghuysen, J. M. (1991). Mechanism of acyl transfer by the class A serine β-lactamase of *Streptomyces albus* G. Biochem J *279*, 213-21.

Lamotte-Brasseur, J., Jacob-Dubuisson, F., Dive, G., Frere, J. M., and Ghuysen, J. M. (1992). *Streptomyces albus* G serine β-lactamase. Probing of the catalytic mechanism via molecular modelling of mutant enzymes. Biochem J *282*, 189-95.

Lampen, J. O., Nielsen, J. B., Izui, K., and Caulfield, M. P. (1980). *Bacillus licheniformis* β-lactamases: multiple forms and their roles. Philos Trans R Soc Lond B Biol Sci *289*, 345-8.

LaVallie, E. R., and McCoy, J. M. (1995). Gene fusion expression systems in *Escherichia coli.* Curr Opin Biotechnol *6*, 501-6.

Leung, Y. C. (1994). Properties of mutant derivatives of β-lactamase I from *Bacillus cereus.* PhD thesis, In Sir William Dunn School of pathology and Linacre college, University of Oxford.

Leung, Y. C., and Errington, J. (1995). Characterization of an insertion in the phage ϕ105 genome that blocks host *Bacillus subtilis* lysis and provides strong expression of heterologous genes. Gene *154*, 1-6.

Leung, Y. C., Robinson, C. V., Aplin, R. T., and Waley, S. G. (1994). Site-directed mutagenesis of β-lactamase I: role of Glu-166. Biochem J *299*, 671-8.

Levy, S. B. (1994). Balancing the drug-resistance equation. Trends Microbiol *2*, 341-2.

Li, X., Robbins, J. W., Jr., and Taylor, K. B. (1992). Effect of the levels of dissolved oxygen on the expression of recombinant proteins in four recombinant *Escherichia coli* strains. J Ind Microbiol *9*, 1-9.

Lietz, E. J., Truher, H., Kahn, D., Hokenson, M. J., and Fink, A. L. (2000). Lysine-73 is involved in the acylation and deacylation of β-lactamase. Biochemistry *39*, 4971-81.

Lin, S., Thomas, M., Shlaes, D. M., Rudin, S. D., Knox, J. R., Anderson, V., and Bonomo, R. A. (1998). Kinetic analysis of an inhibitor-resistant variant of the OHIO-1 β-lactamase, an SHV-family class A enzyme. Biochem J *333*, 395-400.

Livermore, D. M. (1998). Beta-lactamase-mediated resistance and opportunities for its control. J Antimicrob Chemother *41 Suppl D*, 25-41.

Lobkovsky, E., Moews, P. C., Liu, H., Zhao, H., Frere, J. M., and Knox, J. R. (1993). Evolution of an enzyme activity: crystallographic structure at 2 Å resolution of cephalosporinase from the *ampC* gene of *Enterobacter cloacae* P99 and comparison with a class A penicillinase. Proc Natl Acad Sci U S A *90*, 11257-61.

Luck, D. N., Gout, P. W., Sutherland, E. R., Fox, K., Huyer, M., and Smith, M. (1992). Analysis of disulphide bridge function in recombinant bovine prolactin using site-specific mutagenesis and renaturation under mild alkaline conditions: a crucial role for the central disulphide bridge in the mitogenic activity of the hormone. Protein Eng *5*, 559-67.

Machius, M., Wiegand, G., and Huber, R. (1995). Crystal structure of calcium-depleted *Bacillus licheniformis* α-amylase at 2.2 Å resolution. J Mol Biol *246*, 545-59.

Madgwick, P. J., and Waley, S. G. (1987). β-Lactamase I from *Bacillus cereus*. Structure and site-directed mutagenesis. Biochem J *248*, 657-62.

Maina, C. V., Riggs, P. D., Grandea, A. G., 3rd, Slatko, B. E., Moran, L. S., Tagliamonte, J. A., McReynolds, L. A., and Guan, C. D. (1988). An *Escherichia coli* vector to express and purify foreign proteins by fusion to and separation from maltose-binding protein. Gene *74*, 365-73.

Maiti, S. N., Phillips, O. A., Micetich, R. G., and Livermore, D. M. (1998). Beta-lactamase inhibitors: agents to overcome bacterial resistance. Curr Med Chem *5*, 441-56.

Makrides, S. C. (1996). Strategies for achieving high-level expression of genes in *Escherichia coli*. Microbiol Rev *60*, 512-38.

Mas, M. T., Chen, C. Y., Hitzeman, R. A., and Riggs, A. D. (1986). Active human-yeast chimeric phosphoglycerate kinases engineered by domain interchange. Science *233*, 788-90.

Matagne, A., Dubus, A., Galleni, M., and Frere, J. M. (1999). The β-lactamase cycle: a tale of selective pressure and bacterial ingenuity. Nat Prod Rep *16*, 1-19.

Matagne, A., and Frere, J. M. (1995). Contribution of mutant analysis to the understanding of enzyme catalysis: the case of class A β-lactamases. Biochim Biophys Acta *1246*, 109-27.

Matagne, A., Lamotte-Brasseur, J., Dive, G., Knox, J. R., and Frere, J. M. (1993). Interactions between active-site-serine β-lactamases and compounds bearing a methoxy side chain on the α-face of the β-lactam ring: kinetic and molecular modelling studies. Biochem J *293*, 607-11.

Matagne, A., Lamotte-Brasseur, J., and Frere, J. M. (1998). Catalytic properties of class A β-lactamases: efficiency and diversity. Biochem J *330*, 581-98.

Matagne, A., Lamotte-Brasseur, J., and Frere, J. M. (1993). Interactions between active-site serine β-lactamases and so-called β-lactamase-stable antibiotics. Kinetic and molecular modelling studies. Eur J Biochem *217*, 61-7.

