BETA- LACTAMASES

of

Bacillus licheniformis

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A Thesis Presented for
the Degree of
Ph.D.

Department of Molecular Biology
University of Edinburgh

May 1988

A Elisa e Dino
con riconoscenza e affetto

Abstract

During the course of this work the genomic β-lactamase from *Bacillus licheniformis* 6346/c was investigated. A partial genomic DNA library of this bacillary strain was constructed in λNM1149 and a β-lactamase producing recombinant isolated. The 4.7Kb long insert was subcloned into pACYC184, shotgunned into an M13 vector and sequenced by the Sanger dideoxy chain termination method. Making use of the University of Wisconsin package for sequence analysis the assembled DNA sequence was searched for open reading frames and control sequences. The comparison of the deduced protein sequence of the three major open reading frames with the aminoacid sequence of the β-lactamase from *Bacillus licheniformis* 749/c published by Lampen confirmed the presence of a closely related protein. The number of significant aminoacid differences between the two enzymes is quite small, most of them being located in the leader peptide.

These two enzymes present a different spectrum of activity against a range of β-lactams, '6346' being a better cephalosporinase than '749'. From the work of Pollock and Thatcher it was thought that the difference in specificity profile was due to a single substitution or to a small number of tightly linked aminoacid changes. The aim of this work was to investigate which of the aminoacid changes was responsible for the activity difference by substituting 749-like residues in the corresponding positions on the 6346 enzyme.

A 1.6Kb DNA fragment containing the 6346 β-lactamase gene was subcloned from pACYC184 into M13mp18, but was found to be highly unstable and therefore unsuitable as a vector for the site directed mutagenesis (SDM) experiments. A new vector had to be constructed that would allow the maintenance of the gene in a more stable double stranded plasmid and at the same time would be a convenient system for the SDM experiments. This was achieved by partially deleting *amp* from pTZ19R and introducing in its place *tet* from pAT153. The 1.6Kb DNA fragment proved to be stable in the resulting pAD plasmid. Cells carrying the plasmid produce single stranded template in suitable amount both for SDM and sequencing.

The Eckstein method and the gapped-duplex method were used to construct the mutants. Their products were partially purified by FPLC and the K_m

and V_{max} for a range of substrates were estimated spectrophotometrically. The results of this study indicate that the change Gly238 to Ala is responsible for all the differences in substrate profile. All the other substitutions so far introduced do not significantly alter the activity spectrum of the enzyme. This information has been related to the recent X-ray structure of the highly homologous β-lactamase of *Staphylococcus aureus* PC1. Gly238 to Ala is the only substitution close to the active site; this would explain why this highly conservative change has such a profound influence on the substrate profile of the enzyme.

Another mutant, Asn170 to Met, was designed to test a feature of the mechanism of action proposed by Moult for the class A β-lactamases, of which the *Staphylococcus aureus* and the *Bacillus licheniformis* enzymes are members. Preliminary experiments with this mutant protein do not appear to confirm Moult's proposal that the deacylation step of the reaction intermediate makes use of a water molecule bound in the active site at a position which would be blocked by the Asn170 to Met substitution.

Declaration

I hereby declare that I alone have written this thesis and that, except where stated, the work described is my own.

Daniela Anderluzzi

May 1988

Acknowledgements

I wish to thank here all the members of the Molecular Biology Department of the University of Edinburgh for all the help they gave me in so many different ways.

In particular I owe a lot to my supervisor, Dr. Andrew Coulson, for his patience with my initially very poor English and for the unfailing guidance and support he gave me throughout the duration of this project. I am also very grateful to all the people I turned to for help and advice, among them Drs. N.E.Murray, R.P.Ambler, L.Gilmore, D.Finnegan and R.Hayward.

Immense thanks also to Joan Campbell, Ian Scragg, Gill Cowan, Margaret Daniel, Gudmundir Hreggvidsson and Medha Athalye whom, with their friendship, serious discussions and lighthearted chatting made my time in this department so interesting and pleasant.

I am indebted to Miss Pamela Beattie for making and analysing the plasmid heteroduplexes, Miss Joan Fleming for making the anti-β-lactamase antibodies, Graham Brown for the preparation of the photographs used for this thesis, Tam Bruce for growing big batches of cells, to Ruby Docherty, Joan Smail and all the ladies of the media room for providing the indispensable clean glassware and sterile media.

I am grateful to the University of Edinburgh for partly financing this project.

A special thank goes to my family, for their moral and financial support. Without them this work would not have been possible.

Finally, I owe a great deal to Sid, not only for his help with the typing and proofreading of this thesis, but for the boundless support and cooperation he gave me at all times, especially when the going was difficult.

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Abbreviations

A	adenine
Amp	ampicillin
6-APA	6-aminopenicillanic acid
C	cytosine
cfu	colony forming units
Cm	chloramphenicol
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanidine 5'-triphosphate
dNTP	deoxynucleoside 5'-triphosphate
dTTP	deoxythymidine 5'-triphosphate
ddATP etc.	dideoxyadenosine 5'-triphosphate etc.
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DTT	dithiothreitol
EDTA	diaminoethanetetraacetic acid
EEO	electroendoosmosis
g	standard acceleration due to gravity
G	guanine
Kb	kilobase
Km	kanamycin
K_m	affinity constant
MES	2-[N-morpholino]ethanesulfonic acid
moi	multiplicity of infection
M_r	relative molecular mass

mRNA	messenger RNA
ORF	open reading frame
PEG	polyethylene glycol
pfu	plaque forming units
RF DNA	replicative form DNA
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
T	thymine
TEMED	N,N,N',N'–tetramethyl ethylene diamine
Tet	tetracycline
Tris	tris(hydroxymethyl)aminomethane
u.v.	ultraviolet light
V_{max}	rate constant
v:v	volume to volume
w:v	weight to volume
X^r	resistance to antibiotic X

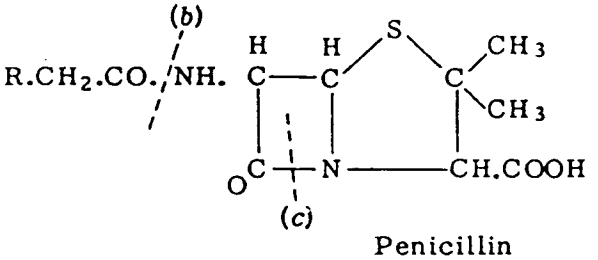
CHAPTER 1

INTRODUCTION

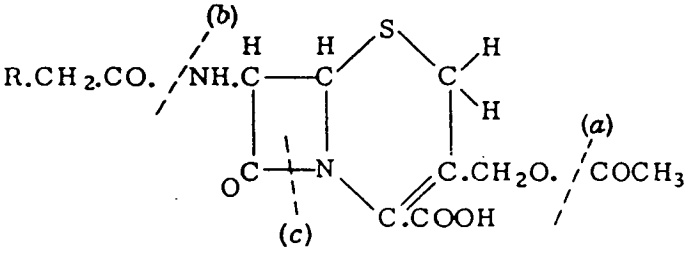
1.1 INTRODUCTION

Since the discovery of penicillin (Fleming 1929) and of cephalosporins (Newton & Abraham 1954) and the first realisation of their potentiality as therapeutic agents (Chain *et al.* 1940) the family of β-lactam antibiotics of which penicillins and cephalosporins (Figure 1.1) are members has grown enormously. Despite the introduction of many competitor drugs, penicillins are still regarded as the most potent of all agents for the treatment of acute infectious diseases. Their action is directed against penicillin-binding proteins (PBPs) located on the bacterial cell surface and interferes with the metabolism of the cell wall. Generally low levels of penicillin cause inhibition of cell division with consequent filamentation of the cells, while lysis occurs at higher antibiotic concentration (Spratt 1975).

FIGURE 1.1 Action of enzymes attacking penicillins and cephalosporins

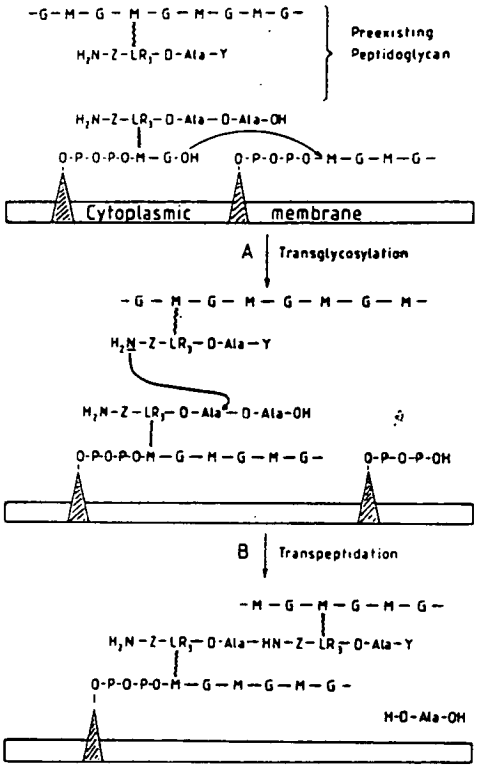


Penicillin



a. Acyl-esterases; b. Amidases; c. Beta-lactamases; (from Pollock 1965a)

FIGURE 1.2 Peptidoglycan synthesis



Possible scheme for the integration of new disaccharide-peptide units into nascent peptidoglycan by transglycosylation (A) and into preexisting peptidoglycan by transpeptidation (B). The peptide in the preexisting peptidoglycan which serves as an acceptor in the transpeptidation reaction might already be engaged in another cross-link (Y = -NH-Z-peptide), be intact (Y = D-Ala-OH), or have been shortened by DD-carboxypeptidase activity (Y = OH). In some cases, the L-R_3-D-Ala-OH peptide bond of the acceptor molecule has been hydrolysed by LD-carboxypeptidase activity (from Frere & Joris 1985).

1.2 MECHANISM OF ACTION OF β-LACTAM ANTIBIOTICS

1.2.1 The bacterial cell wall

All bacterial cells are surrounded by a wall which determines the cell shape and protects the bacterium from osmotic lysis. In Gram positive bacteria the wall, which surrounds the cell membrane, is mainly formed by tiers of peptidoglycan forming a thick layer at the very surface of the cell. In Gram negative microorganisms the peptidoglycan is much thinner and it is contained between the outer and the inner membranes.

All bacterial peptidoglycans are similar, being built of long parallel polysaccharide molecules with alternating units of N-acetylglucosamine and N-acetylmuramic acid and pentapeptides attached by an amide bond to the lactyl side chain of the N-acetylmuramic acid residue. The structure of these peptides varies in different bacterial species, but it always presents a D-alanyl-D-alanine dipeptide at the 3' end and either lysine or diaminopimelic acid as third residue.

During biosynthesis of the cell wall (Figure 1.2) disaccharide-peptide units are transported outside the cell wall and attached to the nascent peptidoglycan by transglycosylation. In the last stage of the synthesis the new peptidoglycan is cross-linked to the preexisting one by a transpeptidation reaction in which the free amino group of the third residue of the peptide on one glycan strand displaces the terminal D-Ala of a second glycan strand (reviewed by Tipper and Wright 1979). It is the final cross-linking step of cell wall biosynthesis that is inhibited by β-lactam antibiotics.

Other transpeptidases might be involved in further maturation of the peptidoglycan (Mirelman 1980), while a carboxypeptidase activity splitting the D-Ala-D-Ala terminal dipeptide could exercise control over the degree of cross-linking. These activities are also penicillin-sensitive.

1.2.2 Penicillin-binding proteins

The enzymes that perform the penicillin sensitive reactions described above are usually membrane bound and they are detected and isolated because of their ability to bind specific β-lactam antibiotics forming stable or slowly degrading complexes.

These penicillin-binding proteins (PBPs) have been studied in many different species and have been numbered according to their relative electrophoretic mobilities. So, although related species may have similar patterns of PBPs, there is no necessary relationship between a particular PBP number of two unrelated organisms.

In *E.coli* PBPs have been divided in two groups depending on their molecular weight. High M_r PBPs, ranging between 60000 and 90000, are numbered 1A, 1B, 2 and 3. They seem to have a strictly synthetic function and are essential to proper cell growth and division.

In vivo, loss of either PBP1A or PBP1B does not affect cell survival, but bacteria lyse when both are inactivated (Suzuki *et al.* 1978). PBP1B has been isolated and extensively studied. It is probably the major transpeptidase involved in the incorporation of new units of peptidoglycan into the cell wall and its activity controls cell elongation (Spratt 1975, Spratt *et al.* 1977).

Inhibition of PBP2, easily achieved with mecillinam, induces loss of the normal rod shape and osmotically stable round cells are found in treated cultures (Iwaya *et al.* 1978). PBP3 is involved in septum formation; its inactivation inhibits cell division inducing filamentation of the cells and eventual death (Spratt 1975, 1977).

PBP1A, 1B and PBP3 have been shown to be constituted by two functional domains, one performing the penicillin sensitive transpeptidase activity mentioned above, the other functioning as a penicillin insensitive transglycosylase (Suzuki *et al.* 1980, Ishino *et al.* 1980, Ishino & Matsuhashi 1981, Hedge & Spratt 1984). On the other hand PBP2 is probably only a transpeptidase (Ishino *et al.* 1982).

All the high M_r PBPs have been cloned and sequenced (Broome-Smith *et al.* 1985, Asoh *et al.* 1986, Nakamura *et al.* 1983), but no crystallographic data are yet available.

Low M_r PBPs (30000-40000) are non-essential to the cell in normal laboratory conditions and mutants with no detectable levels of PBP4, 5 or 6 do not show any loss of viability or morphological alterations (Matsuhashi *et al.* 1977, 1978, Spratt 1980, Broome-Smith & Spratt 1982). They are apparently involved in the maturation of the cell wall, PBP4 performing the secondary transpeptidation reactions (De Pedro & Schwarz 1981), while PBP5 and PBP6 are mainly D-Ala-D-Ala carboxypeptidases (Spratt 1983, Waxman & Strominger 1983). Their role in the metabolism of the peptidoglycan is still not clear (Tamura *et al.* 1976, Spratt & Strominger 1976, Amanuma & Strominger 1980). Sequence information is available for PBP5 (Broome-Smith *et al.* 1983) and its amino terminus was shown to be similar to that of PBP6 (Waxman *et al.* 1982).

Although PBP1A and PBP1B appear to be very closely related, in general little sequence similarity can be found within the two groups of PBPs (high M_r and low M_r) and between them. Nevertheless, in all PBPs a conserved sequence is found around the active site serine (Ser-Xaa-Xaa-Lys) and

Ser-Xaa-Xaa-Lys-Pro is present in all high M_r PBPs. Another region of similarity can be detected at the amino terminus (Asoh *et al.* 1986).

Being membrane bound proteins, PBPs have proved very difficult to crystallize, so that the only structural information available was obtained on soluble D-Ala-D-Ala carboxypeptidases produced by *Streptomyces* R61 (Kelly *et al.* 1982, 1985) and by *Streptomyces albus* G (Dideberg *et al.* 1982).

The wealth of information obtained on PBPs from other organisms as well as from *E.coli* has been extensively reviewed by Frere and Joris (1985).

1.2.3 The substrate analogue hypothesis

Already in 1965 it had been proposed (Tipper & Strominger 1965) that penicillins interfere with the transpeptidation of the nascent peptidoglycan by acting as a substrate analogue of the D-Ala-D-Ala dipeptide. It was suggested that the transpeptidase reaction involves the temporary acylation of an active site residue accompanied by the release of the terminal D-Ala. In a second step of the reaction the acyl-enzyme intermediate would transfer the tetrapeptide to a nucleophile, reconstituting the active enzyme. When the nucleophile is a molecule of water a hydrolysis is performed, while during the transpeptidase reaction the tetrapeptide is transferred to a free amino group.

The β-lactam bond of penicillin is presented to the enzyme in the same way as the peptide bond of the terminal D-Ala-D-Ala and it is cleaved in a similar fashion, but in this case the enzyme is deacylated only very slowly and is therefore inactivated. This theory has been more recently comfirmed by the sequencing of PBPs active site peptides in which a serine residue is acylated both in the presence of normal substrate and of β-lactams (Yocum *et al.* 1979, 1980, Waxman & Strominger 1980).

1.3 RESISTANCE TO β-LACTAM ANTIBIOTICS

1.3.1 Intrinsic resistance

Many factors are involved in the bacterial resistance to β-lactam antibiotics. Among these an altered permeability of the outer cell membrane can cause intrinsic resistance in Gram negative bacteria. Strains of *Neisseria gonorrhoeae*, *Serratia*, *Enterobacter* and *Pseudomonas* have been reported to

have altered cell envelopes that confer resistance to the cells (Neu 1982) and more recently a mutant of *Salmonella paratyphi* A resistant to most β-lactam antibiotics was shown to have decreased quantities of three major porins (Gutmann *et al.* 1988).

Altered susceptibility of target sites can also form the basis of resistance. For example, modified PBPs have been found in a resistant strain of *Streptococcus pneumoniae* and no β-lactam was effective against it (Williamson *et al.* 1981). It is thought that the accumulation of single amino acid substitutions around the active site of PBPs conferring small decreases in affinity to β-lactams could lead to quite considerable levels of resistance. This process has been demonstrated by Hedge & Spratt (1985) who followed the evolution of PBP3 in *E.coli* for increasing levels of resistance to cephalosporins. PBP3 is normally a target for many cephalosporins and its inactivation can lead to cell death, but the cumulation of only four amino acid changes caused a one hundred fold decrease in sensitivity to some cephalosporins, like cefoxitin.

1.3.2 Enzymatic resistance

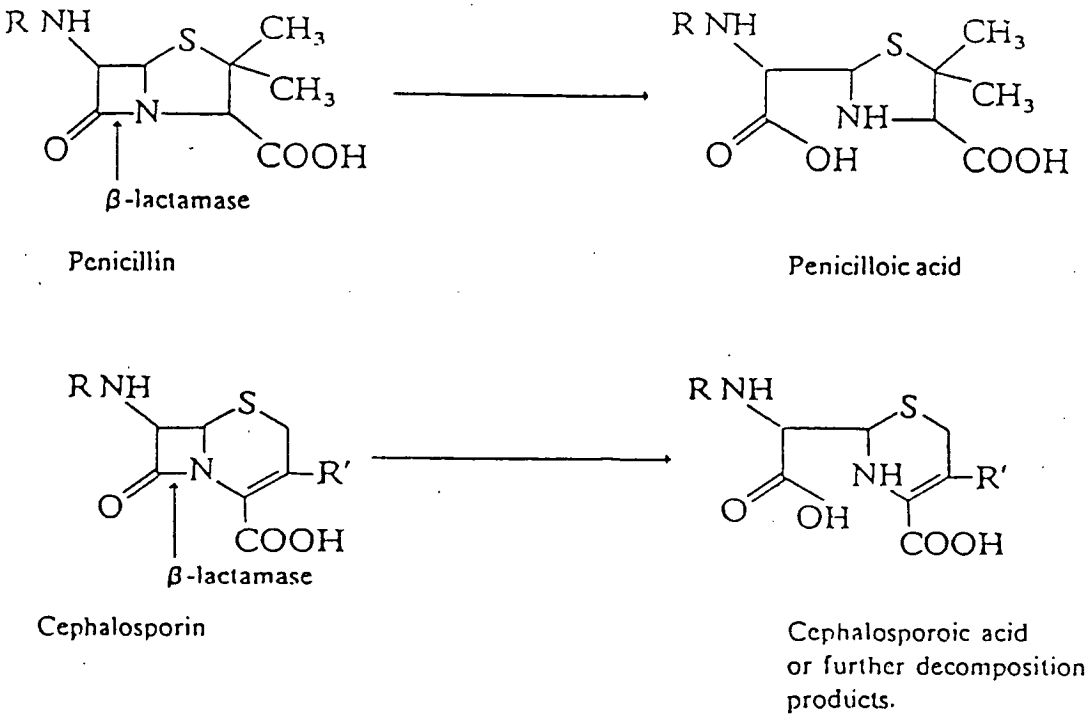
Antibiotic resistance due to the production of β-lactam destroying enzymes was reported very early by a codiscoverer of penicillin (Abraham & Chain 1940). Three types of β-lactam modifying enzymes have since been found to be involved in the destruction of antibiotic efficiency of β-lactams (Figure 1.1).