Matagne, A., Misselyn-Bauduin, A. M., Joris, B., Erpicum, T., Granier, B., and Frere, J. M. (1990). The diversity of the catalytic properties of class A β-lactamases. Biochem J *265*, 131-46.

Maveyraud, L., Pratt, R. F., and Samama, J. P. (1998). Crystal structure of an acylation transition-state analog of the TEM-1 β-lactamase. Mechanistic implications for class A β-lactamases. Biochemistry *37*, 2622-8.

Medeiros, A. A. (1997). Evolution and dissemination of β-lactamases accelerated by generations of β-lactam antibiotics. Clin Infect Dis *24 Suppl 1*, S19-45.

Mezes, P. S., Clarke, A. J., Dmitrienko, G. I., and Viswanatha, T. (1982). 6-β -(Trifluoromethane sulfonyl)-amido-penicillanic acid sulfone: a potent inhibitor for β-lactamases. FEBS Lett *143*, 265-7.

Mezes, P. S., Blacher, R. W., and Lampen, J. O. (1985). Processing of *Bacillus cereus* 569/H β-lactamase I in *Escherichia coli* and *Bacillus subtilis*. J Biol Chem *260*, 1218-23.

Mitchinson, C., and Pain, R. H. (1985). Effects of sulphate and urea on the stability and reversible unfolding of β-lactamase from *Staphylococcus aureus*. Implications for the folding pathway of β-lactamase. J Mol Biol *184*, 331-42.

Mitraki, A., Hass-Petingell, C., and King, J. (1991). Mechanism of inclusion body formation. In Protein Refolding, G. Geogious, and De Bernardez-Clark, E.E., ed. (Washington, DC: American Chemistry Society), pp. 35-49.

Mittl, P. R., Luthy, L., Hunziker, P., and Grutter, M. G. (2000). The cysteine-rich protein A from *Helicobacter pylori* is a β-lactamase. J Biol Chem *275*, 17693-9.

Moews, P. C., Knox, J. R., Dideberg, O., Charlier, P., and Frere, J. M. (1990). Beta-lactamase of *Bacillus licheniformis* 749/C at 2 Å resolution. Proteins *7*, 156-71.

Moosdeen, F. (1996). Impact of β-lactamases on the clinical use of β-lactam antibiotics. In Viewpoints in Medicine, Counting resistance due to beta-lactamases, Worthing, ed.: Cambidge Medical Publication), pp. 6-11.

Mottershead, D. G., Polly, P., Lyons, R. J., Sutherland, R. L., and Watts, C. K. (1996). High activity, soluble, bacterially expressed human vitamin D receptor and its ligand binding domain. J Cell Biochem *61*, 325-37.

Muratani, T., Yokota, E., Nakane, T., Inoue, E., and Mitsuhashi, S. (1993). In-vitro evaluation of the four β-lactamase inhibitors: BRL42715, clavulanic acid, sulbactam, and tazobactam. J Antimicrob Chemother *32*, 421-9.

Naas, T., Vandel, L., Sougakoff, W., Livermore, D. M., and Nordmann, P. (1994). Cloning and sequence analysis of the gene for a carbapenem-hydrolyzing class A β-lactamase, Sme-1, from *Serratia marcescens* S6. Antimicrob Agents Chemother *38*, 1262-70.

Nakamura, K., Furusato, T., Shiroza, T., and Yamane, K. (1985). Stable hyper-production of *Escherichia coli* β-lactamase by *Bacillus subtilis* grown on a 0.5 M succinate-medium using a *B. subtilis* α-amylase secretion vector. Biochem Biophys Res Commun *128*, 601-6.

Nanninga, N. (1991). Cell division and peptidoglycan assembly in *Escherichia coli.* Mol Microbiol *5*, 791-5.

Nathan D. G. (1973) hemolytic Anemias III. Hemoglobinipathic Disorders. In Hematology. *Harvard Pathophysiology series*, Vol 1. edited by Beck W. S. Cambrige: MIT Press; pp. 199-216

Neet, K. E., and Ainslie, G. R., Jr. (1980). Hysteretic enzymes. Methods Enzymol *64*, 192-226.

Neu, H. C. (1992). Structure-activity relationships: biological. In The Chemistry of β-Lactams, M. I. Page, ed. (Blackie: Blackie Academic & professional), pp. 101-128.

Nilsson, J., Stahl, S., Lundeberg, J., Uhlen, M., and Nygren, P. A. (1997). Affinity fusion strategies for detection, purification, and immobilization of recombinant proteins. Protein Expr Purif *11*, 1-16.

Nomine, Y., Ristriani, T., Laurent, C., Lefevre, J. F., Weiss, E., and Trave, G. (2001). Formation of soluble inclusion bodies by HPV E6 oncoprotein fused to maltose-binding protein. Protein Expr Purif *23*, 22-32.

Nomine, Y., Ristriani, T., Laurent, C., Lefevre, J. F., Weiss, E., and Trave, G. (2001). A strategy for optimizing the monodispersity of fusion proteins: application to purification of recombinant HPV E6 oncoprotein. Protein Eng *14*, 297-305.

Nordmann, P., Mariotte, S., Naas, T., Labia, R., and Nicolas, M. H. (1993). Biochemical properties of a carbapenem-hydrolyzing β-lactamase from *Enterobacter cloacae* and cloning of the gene into *Escherichia coli.* Antimicrob Agents Chemother *37*, 939-46.

Nukaga, M., Mayama, K., Crichlow, G. V., and Knox, J. R. (2002). Structure of an extended-spectrum class A β-lactamase from *Proteus vulgaris* K1. J Mol Biol *317*, 109-17.

Nukaga, M., Taniguchi, K., Washio, Y., and Sawai, T. (1998). Effect of an amino acid insertion into the Ω-loop region of a class C β-lactamase on its substrate

specificity. Biochemistry *37*, 10461-8.

Oefner, C., D'Arcy, A., Daly, J. J., Gubernator, K., Charnas, R. L., Heinze, I., Hubschwerlen, C., and Winkler, F. K. (1990). Refined crystal structure of β-lactamase from *Citrobacter freundii* indicates a mechanism for β-lactam hydrolysis. Nature *343*, 284-8.

O'Kane, C., Stephens, M. A., and McConnell, D. (1986). Integrable α-amylase plasmid for generating random transcriptional fusions in *Bacillus subtilis*. J Bacteriol *168*, 973-81.

Olsen, O., Borriss, R., Simon, O., and Thomsen, K. K. (1991). Hybrid *Bacillus* (1-3,1-4)-β-glucanases: engineering thermostable enzymes by construction of hybrid genes. Mol Gen Genet *225*, 177-85.

Orr-Weaver, T. L., and Szostak, J. W. (1983). Multiple, tandem plasmid integration in *Saccharomyces cerevisiae*. Mol Cell Biol *3*, 747-9.

Pain, R. H., and Virden, R. (1979). The structural and conformational basis of β-lactamase activity. In BETA-LCTAMASES, J. M. T. HaMilton-Miller and J. T. Smith, eds. (London: Academic Press), pp. 141-180.

Palva, I., Sarvas, M., Lehtovaara, P., Sibakov, M., and Kaariainen, L. (1992). Secretion of *Escherichia coli* β-lactamase from *Bacillus subtilis* by the aid of α-amylase signal sequence. 1982. Biotechnology *24*, 344-8.

Palzkill, T., and Botstein, D. (1992). Identification of amino acid substitutions that alter the substrate specificity of TEM-1 β-lactamase. J Bacteriol *174*, 5237-43.

Palzkill, T., Le, Q. Q., Venkatachalam, K. V., LaRocco, M., and Ocera, H. (1994). Evolution of antibiotic resistance: several different amino acid substitutions in an active site loop alter the substrate profile of β-lactamase. Mol Microbiol *12*, 217-29.

Paradkar, A. S., Petrich, A. K., Leskiw, B. K., Aidoo, K. A., and Jensen, S. E. (1994). Transcriptional analysis and heterologous expression of the gene encoding β-lactamase inhibitor protein (BLIP) from *Streptomyces clavuligerus*.

Gene *144*, 31-6.

Park, K. (19930 Enviromental Effects on cell Metabolism and recombinant Protein Production by *Bacillus subtilis*. PhD thesis, Colorado State University.

Peracchi, A. (2001). Enzyme catalysis: removing chemically 'essential' residues by site-directed mutagenesis. Trends Biochem Sci *26*, 497-503.

Petit, A., Maveyraud, L., Lenfant, F., Samama, J. P., Labia, R., and Masson, J. M. (1995). Multiple substitutions at position 104 of β-lactamase TEM-1: assessing the role of this residue in substrate specificity. Biochem J *305*, 33-40.

Petrosino, J., Rudgers, G., Gilbert, H., and Palzkill, T. (1999). Contributions of aspartate 49 and phenylalanine 142 residues of a tight binding inhibitory protein of β-lactamases. J Biol Chem *274*, 2394-400.

Plapp, B. V. (1995). Site-directed mutagenesis: a tool for studying enzyme catalysis. Methods Enzymol *249*, 91-119.

Pompon, D., and Nicolas, A. (1989). Protein engineering by cDNA recombination in yeasts: shuffling of mammalian cytochrome P-450 functions. Gene *83*, 15-24.

Pratt, R. F., and Loosemore, M. J. (1978). 6-β-bromopenicillanic acid, a potent β-lactamase inhibitor. Proc Natl Acad Sci U S A *75*, 4145-9.

Pratt, R. F. (1992). Beta-lactamases inhibition. In the Chemistry of Beta-lactams, M. I. page, ed. (Blackie, London: Blackie Academic & Professional), pp. 229-271.

Pryor, K. D., and Leiting, B. (1997). High-level expression of soluble protein in *Escherichia coli* using a His6-tag and maltose-binding-protein double-affinity fusion system. Protein Expr Purif *10*, 309-19.

Rahil, J., and Pratt, R. F. (1994). Characterization of covalently bound enzyme inhibitors as transition-state analogs by protein stability measurements: phosphonate monoester inhibitors of a β-lactamase. Biochemistry *33*, 116-25.

Rahuel, J., Rasetti, V., Maibaum, J., Rueger, H., Goschke, R., Cohen, N. C., Stutz, S., Cumin, F., Fuhrer, W., Wood, J. M., and Grutter, M. G. (2000). Structure-based drug design: the discovery of novel nonpeptide orally active inhibitors of human renin. Chem Biol *7*, 493-504.

Rao, S., and Bodley, J. W. (1996). Expression, purification, and characterization of the G domain of *Saccharomyces cerevisiae* elongation factor 2. Protein Expr Purif *8*, 91-6.

Raquet, X., Lamotte-Brasseur, J., Fonze, E., Goussard, S., Courvalin, P., and Frere, J. M. (1994). TEM β-lactamase mutants hydrolysing third-generation cephalosporins. A kinetic and molecular modelling analysis. J Mol Biol *244*, 625-39.

Rasmussen, B. A., Bush, K., Keeney, D., Yang, Y., Hare, R., O'Gara, C., and Medeiros, A. A. (1996). Characterization of IMI-1 β-lactamase, a class A carbapenem-hydrolyzing enzyme from *Enterobacter cloacae*. Antimicrob Agents Chemother *40*, 2080-6.