Acyl-esterases (Huang *et al.* 1963) are active only against cephalosporins and attack the ester bond of the side chain in position 3' of the β-lactam nucleus, yielding a product with a somewhat reduced antibiotic activity.

Amidases (Batchelor *et al.* 1961) split the amide bond in the side chain of both penicillins and cephalosporins. Their importance in the development of resistance is very limited, but they are now extensively used in the industrial production of semi-synthetic β-lactam antibiotics.

β-lactamases are by far the most important and effective enzymatic activity directed against β-lactams. They break the β-lactam bond of the nucleus of both penicillins and cephalosporins (Figure 1.3). In the case of penicillins the end product is penicilloic acid, which presents no antibiotic activity. The reaction with cephalosporins also involves only the opening of the β-lactam ring, but the resulting cephalosporoate is unstable and undergoes

FIGURE 1.3 β-Lactam bond hydrolysis



further non-catalyzed decomposition to smaller fragments (Hamilton-Miller *et al.* 1970, Faraci & Pratt 1984).

β-lactamases can probably be found in all bacterial groups (Hamilton-Miller 1979). β-lactamase activity has also been reported in the blue-green algae *Coccochloris elabens* and *Anabaena* (Kushner & Breuil 1977), in the yeasts *Candida albicans, Candida boidinii* and *Pichia pinus* (Metha & Nash 1978) as well as in mammalian tissue, including human (Hamilton-Miller 1982), but these non-bacterial enzymes have not been characterized in great detail.

On the other hand the bacterial β-lactamases have been much studied, not only because of their clinical importance in resistance of pathogens but also because their properties make them a convenient system for the studying of more general phenomena.

1.3.3 The spread of resistance linked to β-lactamases

The effectiveness of β-lactamases as a protection against antibiotically active β-lactams is mainly linked to the ease with which these enzymes spread

across bacterial strains and species. The selective pressure that β-lactam antibiotics exercise on target bacterial populations was very quickly recognised as a driving force for the diffusion of resistance (for example Livermore 1986). In the early stages of the clinical application of penicillin very few strains of bacteria were resistant; by 1948 already 50% of the staphylococcal strains isolated in hospitals survived treatment with the drug and by 1967 the incidence of resistance to penicillin in *St.aureus* had risen to 70% in the community at large and to 95% among the hospital population (Ridley *et al.* 1970, Finland 1979).

Many β-lactamase genes were shown to be part of highly mobile genetic elements. Some genes are plasmid-borne and can therefore be easily transferred to other bacterial species by transduction or conjugation. Others are found to be part of transposable elements, e.g. the TEM gene of transposon TnA, and can move from replicon to replicon, be it the bacterial chromosome or a plasmid (Hedges & Jacob 1974, Richmond *et al.* 1980). This type of genetic organization would certainly explain the ease with which β-lactamase activity spreads within and between species. .

Good examples of this phenomenon are given by a plasmid found in *Proteus mirabilis* that carried a β-lactamase identical to the chromosomal enzyme of *E.coli* K12 (Bobrowski *et al.* 1976) or by the transfer of *AmpC* from *E.coli* to *Shigella* clinical isolates (Olsson *et al.* 1983). More recently a TEM-like enzyme (CTX-1), carried on a 84Kb plasmid in *Klebsiella pneumoniae*, was reported to have transferred to five other enterobacterial species isolated from five patients in an intensive care unit (Kitzsis *et al.* 1988).

1.4 CLASSIFICATION Of β-LACTAMASES

1.4.1 The Richmond & Sykes classification

β-lactamases present an enormous variety of enzymic and molecular properties, making a systematic classification based on properties extremely difficult. The first attempt to put some order in the hundreds of distinguishable β-lactamases was that proposed by Richmond & Sykes (Richmond & Sykes 1973, Sykes & Matthew 1976). The classification was based on the substrate profile, that is the activity shown by the enzyme against a series of β-lactams relative to

that of benzylpenicillin, on the molecular weight of the proteins, susceptibility to inhibitors like cloxacillin, as well as on the location of the gene.

Although this classification is still very important and widely used because of the emphasis it puts on the clinically and therapeutically relevant aspects, it is now thought to be incomplete and somewhat misleading (Coulson 1985).

The main objection to the Richmond & Sykes classification is that it is applied only to enzymes produced by Gram negative bacteria. So the staphylococcal and bacillary β-lactamases, that are similar to the Gram negative TEM, could not be accounted for in this scheme. In addition, in view of what has just been said above about the mobility of the β-lactamase genes, their location is no longer considered an indication of relatedness.

Measurement of M_r has also proved unreliable, estimates depending very much on the method used: for example the M_r of the TEM enzyme of *E.coli* has been reported to be 17000 from equilibrium centrifugation studies (Datta & Richmond 1966), 21000 from gel filtration measurements (Dale & Smith 1971) and 27000 from estimates of electrophoretic mobility (Scott 1972).

Finally there is evidence that the substrate profile, on the basis of which β-lactamases are broadly subdivided in penicillinases, cephalosporinases and broad spectrum enzymes, can be altered considerably by a small number of amino acid substitutions (Pollock 1968, this work, Hall & Knowles 1976, Baldwin *et al.* 1980).

1.4.2 Sequence-based classification

Once the amino acid sequence of several β-lactamases had been reported, it became possible to start a classification of the enzymes based on the structure and therefore on likely evolutionary relatedness (Ambler 1980, Jaurin and Grundstrom 1981, Dale *et al.* 1985). To date four classes have been recognized.

CLASS A

This class includes enzymes from both Gram negative and Gram positive bacteria, that differ widely in isoelectric point, molecular weight and substrate

TABLE 1.1 Class A β-lactamases

ORGANISM	NAME	GENETIC LOCATION	REFERENCE[a]
St.aureus PC1	-	plasmid	1,2
B.licheniformis 749/c	-	chromosome	3,4
B.cereus 569/H and 5B	β-lactamase I	chromosome	5,6,7,8
E.coli	TEM	plasmid	9,10
Rps.capsulata SP108	-	unknown	11
Ps.aeruginosa RMS149	-	plasmid	11
Kl.pneumoniae LEN-1	-	chromosome	12
Kl.aerogenes	K1	chromosome	13
Strept.albus G	-	chromosome	14
Strept.cacaoi	-	chromosome	15
B.cereus 569/H	β-lactamase III	chromosome	16
E.coli	PIT-2	plasmid	17

a 1. Ambler 1980, 2. Chan 1986, 3. Ambler & Meadway 1969, 4. Neugebauer et al. 1981, 5. Thatcher 1975a, 6. Sloma & Gross 1983, 7. Wang et al. 1985, 8. Madonna et al. 1987, 9. Ambler & Scott 1978, 10. Sutcliffe 1978, 11. Campbell 1986, 12. Arakawa et al. 1986, 13. Emanuel et al. 1986, 14. Dehottay et al. 1987, 15. De Meester et al. 1987, 16. Hussain et al. 1987, 17. Barthelemy et al. 1988.

profiles as well as in other kinetic parameters. However, about 10% of the amino acid residues are identical in all the enzymes (Figure 1.4) and very few insertions and deletions are required in order to optimize the alignment, indicating that they have all diverged from a common ancestral gene. A list of the enzymes currently assigned to this class is given above (Table 1.1).

The genetic location of the Class A β-lactamases is not consistent, the genes being plasmid-borne in most but not all Gram negative bacteria and normally chromosomal in Gram positive. The enzyme is usually found in the periplasm in Gram negatives, while in Gram positive microorganisms the gene product is either found attached to the cell membrane or it is secreted. In *B.licheniformis* about 50% of the activity is found in the growth media, while the

FIGURE 1.4 Protein sequence alignment of Class A β-lactamases

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                    -46                                                    3
Strept.albus G                                                            VH
Strept.cacaoi       MNRRTRVFDV CPHRTRVFSQ LTAGRPAVVA VFPCAPAYGT AQEVPDTAYS
B.cereus 569/H I                                                          MK
B.cereus 5B I                                                             LK
B.cereus 569/H III                                                      MFVLNKFF
consensus           ..................................................k

                    4                                                     53
Strept.albus G      PSTSRPSRRT LLTATAGAAL AAATLVPGTA HASSGGRGHG SGSVSDAERR
Strept.cacaoi       VPPVVFSSGP PLALVPLVAC GQASGSESGQ QPGLGGADEA HVSADAHEKE
Rps.capsulata                  MRFTATV LSRVATGLAL GLSMATASLA GTPVEALSET
Ps.aeruginosa                  MMKFQCH FLSVPVAILG CVGLICTSAY AMDTGILDLA
E.coli TEM                            MSIQHFRVAL IPFFAAFCLP VFAHPETLVK
St.aureus PC1                            MKKLIF LIVIALVLSA CNSNSSHAKE
B.licheniformis 749  MKLWFSTLK LKKAAAVLLF SCVALAGCAN NQTNASQPAE KNEKTEMKDD
B.cereus 569/H I    NKRMLKIGIC VGILGLSITS LEAFTGESLQ VEAKEKTGQV KHKNQATHKE
B.cereus 5B I       NKKMLKIGMC VGILGLSITS LVTFTGGALQ VEAKEKTGQV KHKNQATHKE
B.cereus 569/H III  TNSHYKKIVP VVLLSCATLI GCSNSNTQSE SNKQTNQTNQ VKQENKRNHA
E.coli PIT2                                                         SPQPLEQ
Kl.pneumoniae                         MRYVRLCV ISLLATLPLV VYAGPQPLEQ
consensus           nkkmlksg.. v.ilg.s.ts l.a..vgl.q ...la.tgl. ..snpatlke

                    54                                          *         103
Strept.albus G      LAGLERASGA RLGVYAYDTG SGRTV.AYRA DELFPMCSVF KTLSSAAVLR
Strept.cacaoi       FRALEKKFDA HPGVYAIDTR DGQEI.THRA DERFAYGSTF KALQAGAILA
Rps.capsulata       VARIEEQLGA RVGLSLMETG TGWSW.SHRE DELFLMNSTV KVPVCGAILA
Ps.aeruginosa       VTQEETTLQA RVGVAVIDTD SGLTW.QHRG DERFPLNSTH KAFSCAAVLA
E.coli TEM          VKDAEDQLGA RVGYIELDLN SGKILESFRP EERFPMMSTF KVLLCGAVLS
St.aureus PC1       LNDLEKKYNA HIGVYALDTK SGKEV.KFNS DKRFAYASTS KAINSAILLE
B.licheniformis 749 FAKLEEQFDA KLGIFALDTG TNRTV.AYRP DERFAFASTI KALTVGVLLQ
B.cereus 569/H I    FSQLEKKFDA RLGVYAIDTG TNQTI.SYRP NERFAFASTY KALAAGVLLQ
B.cereus 5B I       FSQLEKKFDA RLGVYAIDTG TNQTI.AYRP NERFAFASTY KALAAGVLLQ
B.cereus 569/H III  FAKLEKEYNA KLGIYALDTS TNQTV.AYHA DDRFAFASTS KSLAVGALLR
E.coli PIT2         IKLSESQLSG RVGMIEMDLA SGRTLTAWRA DERFPMMSTF KVVLCGAVLA
Kl.pneumoniae       IKQSESQLSG RVGMVEMDLA NGRTLAAWRA DERFPMVSTF KVLLCGAVLA
consensus           faqlEkqlda r.Gvya.dtg .g.tv.ayra derFamaStf Kal.cga.La

                    104                                                   153
Strept.albus G      DLDRNGEFLS RRILYTQDDV EQADGAGPET GKPQNLANAQ LTVE....EL
Strept.cacaoi       QVLRDGREVR RGAEADGMDK VVHYGQDAIL PNSPVTEK.H VADGMSLREL
Rps.capsulata       RW..... .DAGRLSLSD ALPVRKADLV PYAPVTET.R VGGNMTLDEL
Ps.aeruginosa       QA..... .DRHKLNLEQ AIPIERTALV TYSPVTERVP PGGTLTLREL
E.coli TEM          RV..... .DAGQEQLGR RIHYSQNDLV EYSPVTEK.H LTDGMTVREL
St.aureus PC1       QV..... ...PYNKLNK KVHINKDDIV AYSPILEK.Y VGKDITLKAL
B.licheniformis 749 Q..... ..KSIEDLNQ RITYTRDDLV NYNPITEK.H VDTGMTLKEL
B.cereus 569/H I    Q..... ..NSIDSLNE VITYTKEDLV DYSPVTEK.H VDTGMKLGEI
B.cereus 5B I       Q..... ..NSTKKLDE VITYTKEDLV DYSPVTEK.H VDTGMTLGEI
B.cereus 569/H III  Q..... ..NSIEALDE RITYTRKDLS NYNPITEK.H VDTGMTLKEL
E.coli PIT2         RV..... .DAGDEQLER KIHYRQQDLV DYSPVSEK.H LADGMTVGEL
Kl.pneumoniae       RV..... .DAGLEQLDR RIHYRQQDLV DYSPVSEK.H LVDGMTIGEL
consensus           qv..... ..a.ieqld. rihyt.ddlv dyspvtek.h vd.gmtlgel
```

FIGURE 1.4 (continued)

```
                    154                                                 203
Strept.albus G      CEVSLTASDN CAANLMLREL GGPAAVTRFV RSLGDRVTRL DRWEPELNSA
Strept.cacaoi       CDAIVAYSDN TAANLLFDQL GGRRGSTRVL KQLGDHTTSM DRYEQELGSA
Rps.capsulata       CLAAIDMSDN VAANILIGHL GGPEAVTQFF RSVGDPTSRL DRIEPKLNDF
Ps.aeruginosa       CRAAVSISDN TAANLALDAI GGARTFTAFM RSIGDDKTRL DRREPELNEA
E.coli TEM          CSAAITMSDN TAANLLLTTI GGPKELTAFL HNMGDHVTRL DRWEPELNEA
St.aureus PC1       IEASMTYSDN TANNKIIKEI GGIKKVKQRL KELGDKVTNP VRYEIELNYY
B.licheniformis 749 ADASLRYSDN AAQNLILKQI GGPESLKKEL RKIGDEVTNP ERFEPELNEV
B.cereus 569/H I    AEAAVRSSDN TAGNILFNKI GGPKGYEKAL RHMGDRITMS NRFETELNEA
B.cereus 5B I       AEAAVRYSDN TAGNILFHKI GGPKGYEKAL RKMGDRVTMS DRFETELNEA
B.cereus 569/H III  ADASVRYSDS TAHNLILKKL GGPSAFEKIL REMGDTVTNS ERFEPELNEV
E.coli PIT2         CAAAITMSDN SAANLLLTAV GGPAGLTAFL RQIGDNVTRL DRWETELNEA
Kl.pneumoniae       CAAAITLSDN SAGNLLLATV GGPAGLTAFL RQIGDNVTRL DRWETALNEA
consensus           ceaavtySDn tAaNlllkki GGpkglt.fl r..GDrvtrl dR.EpeLnea

                    204                                                 253
Strept.albus G      EPGRVTDTTS PRAITRTYGR LVLGD....A LNPRDRRILT SWLLANTTSG
Strept.cacaoi       VPGDPRDTST PRAFAEDLRA FAVEDGEKAA VAPNDRLQLN DWMSGKPTGD
Rps.capsulata       ASGDERDTTS PAAMSETLRA LLLGD....V LSPEARGKLA EWMRHGGVTG
Ps.aeruginosa       TPGDARDTTT PIAAARSLQT LLLDG....V LSAPARNELT QWMLGDQVAD
E.coli TEM          IPNDERDTTM PAAMATTLRK LLTGE....L LTLASRQQLI DWMEADKVAG
St.aureus PC1       SPKSKKDTST PAAFGKTLNK LIANG....K LSKENKKFLL DLMLNNKSGD
B.licheniformis 749 NPGETQDTST ARALVTSLRA FALED....K LPSEKRELLI DWMKRNTTGD
B.cereus 569/H I    IPGDIRDTST AKAIATNLKA FTVGN....A LPAEKRKILT EWMKGNATGD
B.cereus 5B I       IPGDIRDTST AKAIARNLKD FTVGN....A LPHQKRNILT EWMKGNATGD
B.cereus 569/H III  NPGETHDTST PKAIAKTLQS FTLGT....V LPSEKRELLV DWMKRNTTGD
E.coli PIT2         LPGDARDTTT PASMAATLRK LLTSQ....R LSARSQRQLL QWMVDDRVAG
Kl.pneumoniae       LPGDARDTTT PASMAATLRK LLTAQ....H LSARSQQQLL QWMVDDRVAG
consensus           ipgdarDT.t paa.a.tlr. lllgd....a lsaekr.qLt dwmkgnttgd