Rey M.W, Requadt C., mainzer S.E., lamsa M.H., Ferrari E, Lad P. J. and Gray G. L. (1986) Homologous α-amylases of *Bacillus* and generation of their hybrids *in vivo*. *Bacillus* Molecular Genetic and Biotechnology Application (by Acadmic Press, Inc.), 229-239

Rhazi, N., Galleni, M., Page, M. I., and Frere, J. M. (1999). Peptidase activity of β-lactamases. Biochem J *341*, 409-13.

Richmond, M. H., and Sykes, R. B. (1973). The β-lactamases of gram-negative bacteria and their possible physiological role. Adv Microb Physiol *9*, 31-88.

Rinas, U., Tsai, L. B., Lyons, D., Fox, G. M., Stearns, G., Fieschko, J., Fenton, D., and Bailey, J. E. (1992). Cysteine to serine substitutions in basic fibroblast growth factor: effect on inclusion body formation and proteolytic susceptibility during *in vitro* refolding. Biotechnology (N Y) *10*, 435-40.

Robson, B., and Pain, R. H. (1976). The mechanism of folding of globular proteins. Suitability of a penicillinase from *Staphylococcus aureus* as a model for refolding studies. Biochem J *155*, 325-30.

Rudgers, G. W., and Palzkill, T. (1999). Identification of residues in β-lactamase critical for binding β-lactamase inhibitory protein. J Biol Chem *274*, 6963-71.

Rudgers, G. W., and Palzkill, T. (2001). Protein minimization by random fragmentation and selection. Protein Eng *14*, 487-92.

Rutberg, L., Hoch, J. A., and Spizizen, J. (1969). Mechanism of transfection with deoxyribonucleic acid from the temperate *Bacillus* bacteriophage ϕ105. J Virol *4*, 50-7.

Ryan, W., Parulekar, S. T., and Stark, B. C. (1989). Expression of β-lactamase by recombinant *Escherichia coli* strains containing plasmids of different sizes -- Effects of pH, phosphate, and dissolved oxygen. Biotechnology and Bioengineering *34*, 309-319.

Sachdev, D., and Chirgwin, J. M. (2000). Fusions to maltose-binding protein: control of folding and solubility in protein purification. Methods Enzymol *326*, 312-21.

Samraoui, B., Sutton, B. J., Todd, R. J., Artymiuk, P. J., Waley, S. G., and Phillips, D. C. (1986). Tertiary structural similarity between a class A β-lactamase and a penicillin-sensitive D-alanyl carboxypeptidase-transpeptidase. Nature *320*, 378-80.

Sandanayaka, V. P., and Prashad, A. S. (2002). Resistance to β-Lactam Antibiotics: Structure and Mechanism Based Design of β-Lactamase Inhibitors. Curr Med Chem *9*, 1145-65.

Sanders, C. C., and Sanders, W. E., Jr. (1992). β-Lactam resistance in gram-negative bacteria: global trends and clinical impact. Clin Infect Dis *15*, 824-39.

Sanschagrin, F., Couture, F., and Levesque, R. C. (1995). Primary structure of OXA-3 and phylogeny of oxacillin-hydrolyzing class D β-lactamases. Antimicrob Agents Chemother *39*, 887-93.

Sanschagrin, F., Bejaoui, N., and Levesque, R. C. (1998). Structure of CARB-4

and AER-1 carbenicillin-hydrolyzing β-lactamases. Antimicrob Agents Chemother *42*, 1966-72.

Sanschagrin, F., Theriault, E., Sabbagh, Y., Voyer, N., and Levesque, R. C. (2000). Combinatorial biochemistry and shuffling of TEM, SHV and *Streptomyces albus* omega loops in PSE-4 class A β-lactamase. J Antimicrob Chemother *45*, 517-20.

Sargantanis, I. G., and Karim, M. N. (1998). Adaptive pole placement control algorithm for DO-control in β-lactamase production. Biotechnol Bioeng *60*, 1-9.

Saunders, C. W., Schmidt, B. J., Mirot, M. S., Thompson, L. D., and Guyer, M. S. (1984). Use of chromosomal integration in the establishment and expression of *blaZ*, a *Staphylococcus aureus* β-lactamase gene, in *Bacillus subtilis*. J Bacteriol *157*, 718-26.

Schneider, W. P., Nichols, B. P., and Yanofsky, C. (1981). Procedure for production of hybrid genes and proteins and its use in assessing significance of amino acid differences in homologous tryptophan synthetase α polypeptides. Proc Natl Acad Sci U S A *78*, 2169-73.

Seaman, E. E., Tarmy E. and Marmur J. (1964). Inducible phages of *Bacillus subtilis*. Biochemistry *3*, 607-613.

Skandalis, A., Encell, L. P., and Loeb, L. A. (1997). Creating novel enzymes by applied molecular evolution. Chem Biol *4*, 889-98.

Singh, A., and Hayashi, K. (1995). Construction of chimeric β-glucosidases with improved enzymatic properties. J Biol Chem *270*, 21928-33.

Singh, A., Hayashi, K., Hoa, T. T., Kashiwagi, Y., and Tokuyasu, K. (1995). Construction and characterization of a chimeric β-glucosidase. Biochem J *305*, 715-9.

Smith, D. B. (2000). Generating fusions to glutathione S-transferase for protein studies. Methods Enzymol *326*, 254-70.

Smith, D. B., and Johnson, K. S. (1988). Single-step purification of polypeptides

expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene *67*, 31-40.

Smith, J. C., Derbyshire, R. B., Cook, E., Dunthorne, L., Viney, J., Brewer, S. J., Sassenfeld, H. M., and Bell, L. D. (1984). Chemical synthesis and cloning of a poly(arginine)-coding gene fragment designed to aid polypeptide purification. Gene *32*, 321-7.

Sowek, J. A., Singer, S. B., Ohringer, S., Malley, M. F., Dougherty, T. J., Gougoutas, J. Z., and Bush, K. (1991). Substitution of lysine at position 104 or 240 of TEM-1pTZ18R β-lactamase enhances the effect of serine-164 substitution on hydrolysis or affinity for cephalosporins and the monobactam aztreonam. Biochemistry *30*, 3179-88.

Spizizen, J., and Prestidge, L. (1969). Conditions for competence in the *Bacillus licheniformis* transformation system. J Bacteriol *99*, 70-7.

Spizizen, J., Reilly, B. E., and Evans, A. H. (1966). Microbial transformation and transfection. Annu Rev Microbiol *20*, 371-400.

Stahl, S., and Nygren, P. A. (1997). The use of gene fusions to protein A and protein G in immunology and biotechnology. Pathol Biol (Paris) *45*, 66-76.

Stahl, S., Nygren, P. A., and Uhlen, M. (1997). Detection and isolation of recombinant proteins based on binding affinity of reporter: protein A. Methods Mol Biol *63*, 103-18.

Stahl, S., Nygren, P. A., and Uhlen, M. (1997). Strategies for gene fusions. Methods Mol Biol *62*, 37-54.

Strynadka, N. C., Adachi, H., Jensen, S. E., Johns, K., Sielecki, A., Betzel, C., Sutoh, K., and James, M. N. (1992). Molecular structure of the acyl-enzyme intermediate in β-lactam hydrolysis at 1.7 Å resolution. Nature *359*, 700-5.

Strynadka, N. C., Jensen, S. E., Alzari, P. M., and James, M. N. (1996). A potent new mode of β-lactamase inhibition revealed by the 1.7 Å X- ray crystallographic structure of the TEM-1-BLIP complex. Nat Struct Biol *3*, 290-7.

Strynadka, N. C., Jensen, S. E., Johns, K., Blanchard, H., Page, M., Matagne, A., Frere, J. M., and James, M. N. (1994). Structural and kinetic characterization of a β-lactamase-inhibitor protein. Nature *368*, 657-60.

Summanen, P., Baron, E.J., Citron, D.M. (1993) Wadsworth Anaerobic Bacteriology Manual. Belmont, CA: Star pubilshing Co., 1.

Swaren, P., Maveyraud, L., Guillet, V., Masson, J. M., Mourey, L., and Samama, J. P. (1995). Electrostatic analysis of TEM1 β-lactamase: effect of substrate binding, steep potential gradients and consequences of site-directed mutations. Structure *3*, 603-13.

Swaren, P., Maveyraud, L., Raquet, X., Cabantous, S., Duez, C., Pedelacq, J. D., Mariotte-Boyer, S., Mourey, L., Labia, R., Nicolas-Chanoine, M. H., Nordmann, P., Frere, J. M., and Samama, J. P. (1998). X-ray analysis of the NMC-A β-lactamase at 1.64 Å resolution, a class A carbapenemase with broad substrate specificity. J Biol Chem *273*, 26714-21.

Sykes, R. B., and Matthew, M. (1976). The β-lactamases of gram-negative bacteria and their role in resistance to β-lactam antibiotics. J Antimicrob Chemother *2*, 115-57.

Terry, P. a. B., D.G. (1983). Role of RNA polymease, rho factor and ribosomers in transcription termination. In Gene function in prokaryotes, J. Beckwith, Davies, J. and Gallant, A., ed. (Cold Spring Harbor, N.Y.: Cold Spring Harbor laboratory), pp. 123-161.

Thatcher, D. R. (1975a). Beta-lactamase (*Bacillus cereus*). Methods Enzymol *43*, 640-52.

Thatcher, D. R. (1975b). The partial amino acid sequence of the extracellular β-lactamase I of *Bacillus cereus* 569/H. Biochem J *147*, 313-26.

Therrien, C., Sanschagrin, F., Palzkill, T., and Levesque, R. C. (1998). Roles of amino acids 161 to 179 in the PSE-4 Ωloop in substrate specificity and in resistance to ceftazidime. Antimicrob Agents Chemother *42*, 2576-83.

Thomas, J. G., Ayling, A., and Baneyx, F. (1997). Molecular chaperones, folding

catalysts, and the recovery of active recombinant proteins from *E. coli*. To fold or to refold. Appl Biochem Biotechnol *66*, 197-238.

Thomas, J. G., and Baneyx, F. (1997). Divergent effects of chaperone overexpression and ethanol supplementation on inclusion body formation in recombinant *Escherichia coli*. Protein Expr Purif *11*, 289-96.

Thomas, S., Soriano, S., d'Santos, C., and Banting, G. (1996). Expression of recombinant rat myo-inositol 1,4,5-trisphosphate 3-kinase B suggests a regulatory role for its N-terminus. Biochem J *319*, 713-6.

Thornewell, S. J., East, A. K., and Errington, J. (1993). An efficient expression and secretion system based on *Bacillus subtilis* phage ϕ105 and its use for the production of *B. cereus* β-lactamase I. Gene *133*, 47-53.

Tschantz, W. R., Paetzel, M., Cao, G., Suciu, D., Inouye, M., and Dalbey, R. E. (1995). Characterization of a soluble, catalytically active form of *Escherichia coli* leader peptidase: requirement of detergent or phospholipid for optimal activity. Biochemistry *34*, 3935-41.

Tye, A. J., Siu, F. K., Leung, T. Y., and Lim, B. L. (2002). Molecular cloning and the biochemical characterization of two novel phytases from *B. subtilis* 168 and *B. licheniformis*. Appl Microbiol Biotechnol *59*, 190-7.