                    254                                                 303
Strept.albus G      DRFRAGLPDD WTLGDKTGAG .RYGTNNDAG VTWP.PGRAP IVLTVLTAKT
Strept.cacaoi       ALIRAGVPKD WKVEDKSGQV .KYGTRNDIA VVRP.PGRAP IVVSVMSHGD
Rps.capsulata       ALLRAEAEDA WLILDKSGSG SH..TRNLVA VIQP.EGGAP WIATMFISDT
Ps.aeruginosa       ALLRAGLPRD WQIADKSGAG .GHGSRSIIA VVWP.PKRSA VIVAIYITQT
E.coli TEM          PLLRSALPAG WFIADKSGAG .ERGSRGIIA ALGP.DGKPS RIVVIYTTGS
St.aureus PC1       TLIKDGVPKD YKVADKSGQA ITYASRNDVA FVYPKGQSEP IVLVIFTNKD
B.licheniformis 749 ALIRAGVPDG WEVADKTGAA .SYGTRNDIA IIWP.PKGDP VVLAVLSSRD
B.cereus 569/H I    KLIRAGIPTD WVVGDKSGAG .SYGTRNDIA VVWP.PNRAP IIIAILSSKD
B.cereus 5B I       KLIRAGVPTD WVDADKSGAG .SYGTRNDIA IVWP.PNRSP IIIAILSSKD
B.cereus 569/H III  KLIRAGVPKG WEVADKTGAG .SYGTRNDIA IIWP.PNKKP IVLSILSNHD
E.coli PIT2         PLIRSVLPAG WFIADKTGAG .ERGARGIVA LLGP.NNKAE RIVVIYLRDT
Kl.pneumoniae       PLIRAVLPPG WFIADKTGAG .ERGARGIVA LLGP.DGKPE RIVVIYLRDT
consensus           alirag.p.d wf.aDKsGag .sygtrndia vvwP.pgrap iiv.ilsskd
```

FIGURE 1.4 (continued)

	304			330
Strept.albus G	EQDAARDDGL	VADAARVLAE	TLG	
Strept.cacaoi	TQDAEPHDEL	VAEAGLVVAD	GLK	
Rps.capsulata	DAEFEVRNEA	LKDLGRAVVA	VVRE	
Ps.aeruginosa	AASMSASNQA	VSRIGSALAK	ALQ	
E.coli TEM	QATMDERNRQ	IAEIGASLIK	HW	
St.aureus PC1	NKSDKPNDKL	ISETAKSVMK	EF	
B.licheniformis 749	KKDAKYDDKL	IAEATKVVMK	ALNMNGK	
B.cereus 569/H I	EKEAIYDNQL	IAEATKVIVK	ALR	
B.cereus 5B I	EKEATYDNQL	IKEAAEVVID	AIK	
B.cereus 569/H III	KEDAEYDDTL	IADATKIVLE	TLKVTNK	
E.coli PIT2	PASMAERNQQ	IAGIGAALIE	HWQR	
Kl.aerogenes	PASMAERNQH	IAGIGQR		
consensus	ea.a.ydnql	iaeagkvv.k	alk...K	

In the consensus line completely conserved residues are reported in capital letters, semiconserved aminoacids in lower case.

other half remains cell-bound. This diverse cellular location is reflected in the absence of similarity at the amino terminal part of the preproteins, where the leader peptides are found.

All these β-lactamases share the short sequence Ser-Thr-Xaa-Lys in their active site peptide and other completely conserved residues are scattered along the sequence. It has been shown that Ser70 is the main residue involved in catalysis in all of them (see below).

The crystal structure of the *St.aureus* PC1 β-lactamase has recently been published (Herzberg & Moult 1987) and that of *B.licheniformis* 749 is well advanced (Dideberg *et al.* 1985, Knox *et al.* 1987). Preliminary crystallographic data are also available for other members of this class (TEM: Knox *et al.* 1976, De Lucia *et al.* 1980; *B.cereus* β-lactamase I: Aschaffenburg *et al.* 1978).

CLASS B

So far this class has only one member, the broad spectrum β-lactamase II from *B.cereus*. This is a metallo-enzyme requiring as a cofactor a Zn^{2+} ion liganded by three histidine and a cysteine residue (Baldwin *et al.* 1979). Sequence information (Hussain *et al.* 1985) clearly shows that it is structurally

distinct and that its mechanism of action is unlike that of Class A, C and D enzymes. Its crystallographic structure at low resolution is quite different from that of Class A enzymes, presenting very little α-helical secondary elements and apparently no β-sheet (Sutton, B., Fourth β-lactamase workshop: Holy Island 20th-22nd April 1988).

CLASS C

These are also serine-enzymes, but apart from the serine residue in the active site and the lysine three positions later they do not share detectable similarities with the Class A β-lactamases (Jaurin & Grundstrom 1981, Knott-Hunziker *et al.* 1982). The gene of the *E.coli* enzyme *ampC* which was the first recognized member of this class was used as a probe to screen many Gram negative bacteria, revealing a large number of strains that carry a homologous gene (Bergstrom *et al.* 1983).

Other enzymes have now been assigned to Class C from *Enterobacter cloacae* P99 (Joris *et al.* 1984), *Pseudomonas aeruginosa* (Berks *et al.* 1982, Knott-Hunziker *et al.* 1982), *Citrobacter freundii* and *Shigella sonnei* (Bergstrom *et al.* 1983).

Preliminary crystallographic data have been published for the *Enterobacter cloacae* P99 enzyme (Charlier *et al.* 1983) and the X-ray structure of the *Citrobacter freundii* is now refined to 3 Angstrom resolution, revealing a striking similarity to that of Class A enzymes (Oefner, C., Fourth β-lactamase workshop: Holy Island 20th-22nd April 1988).

CLASS D

To date this class has two members, the OXA-2 β-lactamase and the PSE-2 enzyme. The gene for the OXA-2 β-lactamase was found on the R46 factor of a *Salmonella typhimurium* isolate (Anderson & Datta 1965) and was sequenced by Dale and coworkers (1985). The overall structure of this enzyme presents no similarity to the members of other classes, but their pattern of residues Ser-Thr-Xaa-Lys is identical to that found in the active site of Class A and C enzymes, indicating that they all share the same mechanism of action. On the other hand this enzyme presents very peculiar characteristics that make it quite unlike any other β-lactamase: it can hydrolyse oxacillin and other isoxazolyl

penicillins, it has a dimeric structure and its affinity for cibacron blue and other anthraquinone dyes would suggest that it has a nucleotide binding domain (Dale & Smith 1976, Monaghan *et al.* 1982).

The other member of this class, PSE-2, is found in strains of *Pseudomonas aeruginosa*. Its amino acid sequence (Huovinen *et al.* 1988) is 35% identical to that of OXA-2, with a cluster of identical residues around the putative active site serine.

UNCLASSIFIED

Other β-lactamases have been reported that present sufficiently peculiar characteristics to justify the claim that they are not part of any of the above classes. For example a β-lactamase from *Pseudomonas maltophilia* has been reported to be a tetrameric enzyme that requires Zn^{2+} ions for activity (Saino *et al.* 1982). Its amino terminal 32 amino acid residues have been sequenced and shown to bear no similarity to the other metallo-β-lactamase from *B.cereus*, thus excluding any relation with Class B (Bicknell *et al.* 1985).

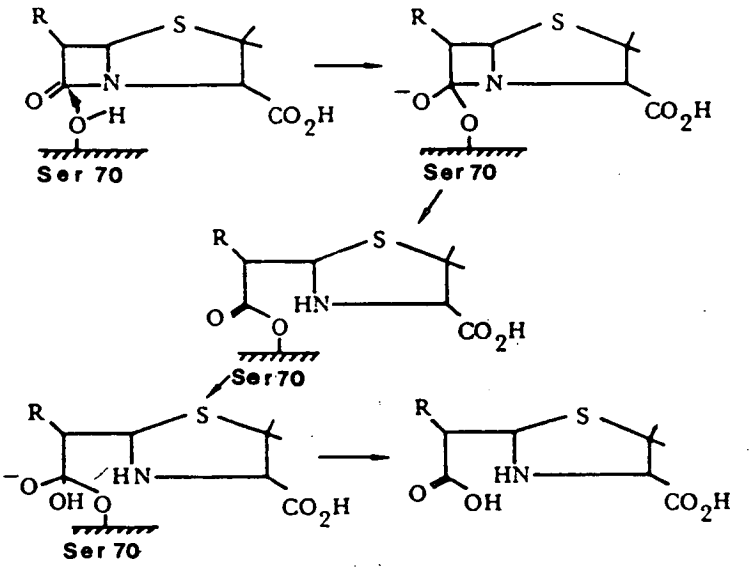
Other novel β-lactamases have also been identified from *Proteus vulgaris* (Matsubara *et al.* 1981), *Bacterioides fragilis* (Yotsuji *et al.* 1983) and *Pseudomonas aeruginosa* (Labia *et al.* 1981). Only information on the structure of these enzymes will tell if they bear any similarity to one of the above classes or if new groups have to be created.

1.5 THE REACTION MECHANISM OF SERINE-β-LACTAMASES

A lot of work has been carried out in the last ten years to try and clarify the detailed mechanism of action of the serine-β-lactamases (reviewed in Coulson 1985).

Both in Class A and Class C enzymes, and in Class D by comparison, the serine contained in the common sequence Ser-Thr-Xaa-Lys (Ser70 in Ambler notation, Ambler 1980) has been repeatedly indicated as the active site residue by chemical modification studies (Fisher *et al.* 1981, Cartwright & Coulson 1980, Knott-Hunziker *et al.* 1979, Cohen & Pratt 1980). This residue is acylated by the β-lactam during the initial part of the reaction and is subsequently hydrolysed (Figure 1.5). In Class C β-lactamases the acyl-intermediate accumulates during

FIGURE 1.5 Acylation of β-lactamases



the course of the reaction, the hydrolysis being the rate determining step, so that evidence of the acylation can be obtained even with good substrates like benzylpenicillin (Knott-Hunziker *et al.* 1982a). On the other hand the rate of the reaction of Class A β-lactamases with good substrates seems to be limited by the formation of the acyl-enzyme itself (Hardy & Kirsch 1984), so that information about the mechanism can only be obtained with bad substrates such as cefoxitin (Fisher *et al.* 1980) or with suicide active site inhibitors such as penicillanic acid sulphone or olivanic acid (mechanisms of inactivation reviewed by Knowles 1985).

Site directed mutagenesis studies confirmed the importance of Ser70: the inversion of the active site dipeptide Ser-Thr to Thr-Ser in the TEM enzyme resulted in an inactive β-lactamase (Dalbadie-McFarland *et al.* 1982), while substitution of Ser70 with Cys gives a protein with low β-lactamase activity that can be inhibited with p-chloromercuribenzoate (Sigal *et al.* 1982).

Specific inactivation studies using phenylpropynal and phenylglyoxal seems to implicate lysine and arginine as possible other active site residues (Schenkein & Pratt 1980, Borders *et al.* 1982). The presence of a carboxyl residue has also been proposed (Hardy *et al.* 1984) and in fact modification of carboxyl groups has been found to inactivate Class A enzymes (Waley 1975).

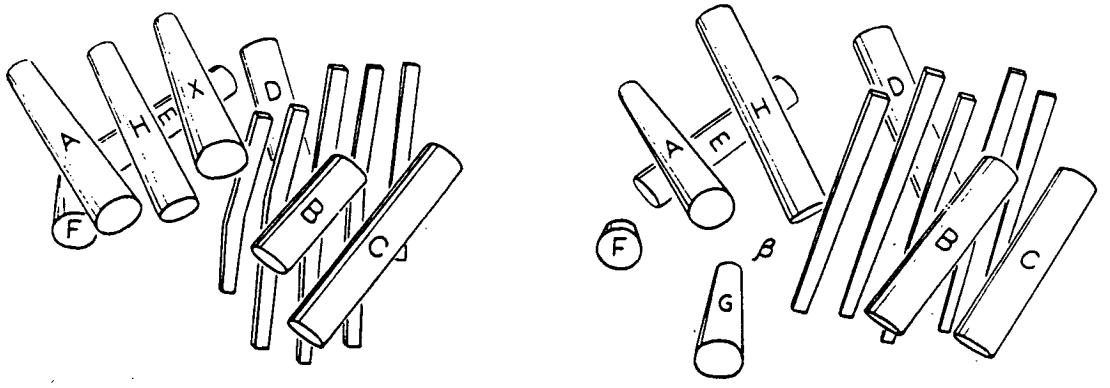
Mutants of *B.cereus* β-lactamase I with Glu166 converted to Gln presented very little activity, confirming the likely involvement of a carboxyl residue. The substitution of the highly conserved Lys73 to Arg greatly affected the activity of the enzyme but did not abolish it, while Glu168 to Asp mutation did not induce any appreciable change in activity (Madgwick & Waley 1987).

1.5.1 Proposed mechanism of action

A detailed mechanism of action has been proposed for the *St.aureus* PC1 enzyme by Herzberg & Moult (1987). In their suggestion the proton of the active site serine, labilized because of the position of the residue at the amino end of an α-helix, would attack the carbonyl carbon of the β-lactam substrate, forming a tetrahedral intermediate. The intermediate is stabilized by the interaction of the negatively charged carbonyl oxygen with an oxyanion hole formed by the main chain amino group of the Ser itself and the side chain of Gln237. This situation is similar to that found in serine-proteases such as subtilisin (Robertus *et al.* 1972, Kraut 1977). The transfer of the proton from the Ser to the β-lactam nitrogen is facilitated by the conserved Lys73, which would provide a potential gradient towards the β-lactam nitrogen, thus reducing the energy barrier for the proton transfer and the formation of the acyl-enzyme intermediate. A water molecule, hydrogen bonded to the main chain carbonyl group of Gln237 and to the side chains of Asn170 and Glu166, probably polarized by Glu166, would act as nucleophile and hydrolyse the acyl enzyme, thus completing the reaction.

This hypothesis, based on the modelling of an ampicillin molecule into the active site of the enzyme, seems to provide an explanation to the impossibility of trapping penicilloyl-enzymes of the Class A β-lactamases with nucleophiles other than water (Brenner *et al.* 1981, Anderson & Pratt 1981, Knott-Hunziker *et al.* 1982a). Although direct observation of the orientation of the β-lactam substrate inside the active site of β-lactamases has yet not been achieved because of the rapid turnover of the substrate, comparisons can be drawn with the behaviour and structure of PBPs.

FIGURE 1.6 Three dimentional structure of *Streptomyces* R61 carboxypeptidase and of β-lactamase of *Bacillus licheniformis*



Secondary structure elements in B.licheniformis beta-lactamase (left) and Streptomyces R61 DD-peptidase (right). Cylinders are alpha-helices and ribbons beta-strands. The two drawings are the results of overlaying computer graphics plots of crystallographically determined polypeptide folding and are to the same scale. The known site of beta-lactam binding in the DD-peptidase from X-ray crystallographic studies is indicated by β (from Kelly et al. 1986).

1.6 β-LACTAMASES AND PBPs

The idea that β-lactamases have evolved from PBPs was suggested by Pollock (1971). Soil bacteria faced with β-lactam antibiotics would be advantaged by the ability to produce increased quantities of target PBPs in order to lower the concentration of the drug around the cell. During evolution PBPs could have evolved into β-lactam hydrolysing enzymes, giving origin to β-lactamases. This proposal has been object of long debates since, but in view of recent findings there seem now to be little doubt that β-lactamases and PBPs do descend from a common ancestor.

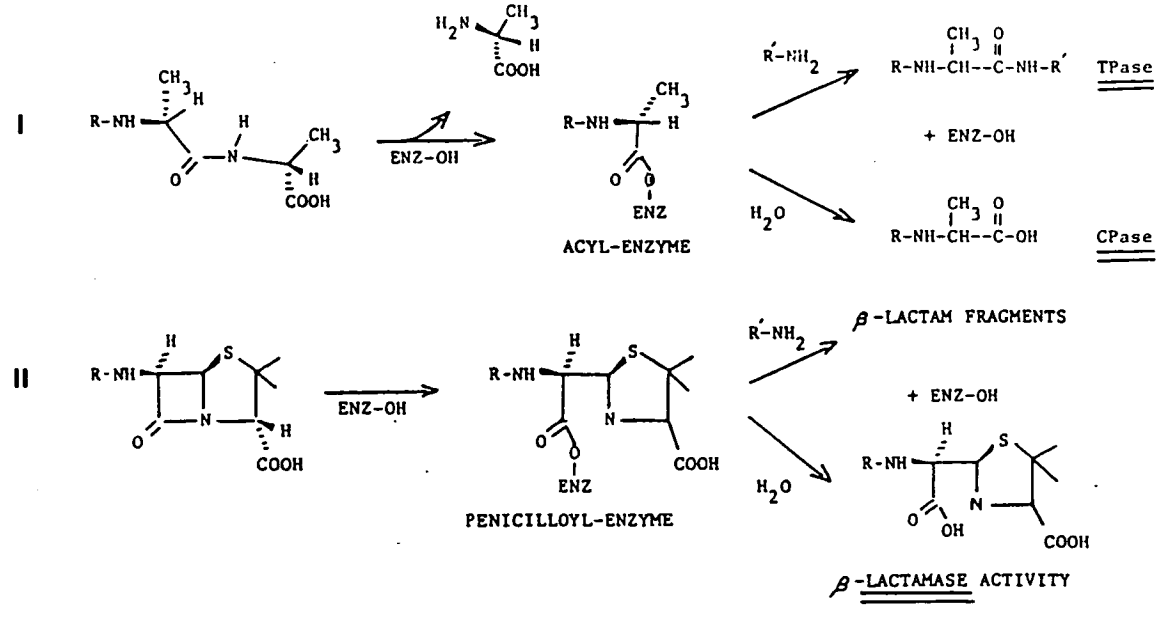
It has already been mentioned that the majority of PBPs and β-lactamases present the sequence Ser-Xaa-Xaa-Lys in their β-lactam binding site. The sequence similarity between these two groups of enzymes has been recently investigated (Joris *et al.* 1988) and several "boxes" of conserved residues identified. Although from this evidence all these enzymes could be considered members of a superfamily of serine-enzymes, the evolutionary distance is obviously great.

The relationship has also been confirmed by the elucidation of four crystal structures: those of the *Streptomyces* R61 carboxypeptidase (Kelly *et al.* 1982, 1985), *B.cereus* β-lactamase I (Samraoui *et al.* 1986), the penicillinase of *B.licheniformis* 749 (Kelly *et al.* 1986) and the *St.aureus* enzyme (Herzberg & Moult 1987). The two papers on the bacillary β-lactamases were published at the same time and independently drew the same comparison between the β-lactamase structures and that of the R61 carboxypeptidase.

In Figure 1.6 a diagram of the tertiary structures of the *B.licheniformis* β-lactamase and of the R61 enzyme is presented. The folding of the two molecules is obviously very similar: both enzymes contain a β-sheet formed by antiparallel strands of similar length covered on both sides by α-helices. The angles and the distances between the helices and between each helix and the β-sheet are comparable, while differences can be found in the connections between the secondary structure elements.

PBPs and β-lactamases share a similar mechanism of action, the first step of the reactions been in both cases the acylation of the active site Ser, but the fate of the acyl-enzyme is different, depending on the enzyme and on the

FIGURE 1.7 Mechanism of action of penicillin-binding proteins (I) and β-lactamases (II)



substrate (Figure 1.7). During the reaction of PBPs with their natural pentapeptide substrate the acylation is accompanied by the liberation of the terminal D-Ala leaving group. When the intermediate is deacylated in the presence of the amino group of a neighbouring pentapeptide, we have a transpeptidation reaction. If the acceptor is instead a molecule of water, the result is a hydrolysis. PBPs are also acylated by β-lactams, but in this case the deacylation is an extremely slow process, normally resulting in the fragmentation of the β-lactam.

β-lactamases also bind β-lactams in an identical fashion and become acylated, but here the intermediate is quickly hydrolysed. Although β-lactamases cannot perform the carboxypeptidation reaction, recent studies of their reaction with peptide analogues have shown that these enzymes can hydrolyse these substrates at a rate similar to that of PBPs (Govardhan & Pratt 1987).

1.7 β-LACTAMASES Of *Bacillus licheniformis*

β-lactamase-producing strains of *B.subtilis* were first isolated by Pollock and coworkers, but only in mid-1960s the taxonomical classification of the *Bacillus* genus led to the distinction between the penicillin-sensitive *B.subtilis* species and the penicillinase-producing *B.licheniformis*.

All the strains of *B.licheniformis* that Pollock isolated from soil samples collected from all over the world, including some soil attached to the roots of plants conserved in a Kew herbarium of 1680, were found to produce β-lactamase (Pollock 1971). The gene is chromosomal and presents two alleles, distinguishable on the basis of the enzymic properties of the proteins. The strains 749 and 6346 (749/c and 6346/c for the magnoconstitutive mutants derived from them) were chosen for further studies as representatives of the two alleles.

The two proteins differ in their electrophoretic mobility and sensitivity to iodine and their activity is influenced to varying degree by antibodies (Pollock 1965a, 1965b). In Table 1.2 the V_{max} and K_m of the 6346/c and 749/c β-lactamases for a range of substrates are reported (Pollock 1965). From these data it is apparent that the two enzymes have a quite distinct specificity of action: the 749/c is a typical penicillinase, breaking down cephalosporins at about 1-2% of the rate it hydrolyses benzylpenicillin; 6346/c on the contrary is relatively more active against cephalosporins, with a hydrolysis rate for

TABLE 1.2 Comparison of β-lactamases from *Bacillus licheniformis* strains 749/c and 6346/c[a]

SUBSTRATE	749	6346
MAXIMUM RATE OF HYDROLYSIS (V_{max})[b]		
Penicillin G	325(100)	54(100)
6-Aminopenicillanic acid	16.2(5.0)	7.0(13)
Cephalosporin C	3.5(1.1)	8.1(15)
Cephalosporin PAC	7.9(2.4)	22.7(42)
DISSOCIATION CONSTANTS (K_m μM)		
Penicillin G	49	9.5
Cephalosporin C	<50	<50
Cephalosporin PAC	<50	<50
PHYSIOLOGICAL EFFICIENCY (V_{max}/K_m)		
Penicillin G	6.6×10^6	5.7×10^6

a Pollock (1965a)

b V_{max} expressed as micromoles of substrate hydrolysed/micrograms of enzyme/hr at 30°C, pH7.0. In parenthesis the values relative to penicillin G are reported.

benzylcephalosporin C of 42%. The V_{max} of the 6346/c β-lactamase for benzylpenicillin is about six times lower than 749/c, but because of a similar variation in K_m the so-called physiological efficiency of the two enzymes, that is the protection against β-lactams conferred to the cells, is comparable.

The structural gene for the 749 β-lactamase has been cloned and sequenced (Brammar *et al.* 1980, Neugebauer *et al.* 1981). The deduced protein sequence is identical to the amino acid sequence published by Ambler & Meadway (1969) except for the leader sequence, which is absent in the latter. The β-lactamase is in fact synthesized as a precursor protein with an amino

terminal signal peptide which is shed during the maturation and secretion of the protein (Neugebauer *et al.* 1981, Nielsen *et al.* 1981). Two forms of the enzyme are found in the cell supernatant, called exo-large and exo-small, differing by eight amino acid residues. The M_r of the smaller form, as calculated from the amino acid composition is 28500 and its isoelectric point is 5.0 (Thatcher 1975).

The amino acid sequence of the 6346 β-lactamase is known to differ from that of 749 at least at four sites. The known differences are reported in Table 1.3.

TABLE 1.3 Amino acid differences between 749/c and 6346/c β-lactamases[a]

POSITION[b]	749/c	6346/c
191	Arg	Gln
287	Met	Val
293	Asn	Glu
294	Gly	Ser

a Thatcher (1975)

b Numbering consistent with Ambler (1980)

The β-lactamases of *B.licheniformis* have been assigned to Class A (Ambler 1980). Within this class the similarity is higher with the other Gram positive enzymes: 749 is 48% similar to the *B.cereus* β-lactamase I and 37% to the *St.aureus* PC1 enzyme. The similarity is somewhat lower but still highly significant with the Gram negative Class A β-lactamases.

Crystallography of the 749 enzyme has been in progress for some years (Dideberg *et al.* 1985) and more recent reports have announced the collection of data at 2 Angstrom resolution (Knox *et al.* 1987).

1.8 OVERCOMING RESISTANCE

Because of the continually growing problem of resistance, mainly connected with the spread of β-lactamases, there has been an enormous development in the field of β-lactam antibiotics.

A great impulse to the field was given by the isolation of the two β-lactam nuclei 6-aminopenicillanic acid and 7-aminocephalosporanic acid, from which many semisynthetic antibiotics with improved stability to β-lactamases were derived.