Udaka, S., Tsukagoshi, N., and Yamagata, H. (1989). *Bacillus brevis*, a host bacterium for efficient extracellular production of useful proteins. Biotechnol Genet Eng Rev *7*, 113-46.

Uhlen, M., Nilsson, B., Guss, B., Lindberg, M., Gatenbeck, S., and Philipson, L. (1983). Gene fusion vectors based on the gene for staphylococcal protein A. Gene *23*, 369-78.

Ulmanen, I., Lundstrom, K., Lehtovaara, P., Sarvas, M., Ruohonen, M., and Palva, I. (1985). Transcription and translation of foreign genes in *Bacillus subtilis* by the aid of a secretion vector. J Bacteriol *162*, 176-82.

van Kampen, M. D., Dekker, N., Egmond, M. R., and Verheij, H. M. (1998). Substrate specificity of *Staphylococcus hyicus* lipase and *Staphylococcus aureus*

lipase as studied by *in vivo* chimeragenesis. Biochemistry *37*, 3459-66.

Vanhove, M., Houba, S., lamotte-Brasseur, J., and Frere, J. M. (1995a). Probing the determinants of protein stability: comparison of class A β-lactamases. Biochem J *308*, 859-64.

Vanhove, M., Raquet, X., and Frere, J. M. (1995b). Investigation of the folding pathway of the TEM-1 β-lactamase. Proteins *22*, 110-8.

Vieille, C., Burdette, D. S., and Zeikus, J. G. (1996). Thermozymes. Biotechnol Annu Rev *2*, 1-83.

Vieille, C., Epting, K. L., Kelly, R. M., and Zeikus, J. G. (2001). Bivalent cations and amino-acid composition contribute to the thermostability of *Bacillus licheniformis* xylose isomerase. Eur J Biochem *268*, 6291-301.

Vos, P., Boerrigter, I. J., Buist, G., Haandrikman, A. J., Nijhuis, M., de Reuver, M. B., Siezen, R. J., Venema, G., de Vos, W. M., and Kok, J. (1991). Engineering of the *Lactococcus lactis* serine proteinase by construction of hybrid enzymes. Protein Eng *4*, 479-84.

Vuilleumier, S., and Fersht, A. R. (1994). Insertion in barnase of a loop sequence from ribonuclease T1. Investigating sequence and structure alignments by protein engineering. Eur J Biochem *221*, 1003-12.

Waley, S. G. (1991). The kinetics of substrate-induced inactivation. Biochem J *279*, 87-94.

Waley, S. G. (1992). Beta-lactamases: mechanism of action. In The Chemistry of Beta-Lactams, M. I. Page, ed. (London: Chapman & Hall), pp. 198-228.

Walker, P. A., Leong, L. E., Ng, P. W., Tan, S. H., Waller, S., Murphy, D., and Porter, A. G. (1994). Efficient and rapid affinity purification of proteins using recombinant fusion proteases. Biotechnology (N Y) *12*, 601-5.

Wall, J. G., and Pluckthun, A. (1995). Effects of overexpressing folding modulators on the *in vivo* folding of heterologous proteins in *Escherichia coli.* Curr Opin Biotechnol *6*, 507-16.

Wallon, G., Kryger, G., Lovett, S. T., Oshima, T., Ringe, D., and Petsko, G. A. (1997). Crystal structures of *Escherichia coli* and *Salmonella typhimurium* 3-isopropylmalate dehydrogenase and comparison with their thermophilic counterpart from *Thermus thermophilus*. J Mol Biol *266*, 1016-31.

Wang, C., Castro, A. F., Wilkes, D. M., and Altenberg, G. A. (1999). Expression and purification of the first nucleotide-binding domain and linker region of human multidrug resistance gene product: comparison of fusions to glutathione S-transferase, thioredoxin and maltose-binding protein. Biochem J *338*, 77-81.

Wang, P. Z., and Novick, R. P. (1987). Nucleotide sequence and expression of the beta-lactamase gene from *Staphylococcus aureus* plasmid pI258 in *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus*. J Bacteriol *169*, 1763-6.

Wang, P. Z., Projan, S. J., Leason, K. R., and Novick, R. P. (1987). Translational fusion with a secretory enzyme as an indicator. J Bacteriol *169*, 3082-7.

Wang, W., and Malcolm, B. A. (2002). Two-stage polymerase chain reaction protocol allowing introduction of multiple mutations, deletions, and insertions, using QuikChange site-directed mutagenesis. Methods Mol Biol *182*, 37-43.

Weber, H., and Weissmann, C. (1983). Formation of genes coding for hybrid proteins by recombination between related, cloned genes in *E. coli*. Nucleic Acids Res *11*, 5661-9.

Wiedemann, B., Kliebe, C., and Kresken, M. (1989). The epidemiology of β-lactamases. J Antimicrob Chemother *24 Suppl B*, 1-22.

Wilchek, M., and Bayer, E. A. (1990). Introduction to avidin-biotin technology. Methods Enzymol *184*, 5-13.

Williams, J. D. (1999). β-Lactamases and β-lactamase inhibitors. Int J Antimicrob Agents *12 Suppl 1*, S3-7; discussion S26-7.

Wong, S. L. (1995). Advances in the use of *Bacillus subtilis* for the expression and secretion of heterologous proteins. Curr Opin Biotechnol *6*, 517-22.

Wong, S. L., Kawamura, F., and Doi, R. H. (1986). Use of the *Bacillus subtilis* subtilisin signal peptide for efficient secretion of TEM β-lactamase during growth. J Bacteriol *168*, 1005-9.