Other β-lactam compounds have been found that exhibit very weak antibacterial activity, but act as inhibitors of many β-lactamases, thus protecting other more active compounds that are susceptible to the enzyme. Clavulanic acid mixed with amoxycillin is already marketed under the name Augmentin and has proven to be active against β-lactamase producing strains of pathogens like *Haemophilus influenzae* and *Neisseria gonorrhoeae* (Hunter *et al.* 1980). The semisynthetic inhibitor sulbactam used in conjunction with ampicillin has also shown synergistic activity against resistant strains, including *Bacterioides fragilis* (Campoli-Richards & Brogden 1987).

Besides fungi, other organisms have been discovered to produce β-lactam compounds with antibiotic activity. *Actinomycetes* produce hydrophilic cephalosporins like cephamycins, nocardicins and carbapenems, which present exceptional stability to hydrolysis. Monocyclic β-lactams, named monobactams, are produced by bacteria such as *Gluconobacter, Chromobacter violaceum* and *Pseudomonas acidophila* (Sykes *et al.* 1981). Because of their structure, monobactams lend themselves to total synthesis for the development of new drugs (for example Sykes and Bonner 1984).

Despite all the efforts spent in the search and development of new antibiotics, there is no certainty that any particular drug of this kind will retain its clinical usefulness indefinitely and although many of the new β-lactams are actually poor substrates of β-lactamases, their antibiotic efficiency is also reduced.

1.9 THE AIM OF THIS WORK

The elucidation of the detailed mechanism of the hydrolysis of β-lactam antibiotics performed by β-lactamases and a deeper understanding of the relationship between structure and specificity of these enzymes are now of paramount importance for the rationalization of antibiotic design.

The two types of β-lactamases produced by *B.licheniformis* are two naturally occurring and biologically significant mutants of the enzyme that seem to differ mainly in their specificity of action. The sequence information available pointed to the presence of very few amino acid differences. In addition *in vivo* recombinants between the two genes give rise to mutant proteins with electrophoretic mobilities intermediate between the two parental enzymes, but the enzymological properties of all the mutants always fall back to those of either of the two types, 749 and 6346 (Dubnau and Pollock 1965). The differences in specificity seem therefore due to either one or to a small number of closely linked amino acid substitutions, making the β-lactamases of *B.licheniformis* a simple model for the study of structure–function relationships.

The work presented here is aimed to the identification of the amino acid (or amino acids) determining the activity profile of the *B.licheniformis* β-lactamases.

CHAPTER 2

SUBCLONING

2.1 CLONING IN λNM1149

The DNA from the constitutive β-lactamase-producing strain of *B.licheniformis* 6346/c was isolated, restricted with *Eco*RI and the obtained fragments separated on a 1% agarose gel. The DNA was then transferred onto nitrocellulose by the method of Southern and probed with λpen; this bacteriophage carries a 4.2Kb *Eco*RI fragment containing the penicillinase gene from *B.licheniformis* 749/c (Brammar *et al.* 1980). A single *Eco*RI fragment of size estimated at about 4.7Kb was found to cross-hybridize with the λpen DNA probe (Figure 2.1), thus confirming that the two genes are highly homologous.

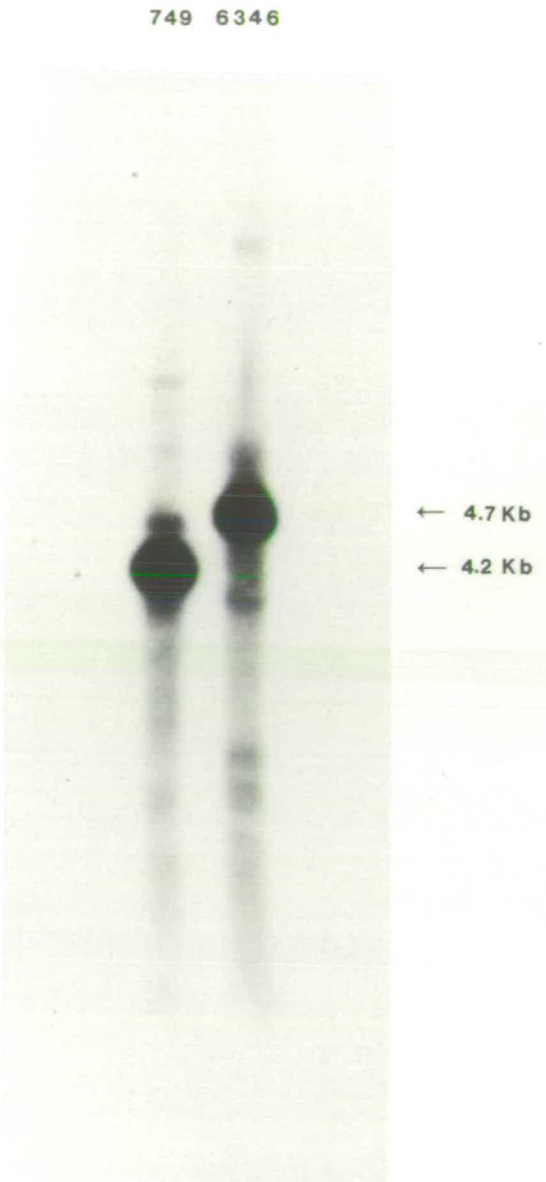
*Eco*RI treated DNA of *B.licheniformis* 6346/c was ligated with λNM1149 restricted with the same endonuclease. This λ vector offers a very convenient way of selecting recombinants, which give clear plaques, while the plaque morphology of the parental phage is turbid. Of those obtained after tranfection of the ligation mixture, 350 clear plaques were replica plated for further analysis. Three recombinants were found to be β-lactamase producing by staining the replica plate with a solution of nitrocefin; plaques of bacteriophages carrying a penicillinase gene are easily recognized because of the pink-red halo developing around them when this substrate is hydrolysed by the enzyme. One of the corresponding plaques on the master plate was picked and purified. The DNA of this recombinant was analysed by restriction and agarose gel electrophoresis. The size of its insert , about 4.7Kb, corresponds to that of the band of *Eco*RI digested DNA from *B.licheniformis* which hybridises with the λpen probe (Figure 2.2 and 2.3).

2.2 SUBCLONING INTO pACYC184

For ease of handling, the 4.7Kb *Eco*RI insert was transferred from λbla into pACYC184; the vector was cut at its single *Eco*RI site and ligated with λbla treated with the same enzyme. The resulting $Cm^sTet^rAmp^r$ plasmid, called pACYCbla, cross-hybridises with λpen (Figure 2.3)

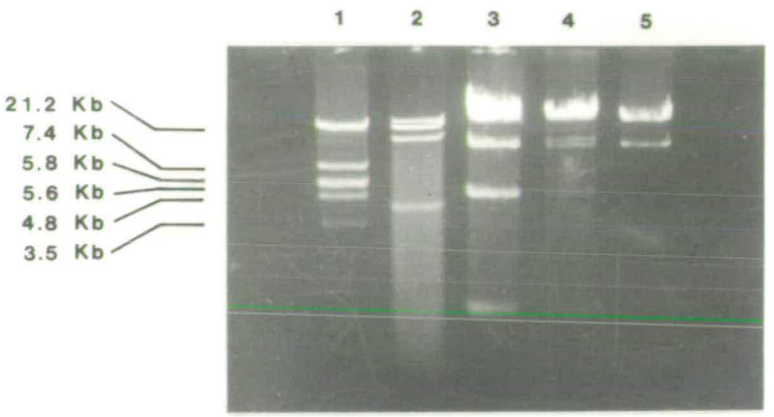
pACYCbla was analysed by restriction mapping (Figure 2.4) and successively used for the shotgunning of its insert into M13mp18 for sequencing. From the combined information given by the restriction analysis of the plasmid

FIGURE 2.1 Probing of chromosomal DNA from *B.licheniformis* 6346/c and 749/c.



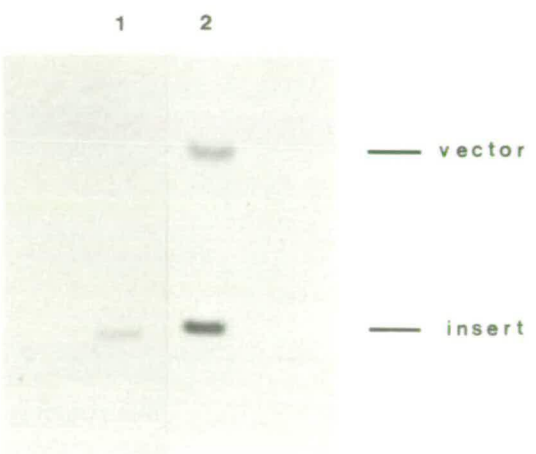
The genomic DNA was digested with EcoRI and probed with λpen.

FIGURE 2.2 Restriction analysis of λ1149 Amp^R recombinants.



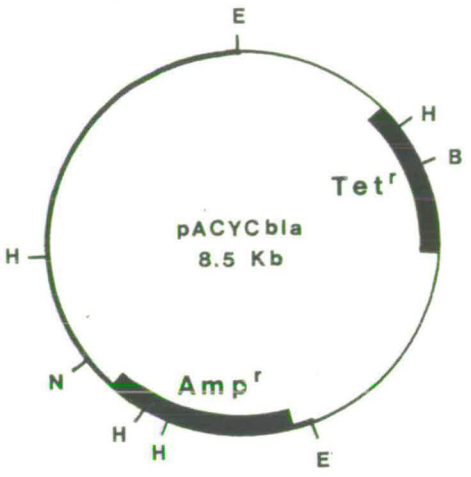
All the DNAs used for this analysis were restricted with EcoRI. In track 1 λcI857 fragments were used as size markers. In track 2 λpen, tracks 3 and 4 two Amp^R λ1149 recombinants, track 5 λ1149. The recombinant phage analysed in track 4 contains one single EcoRI insert of approximately 4.7Kb containing the 6346 bla gene and was used for further subcloning.

FIGURE 2.3 Probing of cloned *bla*.



λ1149 Amp^R recombinant (track 2) and pACYCbla (track 1) DNAs were restricted with EcoRI and probed with λpen.

FIGURE 2.4 Restriction map of pACYCbla



Restriction sites are denoted as follows: E, EcoRI; B, BamHI; H, HindIII; N, NaeI.

and the sequencing of its insert (Chapter 3), it is now known that the β-lactamase is located within the 1.6Kb *Eco*RI–*Nae*I fragment and the direction of transcription is from the *Eco*RI to the *Nae*I site.

2.3 THE MUTAGENESIS VECTOR

2.3.1 Subcloning in M13mp18

For mutagenesis it is convenient to have the gene of choice in a vector that produces single stranded DNA, such as the M13 vectors. Before starting the SDM experiments the β-lactamase gene from *B.licheniformis* 6346/c was excised from pACYCbla by hydrolysis with *Eco*RI and *Nae*I and ligated with M13mp18 vector cut at the *Sma*I and *Eco*RI sites of its polylinker. After transformation of an appropriate host strain (*E.coli* NM522) β-lactamase producing plaques of recombinant bacteriophage could be detected by staining with nitrocefin, but, despite numerous attempts, it was impossible to mantain the insert in its intact form for more then a few rounds of replication. The instability of this DNA fragment was shown not to be influenced by the *recA* dependent pathways of recombination; in fact repeating the experiment with the *recA*$^-$ host strain JM109 did not improve stability of the fragment. It was therefore decided to abandon

the M13 system and look for an alternative vector in which the advantage of double stranded replication, that would increase the stability of the gene, would be combined with the production of single stranded template production.

2.3.2 The phagemid vectors

A new family of plasmids, the pEMBL vectors, was developed a few years ago (Dente *et al.* 1983) to reduce the need for repeated subcloning of genes between the single stranded vectors suitable for SDM and sequencing and the double stranded vectors used for maintenance and further analysis of gene and gene product. These are pUC derived plasmids in which part of the filamentous phage f1 genome that includes the origin of replication has been inserted. When superinfected with f1 helper phage, cells carrying the pEMBL plasmids produce virions containing either the helper phage or the single stranded plasmid DNA.

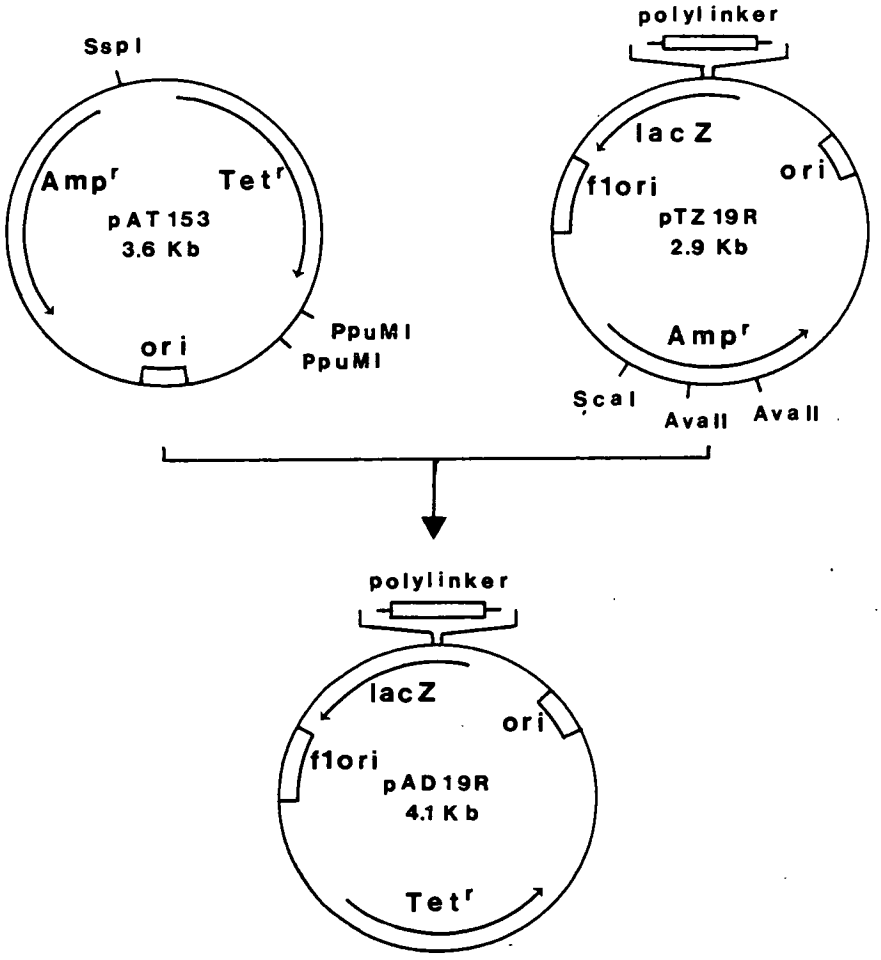
Vectors of the pEMBL family and other chimeras which possess an origin for double stranded replication as well as one for single stranded replication are also called phagemids.

The pTZ series of phagemids (Mead *et al.* 1986) has been constructed on similar lines, by cloning the origin of replication of f1 downstream the β-galactosidase gene of pUC. Depending on the orientation of the f1 origin of replication either of the two strands of the plasmid is packaged: in the U orientation the strand which hybridises to the universal sequencing primer is produced; when the origin is inserted in the opposite R direction, the single stranded DNA produced can be sequenced using the reverse sequencing primer. Other advantageous features of these phagemids are the simple and quick blue to white colour assay for recognition of recombinants constructed by introducing an insert in the polylinker region and the possibility of synthesising mRNA from a T7 promoter also cloned in the β-galactosidase gene.

2.3.3 Construction of pAD19R

The pTZ phagemid, which in my hands seemed to yield more single stranded DNA than the pEMBL vectors when superinfected with the helper phage M13KO7 (Mead *et al.* 1986), was chosen for further work. To create a vector suitable for cloning β-lactamases the ampicillin resistance gene of pTZ was inactivated by deletion and another selectable marker introduced in its stead (Figure 2.5).

FIGURE 2.5 Construction of pAD19R

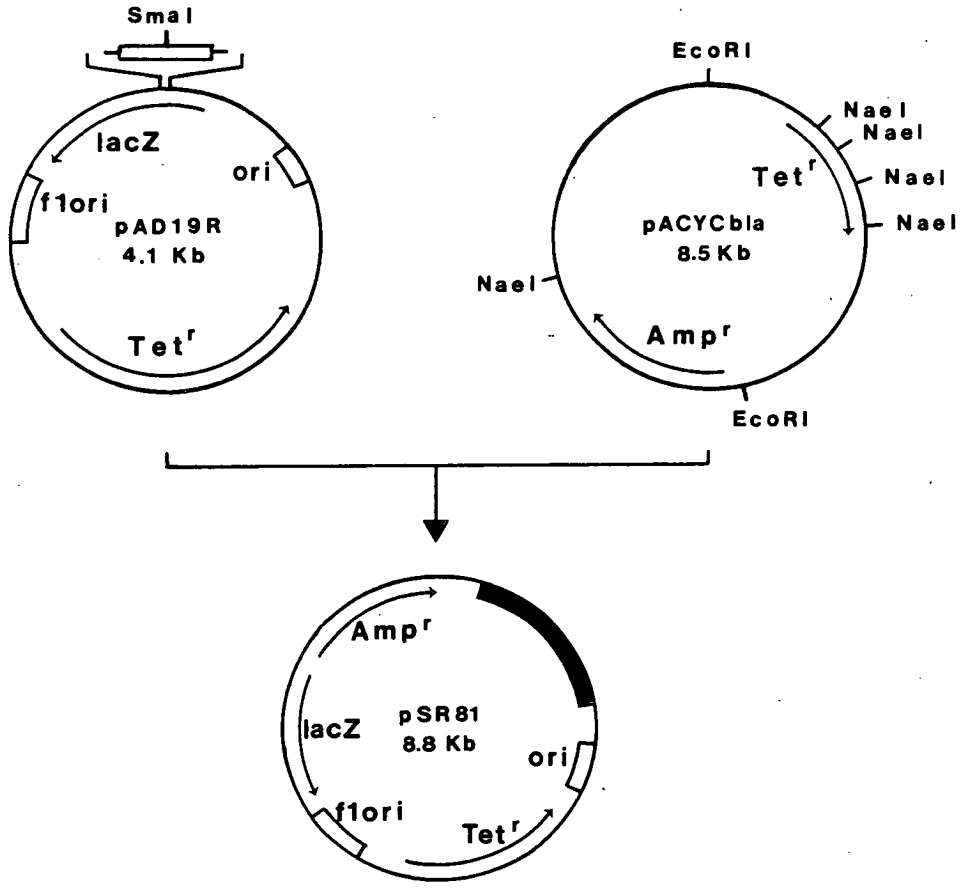


The Amp^r gene of pTZ19R was inactivated by deletion of the ScaI-AvaII and AvaII-AvaII fragments and substituted by the Tet^r gene excised from pAT153 with SspI and PpuMI. In the resulting pAD19R plasmid the Tet^r gene is oriented in the same direction as lacZ.

pAT153, a plasmid derived from pBR322 by deletion of a 705bp section (Twigg and Sherratt 1980), was treated with *Ssp*I and *Ppu*MI and the resulting 1.63Kb fragment comprising the tetracycline resistance gene was separated on agarose gel, extracted, purified and finally ligated with pTZ19R DNA hydrolysed by *Ava*II and *Sca*I endonucleases. In pAT153 the *Ppu*MI target site (5'GGTCCT3') upon hydrolysis gives ends compatible to the *Ava*II site on pTZ19R (5'GGTCC3'), while both *Ssp*I and *Sca*]I are blunt-end cutters. In the resulting pAD19R

plasmid the deleted 340bp in the central part of the Amp^R gene were replaced by the fragment carrying *tet*. The direction of transcription of *tet* in the new plasmid is the same as that of *lacZ*. The calculated length of pAD19R (4165bp) is confirmed by the estimate (4.0Kb σ=0.21) obtained from the analysis of the pTZ19R/pAD19R heteroduplex (Figure 2.7a) (spreadings and microscopy of heteroduplexes carried out by Miss Pamela Beattie).

FIGURE 2.6 Construction of pSR81



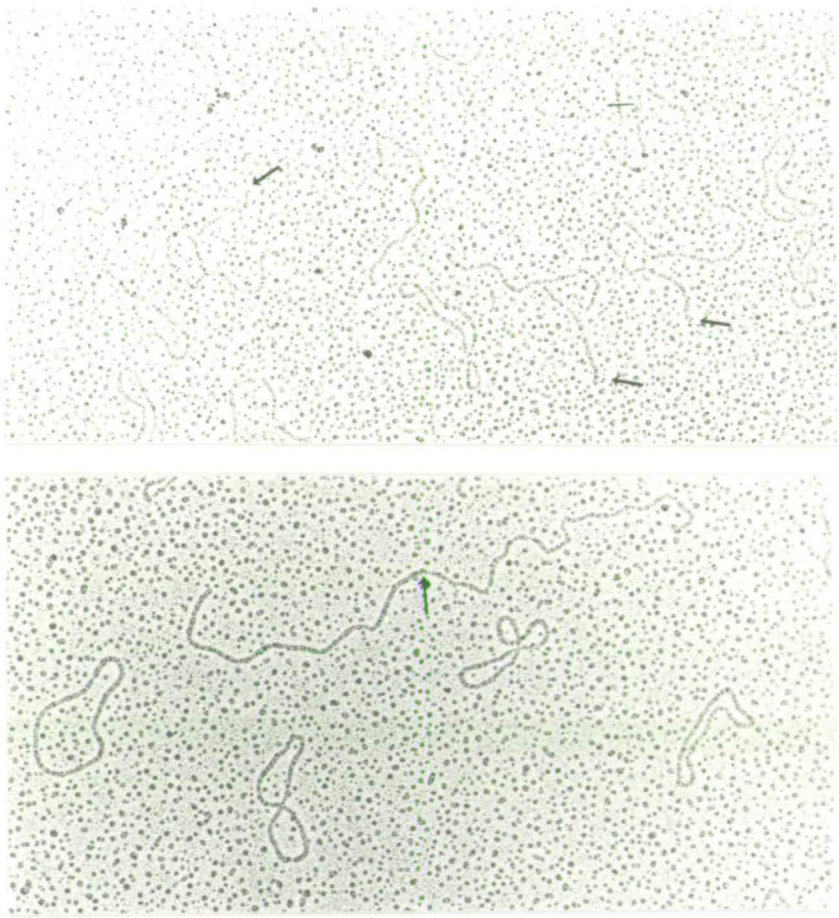
The bla gene of B.licheniformis 6346/c was subcloned from pACYCbla to pAD19R. The 1.6Kb EcoRI-NaeI fragment containing the gene was end-repaired and inserted into the SmaI site of pAD19R. Another fragment of pACYCbla which co-cloned with bla is represented by a black box (see text).

2.3.4 Construction of pSR81

The β-lactamase gene of *B.licheniformis* 6346/c was transferred from pACYCbla onto pAD19R by restricting the *bla* carrying plasmid with *Eco*RI and *Nae*I, filling in the ends and ligating the resulting complex mixture of fragments with *Sma*I treated pAD19R (Figure 2.6). $Amp^R Tet^R$ colonies were selected and their plasmid analysed by restriction. All the isolated plasmids were bigger than the expected pAD19R with the hoped for insertion of the 1.6Kb *Eco*RI-*Nae*I fragment carrying the β-lactamase gene. One of them (pSR81) was chosen for further analysis. Sequencing of pSR81 with the reverse sequencing primer confirmed the presence of the 1.6Kb fragment in the proximal part of the insert. The direction of translation of β-lactamase gene in this construct is opposite to that of *lacZ*. Plasmid sequencing with the universal sequencing primer showed that the distal part of the insert was probably the other 3.1Kb *Eco*RI-*Nae*I fragment, indicating that possibly the whole *Eco*RI fragment, uncut by *Nae*I, had in fact been cloned. The total size of the insert is estimated at about 4.7Kb (Figure 2.7b).

Because the presence of this extra 3.1Kb insert did not in any way hinder the production of single stranded template from pSR81 and did not significantly influence the production of β-lactamase, it was decided to use this construct for the SDM experiments.

FIGURE 2.7 pTZ19R/pAD19R (A) and pAD19R/pSR81 (B) heteroduplexes.



pAD19R and pTZ19R were linearized with SmaI, which cuts once in the polylinker. In pSR81 the SmaI site was destroyed by the insertion of the 6346 β-lactamase gene, so XmaI which also cuts a single time in the polylinker was used instead. The standards used for DNA length estimation were: single stranded M13 (6407 bases) and double stranded pAT153 (3657 bases).

In **A** three heteroduplex molecules are visible. Their left hand arm is indicated by an arrow. Length of left hand double stranded arm: 0.88Kb (σ=0.16); right hand double stranded arm: 1.4Kb (σ=0.05); short single stranded loop: 0.47Kb (σ=0.15); long double stranded loop: 1.72Kb (σ=0.22).

In **B** a single pAD19R/pSR81 heteroduplex is visible. The double stranded part, comprising the whole length of the pAD19R vector, is 4.03Kb long (σ=0.19). The single stranded overhanging insert arm starts at the arrow and measures 4.74Kb (σ=0.27).

Estimated lengths of the plasmids are: pTZ19R 2.73Kb (σ=0.16), pAD19R 4.0Kb (σ=0.21), pSR81 8.77Kb (σ=0.27).

CHAPTER 3

SEQUENCING AND SEQUENCE ANALYSIS OF *Bacillus licheniformis* BETA-LACTAMASE

3.1 INTRODUCTION

The dideoxy chain termination method (Sanger *et al.* 1977, Bankier and Barrell 1983) has been used for the sequencing of the *B.licheniformis* 6346/c β-lactamase. A short description of the procedure is given in Section 6.12.

With minor differences the method can be used both on double stranded and single stranded templates, but because of the practical advantages given by the latter, it is widespread practice to start a sequencing project by introducing the DNA fragment of interest into a single stranded vector like M13.

3.2 SEQUENCING STRATEGY

Two main avenues can be followed when planning a sequencing project: in the first the fragment is introduced as a whole into M13 and the sequence read by "walking" along the fragment with specially synthesized oligonucleotides. This method is generally practicable when the length of DNA to be cloned is limited. Long fragments are in fact known to suffer deletions when carried by single stranded vectors and M13 vectors do not grow well with large inserts. Mutants shortened by deletions tend to quickly outgrow the full length phage, making the maintenance of the intact clone difficult.

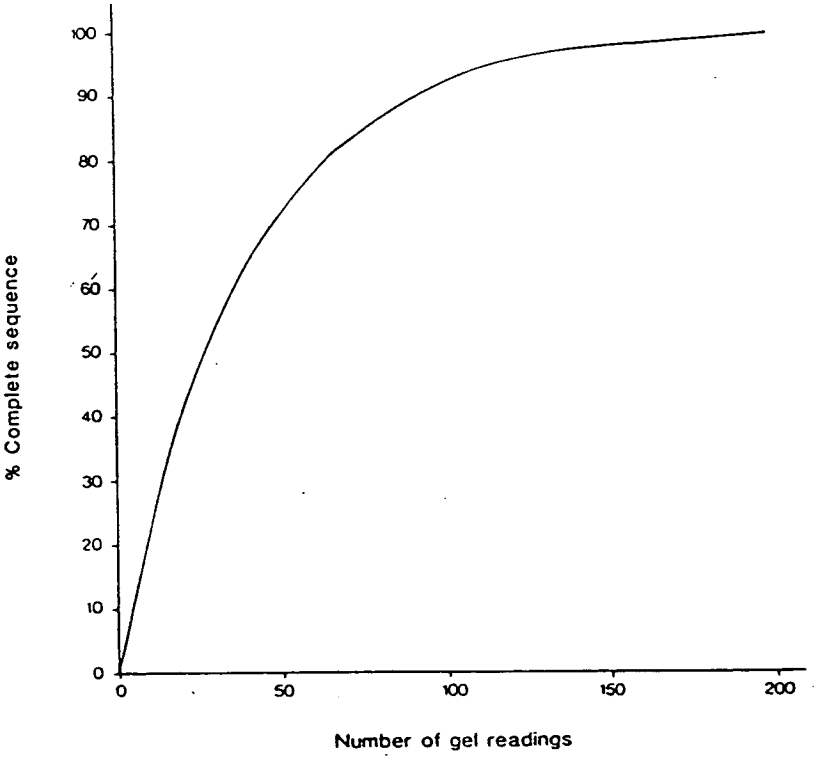
Another point to be borne in mind when considering this approach is the high cost of the purpose-made oligonucleotides: in general to allow at least 50 bases overlap between readings, one primer must be used every 250-300 nucleotides.

In the second approach random segments of the DNA to be sequenced are generated by any of the following methods:

1. nested deletions of the cloned fragment generated with exonuclease III (Henikoff 1984)
2. cleavage with restriction enzymes
3. physical shearing

Method 1 involves the cloning of the DNA fragment in a single stranded vector, which obviously would present the same problems discussed above. In the other two methods shorter fragments are generated prior to cloning, thus avoiding the problems connected with long fragments. Because of the large size (4.7Kb) of

FIGURE 3.1 Rate of sequence accumulation



The percentage sequence accumulation is plotted against the theoretical number of gel readings required to determine the sequence of a 5Kb fragment on both strands, assuming an average of 250bp per clone (from Bankier & Barrell 1983).

the DNA fragment to be sequenced during the course of this work, the choice of sequencing strategy had been narrowed down to these two methods.

The distribution of target sites of four-base restriction endonucleases is known to be not completely random, so that cutting a DNA fragment with these enzymes often generates very small segments, which clone well into M13 and therefore give redundancy of information in that region, and much bigger fragments that sometimes can prove very difficult to retrieve. The sonication procedure followed by size fractionation of the fragments on agarose gel as described by Deininger (1983) seems to give the best distribution of random fragments of a predetermined size range.

If an average of 250 bases are read for each clone, the theoretical number of gel readings (clones) required to determine the sequence of a 5Kb fragment on both strands is given by the plot of Bankier and Barrell (1983) (Figure 3.1). The rate of sequence accumulation is at the beginning proportional

to the number of gel readings, but when 90-95% of the sequence is completed, further sequence data accumulation results in increasing redundancy of information. Hence the acquisition of the remaining 5% requires the sequencing of a number of clones equal to that needed to reach 95% completion. Once this plateau is attained, the employment of more direct methods is more rewarding by far.

3.3 SEQUENCING THE β-LACTAMASE GENE OF *B.licheniformis* 6346/c

In Section 6.12 I will describe in detail the procedure used for the sequencing of the β-lactamase gene of *B.licheniformis* 6346/c. In brief the 4.7Kb DNA fragment comprising the gene was excised from pACYCbla using *Eco*RI and purified after separation from the vector band on a 0.7% low electroendosmosis agarose gel. The fragment was then circularised by self-ligation in order to increase the probability of cloning the ends and sonicated with two bursts of 30 seconds. The single stranded ends were repaired with the Klenow fragment of polymerase I and the sheared DNA was fractionated on a 1% low electroendosmosis agarose gel. Fragments between 300 and 600 bp were eluted from the gel, purified and ligated with *Sma*I treated M13mp18. Single clear plaques obtained from the transformation of the ligation mixture were picked and sequenced by the Sanger method (see Section 6.12.2).

One hundred and forty four clones were used to compile the sequence nearly to completion. Zones of band compression were solved by using sequencing reaction mixtures in which dITP substituted dGTP (Mills & Kramer 1979). Beyond the *Nae*I restriction site the sequence is largely completed except for a few bases in GC rich regions which have been read only on one strand. The sequence of the DNA fragment is presented in Figure 3.2.

3.4 SEQUENCE ANALYSIS

All the analysis of the sequence was done with the help of the Sequence Analysis Software Package of the University of Wisconsin Genetics Computer Group on a VAX computer (Devereux *et al.* 1984).

FIGURE 3.2 Sequence of *Bacillus licheniformis* 6346/c DNA fragment containing the *bla* gene

FIGURE 3.2 (continued)

```
      Ser Asp Asn Thr Ala Gln Asn Leu Ile  Leu Lys Gln Ile  Gly Gly Pro Glu Ser Leu Lys
      AGTGACAATACGGCACAGAACCTCATTCTTAAACAAATTGGCGGACCTGAAAGTTTGAAA
  663 -------+---------+---------+---------+---------+---------+--  722
      TCACTGTTATGCCGTGTCTTGGAGTAAGAATTTGTTTAACCGCCTGGACTTTCAAACTTT

      Lys Glu Leu Arg Lys Ile  Gly Asp Glu Val  Thr Asn Pro Glu Arg Phe Glu Pro Glu Leu
      AAGGAACTGAGGAAGATTGGTGATGAGGTTACAAATCCTGAACGATTCGAACCGGAGTTA
  723 -------+---------+---------+---------+---------+---------+--  782
      TTCCTTGACTCCTTCTAACCACTACTCCAATGTTTAGGACTTGCTAAGCTTGGCCTCAAT

      Asn Glu Val  Asn Pro Gly Glu Thr Gln Asp Thr Ser Thr Ala Arg Ala Leu Ala Thr Ser
      AATGAAGTGAATCCGGGAGAAACTCAGGATACCAGTACAGCAAGAGCACTTGCGACAAGC
  783 -------+---------+---------+---------+---------+---------+--  842
      TTACTTCACTTAGGCCCTCTTTGAGTCCTATGGTCATGTCGTTCTCGTGAACGCTGTTCG

      Leu Gln Ala Phe Ala Leu Glu Asp Lys Leu Pro Ser Glu Lys Arg Glu Leu Leu Ile  Asp
      CTTCAAGCTTTTGCTCTTGAAGATAAACTTCCAAGTGAAAAACGCGAGCTTTTAATCGAT
  843 -------+---------+---------+---------+---------+---------+--  902
      GAAGTTCGAAAACGAGAACTTCTATTTGAAGGTTCACTTTTTGCGCTCGAAAATTAGCTA

      Trp Met Lys Arg Asn Thr Thr Gly Asp Ala Leu Ile  Arg Ala Gly Val Pro Glu Gly Trp
      TGGATGAAACGAAATACCACCGGGGACGCCTTAATCCGCGCCGGTGTGCCGGAAGGCTGG
  903 -------+---------+---------+---------+---------+---------+--  962
      ACCTACTTTGCTTTATGGTGGCCCCTGCGGAATTAGGCGCGGCCACACGGCCTTCCGACC