Wood, H. E., Dawson, M. T., Devine, K. M., and McConnell, D. J. (1990). Characterization of PBSX, a defective prophage of *Bacillus subtilis*. J Bacteriol *172*, 2667-74.

Wu, X. C., Lee, W., Tran, L., and Wong, S. L. (1991). Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. J Bacteriol *173*, 4952-8.

Yamagata, H., Nakahama, K., Suzuki, Y., Kakinuma, A., Tsukagoshi, N., and Udaka, S. (1989). Use of *Bacillus brevis* for efficient synthesis and secretion of human epidermal growth factor. Proc Natl Acad Sci U S A *86*, 3589-93.

Zafaralla, G., Manavathu, E. K., Lerner, S. A., and Mobashery, S. (1992). Elucidation of the role of arginine-244 in the turnover processes of class A β-lactamases. Biochemistry *31*, 3847-52.

Zawadzke, L. E., Chen, C. C., Banerjee, S., Li, Z., Wasch, S., Kapadia, G., Moult, J., and Herzberg, O. (1996). Elimination of the hydrolytic water molecule in a class A β-lactamase mutant: crystal structure and kinetics. Biochemistry *35*, 16475-82.

Zawadzke, L. E., Smith, T. J., and Herzberg, O. (1995). An engineered *Staphylococcus aureus* PC1 β-lactamase that hydrolyses third-generation cephalosporins. Protein Eng *8*, 1275-85.

Zulli, F., Weber, H., and Zuber, H. (1990). Structure and function of L-lactate dehydrogenases from thermophilic and mesophilic bacteria, X. Analysis of structural elements responsible for the differences in thermostability and activation by fructose 1,6- bisphosphate in the lactate dehydrogenases from *B. stearothermophilus* and *B. caldolyticus* by protein engineering. Biol Chem Hoppe Seyler *371*, 655-62.

Appendices

Appendix 1: Reagents

BHY (growth medium for *B. subtilis* for protein expression)

3.7% Brain Heat Infusion Broth (Oxoid), 0.5% Yeast extract (Oxoid)

Celite elution buffer

0.1 M Tris-HCl, 2 M NaCl, 0.1 M tri-sodium citrate, pH 7.0

β-Lactamase assay buffer

50 mM sodium phosphate, 0.5 M NaCl, 0.1 mM EDTA, pH 7.0

LB broth (general growth medium for *E. coli*)

0.1% Bacto-tryptone, 0.05% Bacto-yeast extract, 0.05% NaCl

MBP column buffer (MBP binding buffer)

20 mM Tris-HCl, 0.2 M NaCl, 1 mM EDTA, pH 7.4

SMM (Spizizen minimal medium)

0.2% ammonium sulphate, 1.4% dipotassium phosphate, 0.6 % potassium dihydrogen phosphate, 0.1% sodium citrate dihydrate, 0.02% magnesium sulphate

PBS (Phosphate-buffered saline, served as GST binding buffer)

140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3

PTM (Pre-transformation medium, for the preparation of competent *B. subtilis*)

10 ml SMM, 0.2 ml 40% glucose, 0.1 ml PTM salts (25 mM $CaCl_2$, 50 mM $MgSO_4$, 0.05 mM $MnSO_4$), 0.2 ml 20% casamino acids, 0.1 ml 2 mg/ml tryptophan and 0.1 ml of other supplements (20 mg/ml isoleucine, 20 mg/ml valine, 20 mg/ml leucine, 5 mg/ml methionine)

TBE (running buffer for agarose gel electrophoresis)

90 mM Tris base, 90 mM boric acid, 2.5 mM EDTA

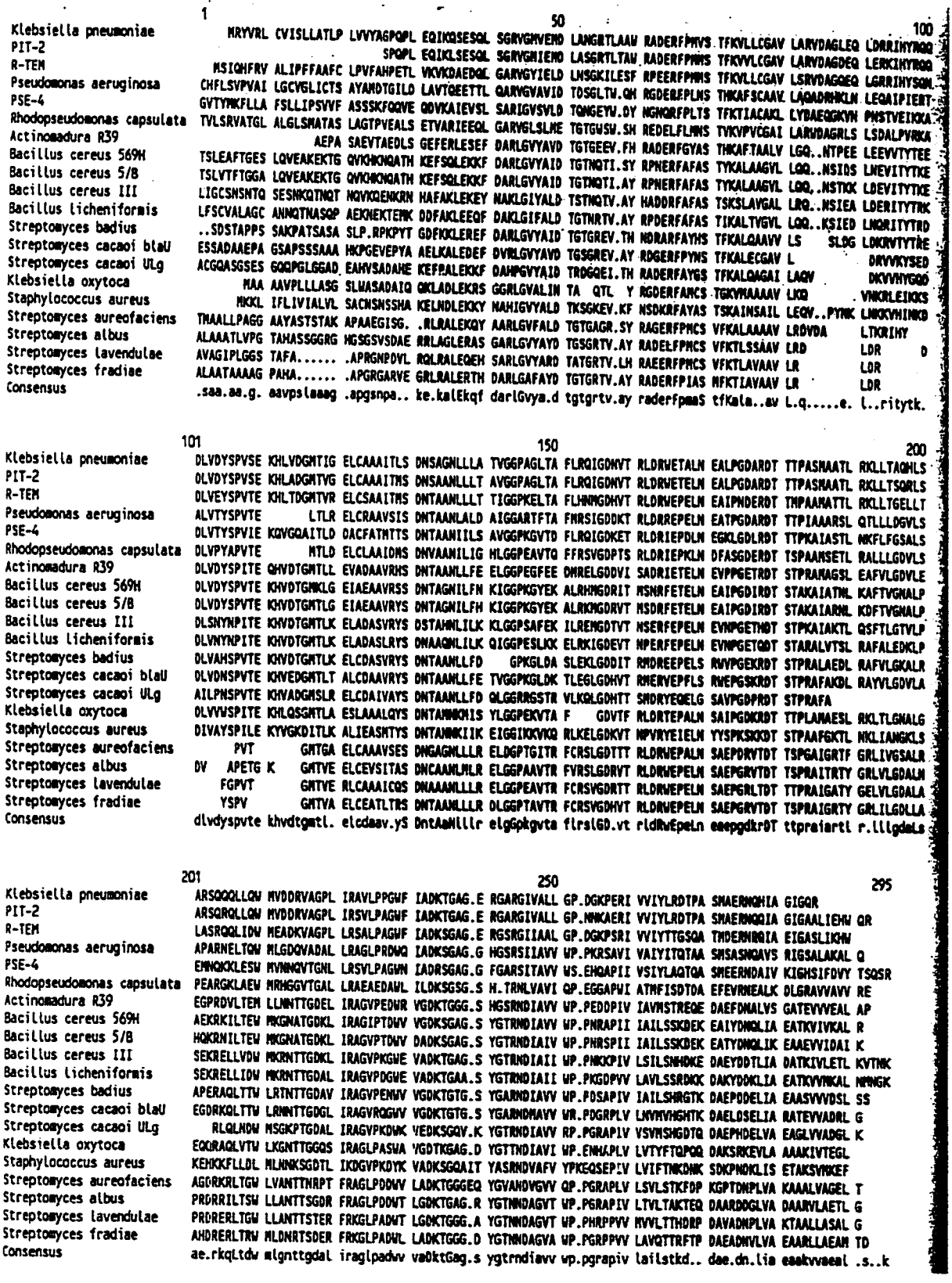
TM (Transformation medium, for the preparation of competent *B. subtilis*)