      Glu Val Ala Asp Lys Thr Gly Ala Gly Ser Tyr Gly Thr Arg Asn Asp Ile  Ala Ile  Ile
      GAAGTGGCTGATAAAACTGGAGCGGGATCATATGGAACCCGGAACGACATTGCCATCATT
  963 -------+---------+---------+---------+---------+---------+--  1022
      CTTCACCGACTATTTTGACCTCGCCCTAGTATACCTTGGGCCTTGCTGTAACGGTAGTAA

      Trp Pro Pro Lys Gly Asp Pro Val Val Leu Ala Val Leu Ser Ser Arg Asp Lys Lys Asp
      TGGCCGCCAAAAGGAGATCCTGTAGTTCTCGCAGTATTATCCAGCCGGGATAAAAAGGAT
 1023 -------+---------+---------+---------+---------+---------+--  1082
      ACCGGCGGTTTTCCTCTAGGACATCAAGAGCGTCATAATAGGTCGGCCCTATTTTTCCTA

      Ala Lys Tyr Asp Asp Lys Leu Ile  Ala Glu Ala Thr Lys Val Val Val Lys Ala Leu Asn
      GCCAAGTATGATGATAAGCTTATTGCAGAGGCAACAAAAGTGGTAGTGAAAGCCTTAAAC
 1083 -------+---------+---------+---------+---------+---------+--  1142
      CGGTTCATACTACTATTCGAATAACGTCTCCGTTGTTTTCACCATCACTTTCGGAATTTG

      Met Glu Ser Lys End
      ATGGAAAGCAAATAAAAAAACATTGCAATACATTTTGTTAAAACATCGTCTTACATAAAG
 1143 -------+---------+---------+---------+---------+---------+--  1202
      TACCTTTCGTTTATTTTTTTGTAACGTTATGTAAAACAATTTTGTAGCAGAATGTATTTC

      TCACTTGGTGATCAAGCTCATATCATTGTCTGGCAATGGTGTGGGCTTTTTTGTTTTCTC
 1203 -------+---------+---------+---------+---------+---------+--  1262
      AGTGAACCACTAGTTCGAGTATAGTAACAGACCGTTACCACACCCGAAAAAACAAAAGAG

      TCTTTAAAGATAATGTGAAGAAAAACGGGAGAATCGGTCTGCGGGAAACGACCGGGTTTT
 1263 -------+---------+---------+---------+---------+---------+--  1322
      AGAAATTTCTATTACACTTCTTTTTGCCCTCTTAGCCAGACGCCCTTTGCTGGCCCAAAA
```

FIGURE 3.2 (continued)

```
      TGTCGAAATCATAGGCGATTGGATTGATTTGCGACAAAATTCGACATATATACTGGCGGA
1323  -------+---------+---------+---------+---------+---------+--  1382
      ACAGCTTTAGTATCCGCTAACCTAACTAAACGCTGTTTTAAGCTGTATATATGACCGCCT

      GTGACTTGATCGACCATGTATCGCGTTTCTTCTCCTATTTGCAATACTTTACAGAACGTT
1383  -------+---------+---------+---------+---------+---------+--  1442
      CACTGAACTAGCTGGTACATAGCGCAAAGAAGAGGATAAACGTTATGAAATGTCTTGCAA

      AATAAATTCTGCCTATAATAGAACCCGATCAATCATTTGTATAGAGAGGGGTAATACGAA
1443  -------+---------+---------+---------+---------+---------+--  1502
      TTATTTAAGACGGATATTATCTTGGGCTAGTTAGTAAACATATCTCTCCCCATTATGCTT

      TGACACATGAAGGCACCGCTGGAGGAATGGATGAAAAAACTGAGCGAGGAAAGCCTCAAG
1503  -------+---------+---------+---------+---------+---------+--  1562
      ACTGTGTACTTCCGTGGCGACCTCCTTACCTACTTTTTTGACTCGCTCCTTTCGGAGTTC

      GACAATACGTTTGACCGCCGCCGCTTTATTCAAGGGGCCGGCAAAATAGCCGGGTTTCGC
1563  -------+---------+---------+---------+---------+---------+--  1622
      CTGTTATGCAAACTGGCGGCGGCGAAATAAGTTCCCCGGCCGTTTTATCGGCCCAAAGCG

      TCGGACTTGCGATCGCGCAATCGATGGGGGCAATGGAAGTCAATGCAGCACCGAGGTTCT
1623  -------+---------+---------+---------+---------+---------+--  1682
      AGCCTGAACGCTAGCGCGTTAGCTACCCCCGTTACCTTCAGTTACGTCGTGGCTCCAAGA

      CCGAATATCCGTTTACACTTGGCGTTGCATCGGGAGATCCGCTTTCTGACAGACGTCGTA
1683  -------+---------+---------+---------+---------+---------+--  1742
      GGCTTATAGGCAAATGTGAACCGCAACGTAGCCCTCTAGGCGAAAGACTGTCTGCAGCAT

      TTGTGGACAAGGCTGGCGCCCGATCCGCTAAACGGGGGCGGGATGCCAAATGAAGCTGTA
1743  -------+---------+---------+---------+---------+---------+--  1802
      AACACCTGTTCCGACCGCGGGCTAGGCGATTTGCCCCCGCCCTACGGTTTACTTCGACAT

      TCCGTGAAATGGGAGCTCGCAGAAGACGAACGCTTCCGCCGTGTCGTCAAACGGGGAACC
1803  -------+---------+---------+---------+---------+---------+--  1862
      AGGCACTTTACCCTCGAGCGTCTTCTGCTTGCGAAGGCGGCACAGCAGTTTGCCCCTTGG

      GAAAAAGCAACACCTCACCTTGCACATTCGGTACATGCCGAGGTATCCGGGCTGAAACCG
1863  -------+---------+---------+---------+---------+---------+--  1922
      CTTTTTCGTTGTGGAGTGGAACGTGTAAGCCATGTACGGCTCCATAGGCCCGACTTTGGC

      AATCATGTATACTATTACCGCTTTAAATGCGGCAACCAGCTGAGTCCTGTCGGCAAGACA
1923  -------+---------+---------+---------+---------+---------+--  1982
      TTAGTACATATGATAATGGCGAAATTTACGCCGTTGGTCGACTCAGGACAGCCGTTCTGT

      AAGACGCTTCCGGCGCCCGGGGCAGATGTTGCTAAATTCACATTCGCTTTTGCTTCATGC
1983  -------+---------+---------+---------+---------+---------+--  2042
      TTCTGCGAAGGCCGCGGGCCCCGTCTACAACGATTTAAGTGTAAGCGAAAACGAAGTACG

      CAGCAGTACGAACACGGCTATTATACCGCCTATCAGCATATGGCAAAGGAAAAGCTCGAT
2043  -------+---------+---------+---------+---------+---------+--  2102
      GTCGTCATGCTTGTGCCGATAATATGGCGGATAGTCGTATACCGTTTCCTTTTCGAGCTA
```

FIGURE 3.2 (continued)

```
      CTTGTTTTTCATCTCGGCGACTATATTTATGAGTACGGTCCGAATGAATATGTTTCGAAA
2103  -------+---------+---------+---------+---------+---------+--  2162
      GAACAAAAAGTAGAGCCGCTGATATAAATACTCATGCCAGGCTTACTTATACAAAGCTTT

      ACAGGAAATGTCAGAACACACAGCGGTCCTGAAATCATGTCACTGCTCGATTACCGAAAC
2163  -------+---------+---------+---------+---------+---------+--  2222
      TGTCCTTTACAGTCTTGTGTGTCGCCAGGACTTTAGTACAGTGACGAGCTAATGGCTTTG

      CGTCATGCCCAATACCGTTCAGATGCAAACCTGAAAGCTGCGCATGCCGCCTTCCCGTGG
2223  -------+---------+---------+---------+---------+---------+--  2282
      GCAGTACGGGTTATGGCAAGTCTACGTTTGGACTTTCGACGCGTACGGCGGAAGGGCACC

      GTTGTCACATGGGACGACCATGAAGTGGAGAACAACTATGCGAATGTCATCCCGGAAAAA
2283  -------+---------+---------+---------+---------+---------+--  2342
      CAACAGTGTACCCTGCTGGTACTTCACCTCTTGTTGATACGCTTACAGTAGGGCCTTTTT

      GGCCAGTCAGTTGAAGCGTTTATTAAACGGCGCGCCGCATACCAAGCTTATTACGAGCAT
2343  -------+---------+---------+---------+---------+---------+--  2402
      CCGGTCAGTCAACTTCGCAAATAATTTGCCGCGCGGCGTATGGTTCGAATAATGCTCGTA

      ATGCCGCTCCGCCGCCTAATCCTTGCCGAACGGTCCTGATATGCAATTGTACCGGAATTT
2403  -------+---------+---------+---------+---------+---------+--  2462
      TACGGCGAGGCGGCGGATTAGGAACGGCTTGCCAGGACTATACGTTAACATGGCCTTAAA

      TTCCTACGGCTAATTTAGCTGACATTAGCGTGCTGGATACCGTCAGTATCGCGATGACCA
2463  -------+---------+---------+---------+---------+---------+--  2522
      AAGGATGCCGATTAAATCGACTGTAATCGCACGACCTATGGCAGTCATAGCGCTACTGGT

      GGCTAACGGCGATGGCAATAAGCCGCCTTCTGATGAATCGAATGATCCGAAGCGGACGCT
2523  -------+---------+---------+---------+---------+---------+--  2582
      CCGATTGCCGCTACCGTTATTCGGCGGAAGACTACTTAGCTTACTAGGCTTCGCCTGCGA

      GCTTGGAGCGGAGCAGGAGGCTTGGCTGTTTGACAATCTGAGTCGCTCCGAGGCGCACTG
2583  -------+---------+---------+---------+---------+---------+--  2642
      CGAACCTCGCCTCGTCCTCCGAACCGACAAACTGTTAGACTCAGCGAGGCTCCGCGTGAC

      GAACATTATCGCACAGCAGATTTTCTTTGCGCAGTGGAACTTTGGGACAAGCGCGGCGCC
2643  -------+---------+---------+---------+---------+---------+--  2702
      CTTGTAATAGCGTGTCGTCTAAAAGAAACGCGTCACCTTGAAACCCTGTTCGCGCCGCGG

      GATTTACAGCATGGATTCGTGGGACGGATATCCCGCTCAGCGCAAGCGGGTGATCGACTT
2703  -------+---------+---------+---------+---------+---------+--  2762
      CTAAATGTCGTACCTAAGCACCCTGCCTATAGGGCGAGTCGCGTTCGCCCACTAGCTGAA

      CATCAAATCTCAAAAACTGAACAACATCGTCGTCCTTACCGGTGATGTGCATGCCAGCTG
2763  -------+---------+---------+---------+---------+---------+--  2822
      GTAGTTTAGAGTTTTTGACTTGTTGTAGCAGCAGGAATGGCCACTACACGTACGGTCGAC

      GGCAAATAATCTGCTCGTTGATTTTGACAATCCGAAATCGGACATCTTTGGCGTGGAGTT
2823  -------+---------+---------+---------+---------+---------+--  2882
      CCGTTTATTAGACGAGCAACTAAAACTGTTAGGCTTTAGCCTGTAGAAACCGCACCTCAA
```

FIGURE 3.2 (continued)

```
                CGTCGGGACGTCGATTACATCGGGAGGAAACGGCGCTGATAAAAGAGCGGATACGGATAA
2883  --------+---------+---------+---------+---------+---------+---------+--  2942
                GCAGCCCTGCAGCTAATGTAGCCCTCCTTTGCCGCGACTATTTTCTCGCCTATGCCTATT

                GATTTTATCGAACAATCCTCATATAAAGTTTTTCAATGATTACCGCGGCTATGTGCGCTG
2943  --------+---------+---------+---------+---------+---------+---------+--  3002
                CTAAAATAGCTTGTTAGGAGTATATTTCAAAAAGTTACTAATGGCGCCGATACACGCGAC

                TACGGTCACCCCGGAAGAATGGCGGACAGATTACCGCGTCGTCCCGTATGTGACAGAGCC
3003  --------+---------+---------+---------+---------+---------+---------+--  3062
                ATGCCAGTGGGGCCTTCTTACCGCCTGTCTAATGGCGCAGCAGGGCATACACTGTCTCGG

                GGGTGCGGCGGTTTCGACAAGAGCTTCCTATGTTTATCATAAAGACTACACAGGGCTGAA
3063  --------+---------+---------+---------+---------+---------+---------+--  3122
                CCCACGCCGCCAAAGCTGTTCTCGAAGGATACAAATAGTATTTCTGATGTGTCCCGACTT

                ACGGACCGCTTCCAACATTGTGCCTGGAGGCGTGAAGAAGTCAAACGAAGTAGAAGAAGA
3123  --------+---------+---------+---------+---------+---------+---------+--  3182
                TGCCTGGCGAAGGTTGTAACACGGACCTCCGCACTTCTTCAGTTTGCTTCATCTTCTTCT

                CCGTTTCCTCGCACATACAAGGGCCCATCAGAAGCAAGTGAAGCAAAAGGAGAAAAAAGT
3183  --------+---------+---------+---------+---------+---------+---------+--  3242
                GGCAAAGGAGCGTGTATGTTCCCGGGTAGTCTTCGTTCACTTCGTTTTCCTCTTTTTTCA

                AACCCAGTAATCAATGAAAGGATGACAGCAAATGCTTTCAAATATCGGTATTCCTGGTCT
3243  --------+---------+---------+---------+---------+---------+---------+--  3302
                TTGGGTCATTAGTTACTTTCCTACTGTCGTTTACGAAAGTTTATAGCCATAAGGACCAGA

                CATCTTGGTTCTGGTAATTAGCGCTGATCATTTTCGGGCCTTCAAAGCTCCCGGAAATCG
3303  --------+---------+---------+---------+---------+---------+---------+--  3362
                GTAGAACCAAGACCATTAATCGCGACTAGTAAAAGCCCGGAAGTTTCGAGGGCCTTTAGC
                                                    End Leu Glu Arg Phe Asp
                                    (EcoRI)
                GCCGGGCTTTTGGAAAAACGCTGACCGAATTCAGAAGCGGCTACCAAAGATGACGTTCTT
3363  --------+---------+---------+---------+---------+---------+---------+--  3422
                CGGCCCGAAAACCTTTTTGCGACTGGCTTAAGTCTTCGCCGATGGTTTCTACTGCAAGAA
                Ala Pro Ser Lys Ser Phe Arg Gln Gly Phe Glu Ser Ala Ala Val Leu Ser Ser Thr Arg

                TGCTTAAGTGAAGTGAATTTTAAAATCACCTCGCCATATTTGAGATGCAATTTTTTATCA
3423  --------+---------+---------+---------+---------+---------+---------+--  3482
                ACGAATTCACTTCACTTAAAATTTTAGTGGAGCGGTATAAACTCTACGTTAAAAAATAGT
                Gln Lys Leu Ser Thr Phe Lys Leu Ile Val Glu Gly Tyr Lys Leu His Leu Lys Lys Asp

                AGCGTTTTTAATACGGCAAAGTCGCAAGAAATCTCCATCTCTGTTTTTGCTTCCTTTGAT
3483  --------+---------+---------+---------+---------+---------+---------+--  3542
                TCGCAAAAATTATGCCGTTTCAGCGTTCTTTAGAGGTAGAGACAAAAACGAAGGAAACTA
                Leu Thr Lys Leu Val Ala Phe Asp Cys Ser Ile Glu Met Glu Thr Lys Ala Glu Lys Ser

                AAATACCACACGAGAGGATTAAACCAATAGACGATTTTGGACAGGCAAAGAAAATAGTTG
3543  --------+---------+---------+---------+---------+---------+---------+--  3602
                TTTATGGTGTGCTCTCCTAATTTGGTTATCTGCTAAAACCTGTCCGTTTCTTTTATCAAC
                Leu Tyr Trp Val Leu Pro Asn Phe Trp Tyr Val Ile Lys Ser Leu Cys Leu Phe Tyr Asn
```

FIGURE 3.2 (continued)

```
        ATGAGCATATCTTTTCGTTTACAATGGTACAGTTCATGAAGCAAAACACATTTCATCTCG
3603    -------+---------+---------+---------+---------+---------+--  3662
        TACTCGTATAGAAAAGCAAATGTTACCATGTCAAGTACTTCGTTTTGTGTAAAGTAGAGC
        Ile Leu Met Asp Lys Arg Lys Cys His Tyr Leu Glu His Leu Leu Val Cys Lys Met Glu

        TCGGCAGAAAACATTGAAATATCCTTTGGCAAGATAATATATGGACGGACAACCCCAAAT
3663    -------+---------+---------+---------+---------+---------+--  3722
        AGCCGTCTTTTGTAACTTTATAGGAAACCGTTCTATTATATACCTGCCTGTTGGGGTTTA
        Asp Ala Ser Phe Met Ser Ile Asp Lys Pro Leu Ile Ile Tyr Pro Arg Val Val Gly Phe

        GTTATGGGGGATTTTATCAATGGGGAACAGCCTAAAATCACTTTTTGATGAAAGCGTATT
3723    -------+---------+---------+---------+---------+---------+--  3782
        CAATACCCCCTAAAATAGTTACCCCTTGTCGGATTTTAGTGAAAAACTACTTTCGCATAA
        Thr Ile Pro Ser Lys Ile Leu Pro Ser Cys Gly Leu Ile Val Lys Gln His Phe Arg Ile

        TCCTCTTTACATGTACGAAAAAGGGTTAATAGCTCTTTGTTGTTAACCATTTGCAGGCTC
3783    -------+---------+---------+---------+---------+---------+--  3842
        AGGAGAAATGTACATGCTTTTTCCCAATTATCGAGAAACAACAATTGGTAAACGTCCGAG
        Glu Glu Lys Cys Thr Arg Phe Leu Thr Leu Leu Glu Lys Asn Asn Val Met Gln Leu Ser

        TTTTTTATTTTGCCGATTTTTCGATTGCTATAAAGAGTAGCGATAAGCATAACAGCAATG
3843    -------+---------+---------+---------+---------+---------+--  3902
        AAAAAATAAAACGGCTAAAAAGCTAACGATATTTCTCATCGCTATTCGTATTGTCGTTAC
        Lys Lys Ile Lys Gly Ile Lys Arg Asn Ser Tyr Leu Thr Ala Ile Leu Met Val Ala Ile

        CCCAATATCCAGACTGCGAAAAAGGCTGAATCTATCATTTTGAAGGACGATTGTTCAATC
3903    -------+---------+---------+---------+---------+---------+--  3962
        GGGTTATAGGTCTGACGCTTTTTCCGACTTAGATAGTAAAACTTCCTGCTAACAAGTTAG
        Gly Leu Ile Trp Val Ala Phe Phe Ala Ser Asp Ile Met Lys Phe Ser Ser Gln Glu Ile

        GACATCGAAAAATCCTGCACCCAGTTTGCATGTTGGCCAATTGCTTCCGCCGTCTTATCT
3963    -------+---------+---------+---------+---------+---------+--  4022
        CTGTAGCTTTTTAGGACGTGGGTCAAACGTACAACCGGTTAACGAAGGCGGCAGAATAGA
        Ser Met Ser Phe Asp Gln Val Trp Asn Ala His Gln Gly Ile Ala Glu Ala Lys Asp Thr

        GTGGAACGGGTGTGAGATAGCGTTGAGCTAGCTCCTCCTAAATTCAGAATCCAGTCAAAA
4023    -------+---------+---------+---------+---------+---------+--  4082
        CACCTTGCCCACACTCTATCGCAACTCGATCGAGGAGGATTTAAGTCTTAGGTCAGTTTT
        Thr Ser Arg Thr His Ser Leu Thr Ser Ser Ala Gly Gly Leu Asn Leu Ile Trp Asp Phe

        TGATTGCTTTTTAGATAATGAAAGGGGAGAAAAGGAGCGATCAATGCCAAAAGAGAAATC
4083    -------+---------+---------+---------+---------+---------+--  4142
        ACTAACGAAAAATCTATTACTTTCCCCTCTTTTCCTCGCTAGTTACGGTTTTCTCTTTAG
        His Asn Ser Lys Leu Tyr His Phe Pro Leu Phe Pro Ala Ile Leu Ala Leu Leu Ser Ile

        ACGCTAAGATGATAGTGGGTGCCTACAGTGATTTGAGCCTTCAGCAGTTTTTTTATGAGT
4143    -------+---------+---------+---------+---------+---------+--  4202
        TGCGATTCTACTATCACCCACGGATGTCACTAAACTCGGAAGTCGTCAAAAAAATACTCA
        Val Ser Leu His Tyr His Thr Gly Val Thr Ile Gln Ala Lys Leu Leu Lys Lys Ile Leu

        ATGATGAGAGAAAAAAACAGAGACAATAGAATTTGACTAACCAAAAAGGGAATAAAGAAA
4203    -------+---------+---------+---------+---------+---------+--  4262
        TACTACTCTCTTTTTTTGTCTCTGTTATCTTAAACTGATTGGTTTTTCCCTTATTTCTTT
        Ile Ile Leu Ser Phe Phe Leu Ser Leu Leu Ile Gln Ser Val Leu Phe Pro Ile Phe Phe
```

FIGURE 3.2 (continued)

```
        GAACTGCTCATTTCATTCCTTCTTTCTGTTCTTATGTTCTTCTAGTATTTGATACAACTC
4263    -------+---------+---------+---------+---------+---------+--  4322
        CTTGACGAGTAAAGTAAGGAAGAAAGACAAGAATACAAGAAGATCATAAACTATGTTGAG
        Ser Ser Ser Met  End Glu Lys Lys Arg Asn Lys His Glu Glu Leu  Ile Gln Tyr Leu Glu
                         ------
                          SD

        ATTAATTTCTTCACCTGACAGCTGATCATTCTCCAAAAAGTTTAATACCATCGAATTAAG
4323    -------+---------+---------+---------+---------+---------+--  4382
        TAATTAAAGAAGTGGACTGTCGACTAGTAAGAGGTTTTTCAAATTATGGTAGCTTAATTC
        Asn  Ile Glu Glu Gly Ser Leu Gln Asp Asn Glu Leu Phe Asn Leu  Val Met Ser Asn Leu

        AGTGCCATTGTAAAACCGGTTTAAAAAACTGTGACTTTTCACTTCTATATAATCATTTTC
4383    -------+---------+---------+---------+---------+---------+--  4442
        TCACGGTAACATTTTGGCCAAATTTTTTGACACTGAAAAGTGAAGATATATTAGTAAAAG
        Thr Gly Asn Tyr Phe Arg Asn Leu Phe Ser His Ser Lys Val Glu  Ile Tyr Asp Asn Glu

        GTCTATATTTGGCGTATAAACGAAAACCCGGCCTTCTTTATGGTGGTTTAATGCTCCTTT
4443    -------+---------+---------+---------+---------+---------+--  4502
        CAGATATAAACCGCATATTTGCTTTTGGGCCGGAAGAAATACCACCAAATTACGAGGAAA
        Asp  Ile Asn Pro Thr Tyr Val Phe Val Arg Gly Glu Lys His His Asn Leu Ala Gly Lys

        TTTAATGAGGCACAGCAGCATGGTTTGGATGGTTTTAGGGCTCCATGTACTGGTTTTGGA
4503    -------+---------+---------+---------+---------+---------+--  4562
        AAATTACTCCGTGTCGTCGTACCAAACCTACCAAAATCCCGAGGTACATGACCAAAACCT
        Lys  Ile Leu Cys Leu Leu Met Thr Gln  Ile Thr Lys Pro Ser Trp Thr Ser Thr Lys Ser

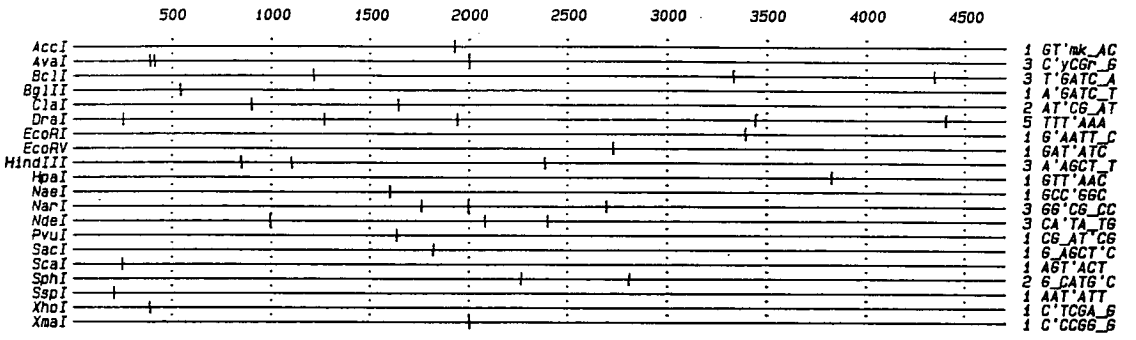
        CAACTCTTTAATCACCTCATTGGTATTGATCGAAGAATGCTTCCAGATGACTTTCATCAC
4563    -------+---------+---------+---------+---------+---------+--  4622
        GTTGAGAAATTAGTGGAGTAACCATAACTAGCTTCTTACGAAGGTCTACTGAAAGTAGTG
        Leu Glu Lys  Ile Val Glu Asn Thr Asn  Ile Ser Ser His Lys Trp  Ile Val Lys Met Val

        TTCTAATTCCGCATCAGAGATTTGAGGTATTTTTTTCATTTCCATCATCCTATACTTACA
4623    -------+---------+---------+---------+---------+---------+--  4682
        AAGATTAAGGCGTAGTCTCTAAACTCCATAAAAAAAGTAAAGGTAGTAGGATATGAATGT
        Glu Leu Glu Ala Asp Ser  Ile Gln Pro  Ile Lys Lys Met                SD