10 ml SMM, 0.15 ml 40% glucose, 0.2 ml 250 mM $MgSO_4$, 5 μl 20% casamino acids, 0.1 ml 2 mg/ml tryptophan and 0.1 ml of other supplements (20 mg/ml isoleucine, 20 mg/ml valine, 20 mg/ml leucine, 5 mg/ml methionine)

2 X TY (rich growth medium for *E. coli*)

0.16% Bacteriotryptone, 0.1% Yeast extract, 0.05% NaCl

Appendix 2: Alignment of 20 class A β-lactamases numbered according to the ABL scheme after Ambler *et al.*, 1991)



The sequences are referred to by their most familiar names. '.' indicates a postulated deletion; blank spaces indicate one or more residues omitted from the alignment. Leader sequences before position 1 are omitted. Note that single tyrosine residues have been omitted from the *Streptomyces badius* and *Streptomyces cacaoi* sequences at position 241. Publication references are as follows: ***Klebsiella pneumoniile***: Arakawa, Y., Ohta, M.IKido, N., Fujii, Y., Komatsu, T. & Kato, N. (1986) FEBS Lett. 207, 69-74; **PIT-2**: Barthelemy, M., Peduzzi, J. & Labia, R. (1988) Biochem. J; 251, 73-79; R- TEM: Sutcliffe, J. G. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 3737-3741; ***Pseudomonas aeruginosa and Rhodopseudomonai capsulata***: Campbell, J. I. A., Scahill, S. A., Gibson, T. & Ambler, R. P. (1989) Biochem. J. 200, 803-812; **PSE-4**: Boissinot, M. & Levesque, R. C. (1990) J. Bioi. Chem. 265, 1225-1230; ***Actinomadura* R39**: Houba, S., Molitor, C., Willem, S., Ghuysen, J.-M., Frère, J.-M., Duez, C. & Du, J. (1989) FEMS Microbiol. Lett. 65, 241-246; ***Bacillus cereus* 569H and 5/B**; Madgwick, P. J. & Waley, S. G. (1987) Biochem. J. 248, 657, and Madonna, M. J., Zhu, Y. F. & Lampen, J. O. (1987) Nucleic Acids Res. 15,1877; ***Bacillus cereus* III**: Husain, M., Pastor, F. I. J. & Lam, J. O. (1987) J. Bactenol. 169, 579-586; ***Bacillus licheniformis***: Neugebauer, K., Sprengel, R. & Schaller, H. (1981) Nucleic Acids Res. 9, 2577-2588, ***Streptomyces badius, cacaoi* blaU**, ***lavendulae*** and ***fradiae***: Forsman, M., Haggstrom, B., Lindgren, L. & Jaurin, B. (1990) J. Gen. Microbiol. I. 589-598; ***Streptomyces cacaoi* ULg**: Lenzini, M. V., Ishihara, H., Dusart, J., Ogawara, H., Jons, B., Van Beeumen, J., Frère, J.-M. & Ghuy, J.-M. (1988) FEMS Microbiol. Lett. 49, 371-376; ***Klebsiella oxytoca***: Arakawa, Y., Ohta, M., Kido, N., Mon, M., Ito, H., Komatsu, T., Fujii, Y. & Kato, N. (1989) Antimicrob. Agents Chemother. 33, 63-70; ***Staphylococcus aureus***: Ambler, R. P. (1975) Biochem. J. 151, 197-218 and McLaughlin, J. R., Murray, C. J. & Rabinowitz, J. C. (1981) J. Bioi. Chem. 256, 11273-11282; ***Streptomyces aureofaciens***: Tiraby, G. unpublished work; ***Streptomyces albus* G**: Dehottay, P., Dusart, J., De Meester, F., Jons, B., Van Beeumen, J., Erpicum, T., Frère, J.-M. & Ghuysen, J.-M. (1987) Eur. J. Biochem. 166, 345.