        AATGTAATACTTTCATTATAGGTTTGCC
4683    -------+---------+---------+  4710
        TTACATTATGAAAGTAATATCCAAACGG
```

The deduced protein sequences of the blaP, ORFe and ORFf are reported.

FIGURE 3.3 Restriction map of the 4.7Kb *Eco*RI fragment containing the *bla* gene

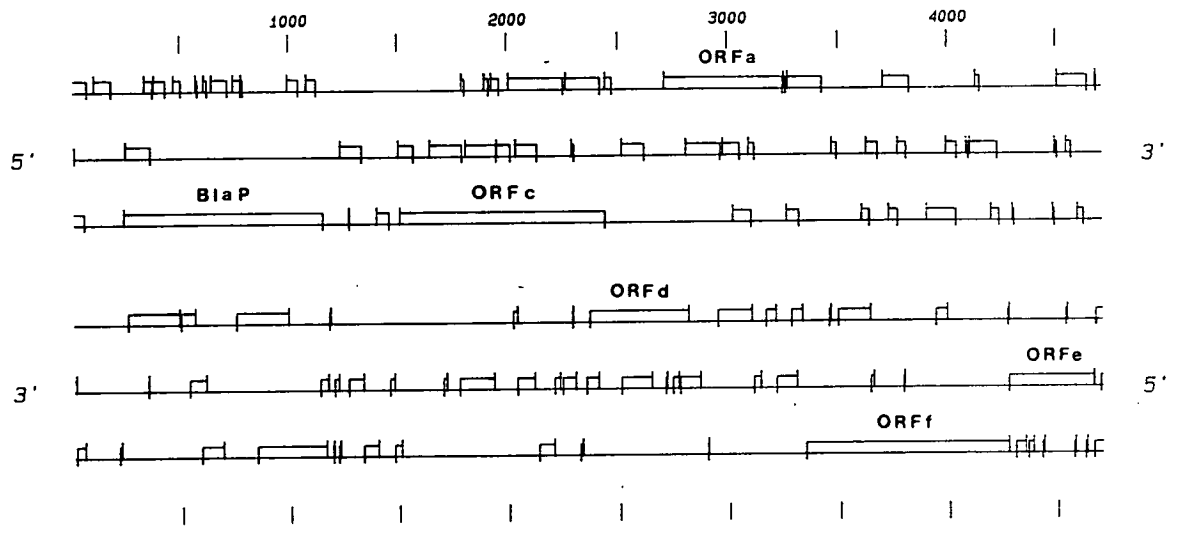


Restriction map output of the University of Wisconsin program MAPPLOT.

A search for restriction sites with the program MAP and MAPPLOT largely confirmed the results of the preliminary restriction analysis of the fragment (see Figure 3.3) with the exception of *Eco*RI site surprisingly found at position 3389. Upon digestion with *Eco*RI neither λbla nor pACYCbla were ever seen to produce more than one extra fragment with respect to their corresponding vectors (see Figure 2.2), possibly indicating that this site is cryptic. Other *Eco*RI restriction sites are known to cut at variable frequencies (Halford *et al*. 1980), but no clear reasons have been found to explain this phenomenon. It is possible that the shorter fragments of DNA (3.4Kb and 1.3Kb), expected as products of the reactions of the restriction enzymes on this site, are generated at such a low level that they are not visible as a band on agarose gels, nor as a detectable signal after probing the digestion products with λpen. The latter should hybridise at least to the larger product, the 3.4Kb fragment, which includes the β-lactamase gene known to cross-hybridise to λpen (see Figure 2.3).

Alternatively, this *Eco*RI site could be interpreted as an artifact resulting from the repeated reading of M13 clones in which the real ends of the original *Eco*RI fragment joined together had been cloned. This possibility is discussed in Section 3.10.

FIGURE 3.4 Open reading frames (ORFs) of the 4.7Kb *Eco*RI fragment containing the *bla* gene



Graphic output of the University of Wisconsin sequence analysis program FRAMES.

The program FRAMES was used to detect open reading frames (ORFs), that is long stretches of DNA sequence between a start and a stop codon that could potentially code for a polypeptide. Five significantly long (more than 300 nucleotides) ORFs were found as depicted in Figure 3.4.

3.5 THE β-LACTAMASE GENE

As already mentioned the gene for the β-lactamase of 6346/c was found to lie at one end of the sequenced fragment, in the 1.6Kb *Eco*RI-*Nae*I fragment. The only ORF present in this section of DNA is on frame C. It spans bases 234 to 1154 and codes for a protein of 307 amino acids. The nucleotide composition of the gene is the same as that of the whole 4.7Kb sequenced: it is slightly richer in As and Ts (A+T 56%, G+C 44%) which results in a bias toward A and T in the third codon position. A table of codon usage in the β-lactamase gene is given in Table 3.1.

TABLE 3.1 Codon usage in the *Bacillus licheniformis* 6346/c *bla* gene

AMINOACID	CODON	NUMBER[a]	/1000[b]	FRACTION[c]
Gly	GGG	2.00	6.51	0.12
Gly	GGA	8.00	26.06	0.47
Gly	GGT	3.00	9.77	0.18
Gly	GGC	4.00	13.03	0.24
Glu	GAG	10.00	32.57	0.42
Glu	GAA	14.00	45.60	0.58
Asp	GAT	21.00	68.40	0.88
Asp	GAC	3.00	9.77	0.13
Val	GTG	7.00	22.80	0.39
Val	GTA	6.00	19.54	0.33
Val	GTT	4.00	13.03	0.22
Val	GTC	1.00	3.26	0.06
Ala	GCG	3.00	9.77	0.10
Ala	GCA	13.00	42.35	0.43
Ala	GCT	7.00	22.80	0.23
Ala	GCC	7.00	22.80	0.23
Arg	AGG	1.00	3.26	0.07
Arg	AGA	2.00	6.51	0.14
Ser	AGT	5.00	16.29	0.28
Ser	AGC	4.00	13.03	0.22
Lys	AAG	8.00	26.06	0.28
Lys	AAA	21.00	68.40	0.72
Asn	AAT	7.00	22.80	0.50
Asn	AAC	7.00	22.80	0.50
Met	ATG	5.00	16.29	1.00
Ile	ATA	2.00	6.51	0.14
Ile	ATT	8.00	26.06	0.57
Ile	ATC	4.00	13.03	0.29
Thr	ACG	8.00	26.06	0.33
Thr	ACA	7.00	22.80	0.29
Thr	ACT	4.00	13.03	0.17
Thr	ACC	5.00	16.29	0.21
Trp	TGG	4.00	13.03	1.00
End	TGA	0.00	0.00	0.00
Cys	TGT	0.00	0.00	0.00
Cys	TGC	2.00	6.51	1.00
End	TAG	0.00	0.00	0.00
End	TAA	0.00	0.00	0.00
Tyr	TAT	5.00	16.29	0.83
Tyr	TAC	1.00	3.26	0.17
Leu	TTG	2.00	6.51	0.06
Leu	TTA	10.00	32.57	0.30
Phe	TTT	5.00	16.29	0.56
Phe	TTC	4.00	13.03	0.44
Ser	TCG	3.00	9.77	0.17
Ser	TCA	3.00	9.77	0.17
Ser	TCT	2.00	6.51	0.11
Ser	TCC	1.00	3.26	0.06
Arg	CGG	3.00	9.77	0.21
Arg	CGA	4.00	13.03	0.29
Arg	CGT	1.00	3.26	0.07
Arg	CGC	3.00	9.77	0.21
Gln	CAG	4.00	13.03	0.50
Gln	CAA	4.00	13.03	0.50
His	CAT	2.00	6.51	0.67
His	CAC	1.00	3.26	0.33
Leu	CTG	3.00	9.77	0.09
Leu	CTA	0.00	0.00	0.00
Leu	CTT	14.00	45.60	0.42
Leu	CTC	4.00	13.03	0.12
Pro	CCG	6.00	19.54	0.55
Pro	CCA	2.00	6.51	0.18
Pro	CCT	3.00	9.77	0.27
Pro	CCC	0.00	0.00	0.00

a Number of times codon is found in the gene

b Number of times codon would occur per 1000 codons

c Fraction of each aminoacid coded by a particular triplet

Six bases upstream from the translation start codon there is a possible Shine-Dalgarno sequence (GAGACG) which presents some complementarity to the 3' end of *Bacillus subtilis* 16S rRNA, 3'-UCUUUCCUCCACUAG-5' (McLaughlin *et al*. 1981, Moran *et al*. 1982). About 180 bases upstream from the Shine-Dalgarno sequence there is a typical promoter with a TTGCAT sequence at the -35 region, a spacer of 17 nucleotides and a TAATAC sequence at the -10 region; this arrangement closely resembles that of the consensus sequence (TTGACA for the -35 region, a spacer of 17-18 bases and TATAAT for the -10 region) observed by Moran (Moran *et al.* 1982) for the RNA polymerase $E\sigma^{55}$ of *Bacillus subtilis*.

A typical Rho-independent terminator (Platt 1986) can be found 57 nucleotides downstream from the translation stop codon of the gene. It has a hairpin with a stem formed by 15bp and a loop of 5 bases; the free energy of this secondary structure, as calculated following the rules of Tinoco (Tinoco *et al.* 1973) for RNA folding, was -15.0 Kcal/mol. A region extremely rich in thymine residues follows immediately the hairpin loop; it is probably in this region that the RNA polymerase falls off the messenger RNA giving rise to the main mRNA population of 1.2Kb (Salerno & Lampen 1986).

3.6 SEQUENCE COMPARISON

The first 1300 bases of the sequence, including the *B.licheniformis* 6346/c β-lactamase gene and the flanking sequences, was compared to the sequence published by Neugebauer (Neugebauer *et al.* 1981) of the corresponding region cloned from *B.licheniformis* 749/c. In the DOTPLOT comparison output (Figure 3.5) three distinct zones of homology can be found.

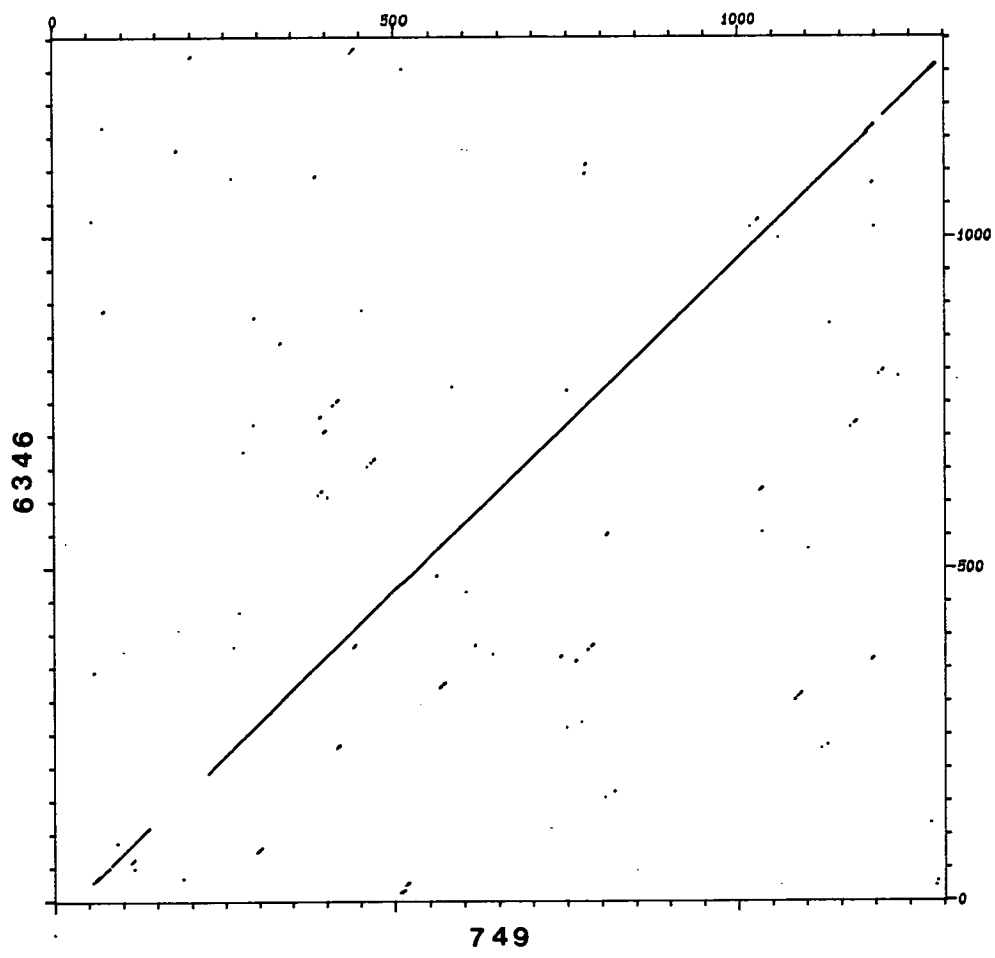
The central long region of matching, corresponding to the coding sequence and the ribosomal binding site, is preceded by a short zone of homology centered on the promoter. The other short match, which follows the gene, coincides with the terminator site.

Of the 53 nucleotide differences found between the two coding sequences, 33 (62% of the total) are silent, while the other 20 (38%) give rise to 17 amino acid substitutions.

3.7 ORFe and ORFf

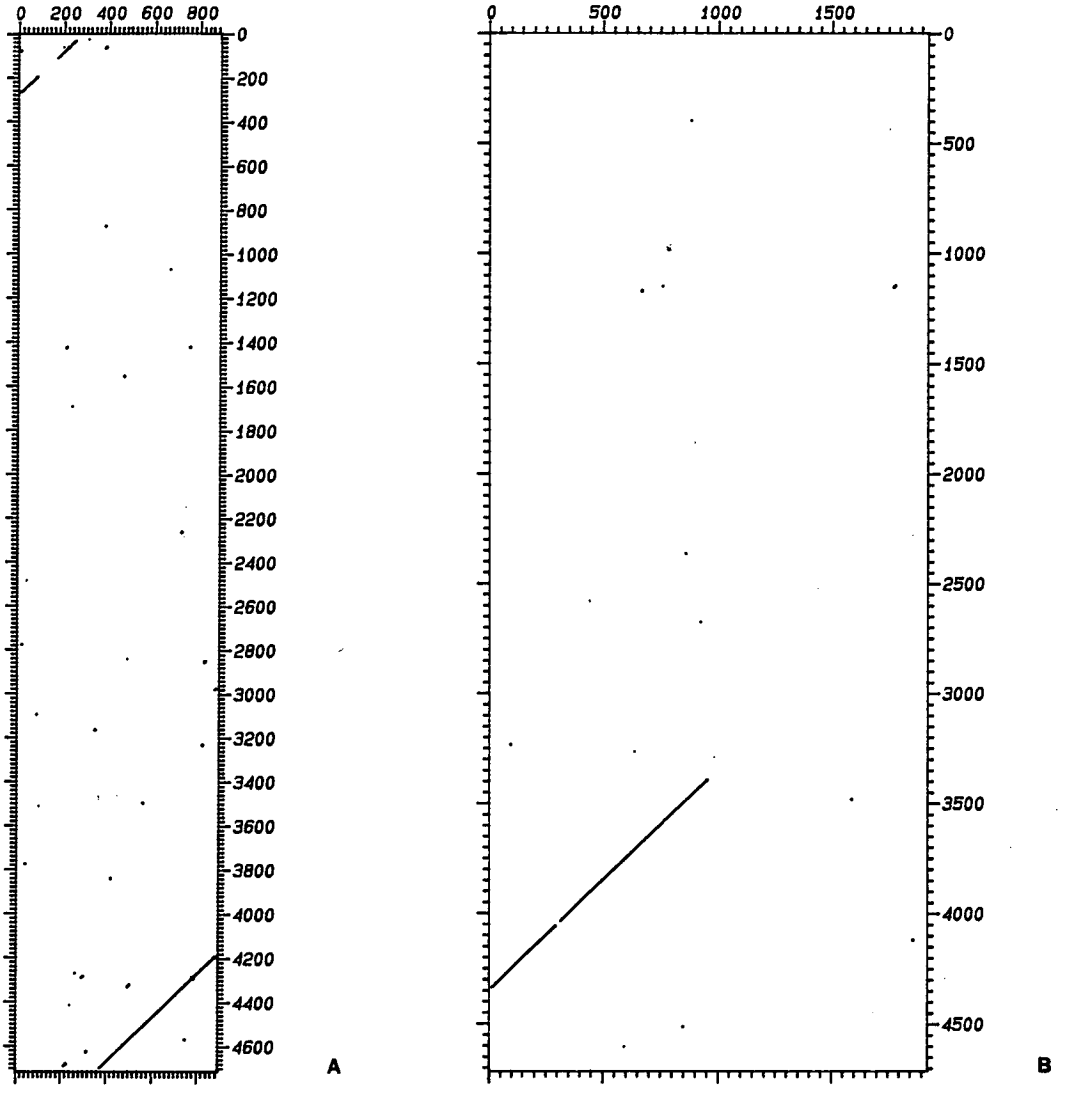
At the other end of the sequenced fragment and on the opposite strand with respect to the β-lactamase gene, two open reading frames can be found, designated ORFe and ORFf from the frame they lie on. ORFf (bases 4273-3347) follows closely ORFe (bases 4661-4278). ORFf's translation start codon is separated from the stop codon of ORFe by only one nucleotide and its translation stop codon lies just beyond the cryptic *Eco*RI site.

FIGURE 3.5 Dotplot comparison of *B.licheniformis* 6346/c and 749/c β-lactamases



Window: 21; Stringency: 14.

FIGURE 3.6 Dotplot comparison of the 4.7Kb *Eco*RI fragment with *penI* and *penRI*



The sequence of the 4.7Kb EcoRI fragment (vertical sequence) is compared with penI (A) and penRI (B). Window: 21; Stringency: 14.

Both ORFs are preceded by good Shine-Dalgarno sequences (AGGATG for ORFe and AGAAGG for ORFf) situated six bases upstream from their respective translation start codon; ORFe codes for a polypeptide of 128 amino acids, while the protein encoded by ORFf is 309 amino acids long.

Comparison of these open reading frames with the sequences of the regulatory genes of the penicillinase operon in *B.licheniformis* 749 and 9945a (Imanaka *et al*. 1987, Kobayashi *et al*. 1987, Himeno *et al*. 1986) showed extensive homology between ORFe and the penI gene and between ORFf and the first half of the penR1 gene. The DOTPLOT comparisons are shown in Figures 3.6 A and B.

3.8 THE PENICILLINASE OPERON IN *B.licheniformis* 749

B.licheniformis 749 is a β-lactamase inducible strain; on induction by a β-lactam antibiotic such as cephalosporin C the enzyme secretion increases very slowly, reaching a maximum level after about two hours and remains high for a few hours (Collins 1979).

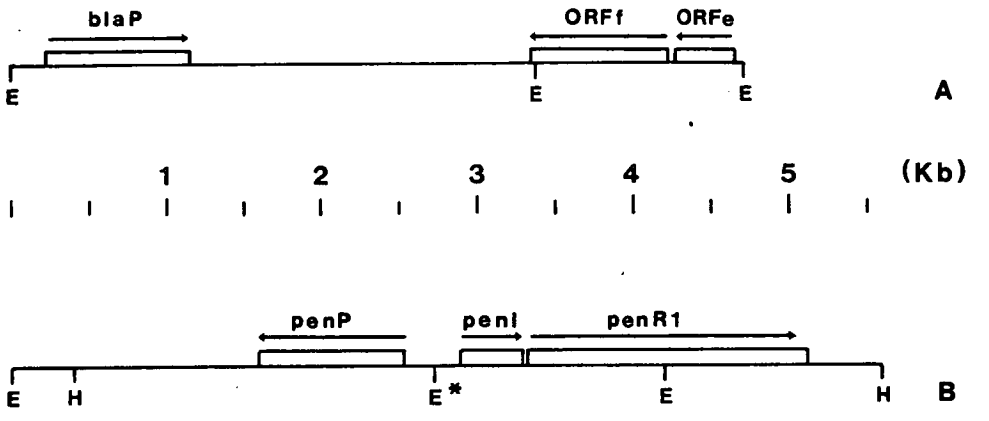
According to Sherratt and Collins (1973) three regulatory genes are required for β-lactamase induction in *B.licheniformis*. A negative regulatory gene (*penI*) is 90% linked to the structural gene (*penP*); *penP* is also 50% linked to a second locus involved in regulation, called R1, and not linked at all to the further regulatory gene R2.

The *penP* structural gene and the two regulatory genes *penI* and *penR1* have been cloned and sequenced (Neugebauer *et al*. 1981, Himeno *et al*. 1986, Imanaka *et al*. 1987, Kobayashi *et al*. 1987, Nicholls 1986). *PenR1* follows closely *penI* in the genome and it is transcribed from the same promoter situated just upstream of *penI*. The structural gene *penP* is transcribed divergently to *penI* and *penR1*, from a promoter which lies 5' of the two regulatory genes (Figure 3.7b).

PenI gene product is a regulatory protein which binds to operator sequences upstream of *penP* and of its own gene, repressing transcription (Grossman & Lampen 1987). *PenR1* codes for a membrane protein of 601 amino acids with a transmembrane region at its amino terminus and a putative β-lactam binding site (Kobayashi *et al*. 1987). This protein is believed to be the β-lactam receptor.

It is current thought that β-lactam antibiotics would bind irreversibly to the receptor protein, causing the production of an intracellular stimulus, maybe mediated by the still unidentified *penR2* gene product, which would lift the repression of both *penI* and *penP* and therefore increase the production of

FIGURE 3.7 The *B.licheniformis* β-lactamase operon



In A the beta-lactamase operon as found in the cloned 4.7Kb EcoRI fragment carrying the B.licheniformis 6346/c beta-lactamase. In B the beta-lactamase operon of B.licheniformis 749 (Himeno et al. 1986; Kobayashi et al. 1987; Imanaka et al.1987). Restriction sites are marked below the line and denoted as follows: E, EcoRI; E*, EcoRI*; H, HindIII. Above the line genes are represented by boxes. The direction of transcription in indicated by the arrows.

penicillinase. In the absence of inducer both *penI* and *penP* are repressed by *penI* gene product.

In the magnoconstitutive producer of penicillinase *B.licheniformis* 749/c the defect in induction has been found to be caused by a nonsense mutation in codon 32 of the *penI* gene, resulting in the production of a non-functional truncated repressor (Grossman & Lampen 1987).

3.9 THE PENICILLINASE OPERON IN *B.licheniformis* 6346/c

From the nucleotide sequence of the DNA fragment carrying the β-lactamase gene cloned from the magnoconstitutive strain of *B.licheniformis* 6346/c, it was found that the arrangement of the penicillinase operon is different from that described above for the 749/c strain. In this case the genes of the penicillinase operon (referred to as *bla* for the 6346/c strain) *blaP* and *blaI/blaR1* are still situated on different strands, but they are transcribed convergently, the *blaI/blaR1* unit lying at the 3' side of *blaP* (see Figure 3.7a).

As just mentioned above, the non-inducibility of the β-lactamase production in strain 749/c is due to the presence of an inactive repressor. In the case of 6346/c no defect has yet been found that would explain its magnoconstitutive production of β-lactamase. Comparison of the deduced sequence of the ORFe gene product with that of *penI* shows that these two proteins have the same length (128 aa) and present only two amino acid differences: in position 49 the Arg found in the 749 repressor is substituted by Cys in the ORFe gene product; in position 75 Ser is substituted by Asn. It is therefore clear that ORFe is in fact the gene of the penicillinase repressor in *B.licheniformis* 6346/c and will be referred to as *blaI*.

In view of what has just been said about the *blaI* gene product, it is obvious that, whatever the defect in regulation of β-lactamase production in 6346/c might be, it is not similar to that of the 749/c strain where a truncated repressor was found. However at this stage it is not possible to rule out the hypothesis that one or both the amino acid substitutions could still result in an inactive full length repressor.

In the 6346/c derived clone ORFf was found to be very similar to the first half of the *blaR1* gene; so instead of a full length protein of 601 amino acids in 6346/c a shortened protein of 309 amino acids is found. This part corresponds to most of the transmembrane part of the β-lactam receptor but not the β-lactam binding site, which lies in the carboxy terminal half of the protein. The similarity between the two proteins is very good up to the amino acid whose codon superimposes the *Eco*RI site internal to the *blaR1* gene of 749. In ORFf, the cryptic *Eco*RI site is found at the same position and any similarity to *blaR1* ceases at this point. ORFf gene product is truncated at a stop codon about 40 bases downstream from the cryptic *Eco*RI site. This truncated protein could conceivably still take up its proper transmenbrane position in the cell, but it could not act as a receptor for β-lactams, thereby hindering the induction process at the very first stage.

It is possible that this organisation of the operon does not reflect that on the genome of *B.licheniformis* 6346/c, but it is due to a rearrangement of DNA fragments during the first cloning step. This would imply that during the creation of the genomic library in λNM1149 (see Section 2.1) *Eco*RI endonuclease cut also at a site inside the fragment containing this part of the operon, corresponding to the 4.2Kb fragment derived from 749/c (Salerno & Lampen

1986) (see Figure 3.7b) and that during the cloning the two segments were rescued together, but in a different order to that on the genome. In the 749/c derived 4.2Kb *Eco*RI fragment there is an *Eco*RI* site (GAATTA) lying between the *blaP* and the *blaI* promoters. If a similar situation were present in the 6346/c operon, it is conceivable that accidental cutting at this site could have caused the rearrengement.

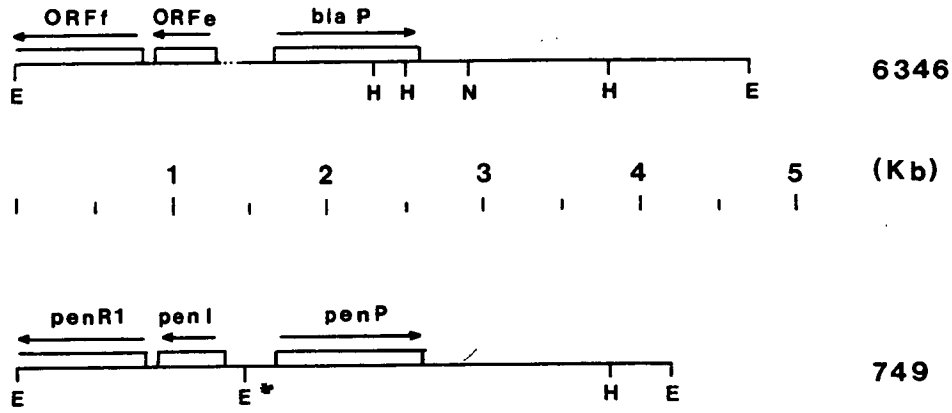
More work is needed in order to clarify the situation; possibly repeating the original cloning step could give us the answer. However during the course of this work sequencing of the regulatory region together with the sought for β-lactamase gene was purely accidental. So, despite the interest that these findings aroused, it was decided not to pursue the matter any further.

3.10 DISCUSSION

Some of the inconsistencies found during the analysis of the sequencing results could have an alternative explanation. The *Eco*RI site found at position 3389 could be an artifact which arose during the sequencing of the 4.7Kb *Eco*RI fragment containing the β-lactamase gene of *B.licheniformis* 6346/c.

In the shotgunning procedure used to start this sequencing project, a step was included which involved the partial circularisation of the fragment in order to increase the probability of reading the ends. Normally, unless the joining of the ends creates artificial sequences that are difficult to clone, this approach leads to the definition of a circular contig (consensus sequence obtained from the overlapping of individual readings). In the experiments reported here the contig did not reflect a circular molecule and this was assumed to be due to difficulties in the cloning of the ends of the fragment. However, it could be envisaged that the apparent reorganisation of the penicillinase operon of *B.licheniformis* 6346/c with respect to that of 749 was due to an erroneous joining of the contig. The *Eco*RI site found in position 3389 could be an artifact due to the ligation of the original ends of the fragment. Lack of clones covering the region between ORFe and *blaP*, which should now be thought to be contiguous as in the 749 operon (Figure 3.8), could be related to the particular nature of this region, which includes the promoters and operator regions of both *blaI* and *blaP*. M13 recombinants containing a fragment covering this region could be difficult to obtain because of the many inverted repeats (the operator sequences) which could form stemloop structures. On the other hand,

FIGURE 3.8 An alternative arrangement of the penicillinase operon in *Bacillus licheniformis* 6346/c



The EcoRI fragment comprising part of the penicillinase operon of B.licheniformis 749 is presented here in the opposite orientation with respect to Figure 3.7 for ease of comparison. Restriction sites are denoted as follows: E, EcoRI; E*, EcoRI*; N, NaeI; H, HindIII.

even if these M13 recombinants were made, transcription of the *lacZ* gene of the vector could start from the cloned promoters, thus giving rise to blue plaques that could be mistaken for wild type M13 and therefore not sequenced.

The arrangement of the penicillinase operon in *B.licheniformis* 6346/c would be similar to that of 749 and the truncation of *blaRI* (ORFf) be due to the presence of an *Eco*RI site in the middle of the gene, as in 749. The short extension of ORFf beyond the *Eco*RI site at position 3389 (see above Section 3.7) would be accidental.

Whatever the cause of the rearrangement (real or apparent) of the 6346/c operon, the two short stretches of similarity found at the top of the dotplot comparison between the contig and the *penI* sequence (Figure 3.6a) must be thought of as continuous with the major similarity between *blaI* and *penI*. These two regions of similarity corresponding to ca. bases 1-260 of the contig are due to the homology of the promoter regions of *blaI* and *blaP* (separated in the figure from *blaI* coding sequence) and the corresponding region as found immediately upstream of *penI*.

3.11 OTHER OPEN READING FRAMES

Three more open reading frames of considerable length were highlighted by the program FRAMES (Figure 3.4). Two of them, ORFc and ORFa, follow the β-lactamase gene. The third lies on the opposite strand, about 0.5Kb downstream of ORFf.

ORFc — This ORF follows the β-lactamase gene on the same reading frame. It has two possible starting points, one at base 1509 and the other at base 1533 and terminates at a stop codon at position 2439. Only one possible Shine -Dalgarno sequence (TGGAGG) was found, five bases upstream of the second initiation codon, which is therefore the more likely of the two to be the real starting point of translation. The gene product of ORFc would then be a protein of 302 amino acids.

ORFa — The third ORF in the leading strand, ORFa spans nucleotides 2713 to 3248. No similarity to the Shine-Dalgarno sequence was found immediately upstream of its translation start codon.

ORFd — This reading frame runs in the opposite direction from base 2814 to 2368. No possible ribosomal binding site was found upstream of its start codon.

A protein sequence comparison search between the deduced amino acid sequences of these ORFs and the protein database assembled by Claverie & Bricault (1986) was undertaken. The search, carried out on a ICL 64x64 DAP computer as described by Collins (Collins *et al.* 1988) and Coulson (Coulson *et al.* 1987), failed to uncover any significant similarity between the putative gene products of these ORFs and the protein sequences stored in the database.

CHAPTER 4

MUTAGENESIS

4.1 INTRODUCTION

The aim of this work was to study the influence of protein structure on the specificity of β-lactamases. The system used, that of *B.licheniformis* β-lactamases, was chosen because it was known that a biologically significant change in substrate specificity was caused by a small number of amino acid substitutions.

Each natural isolate of *B.licheniformis* produces one or the other of two types of β-lactamases, which can be called 749 and 6346 type from the two magnoconstitutive strains chosen to represent them. The difference in activity against a range of β-lactams is the main feature of the system (Pollock 1965a), the 749 type enzymes being more a penicillinase than the 6346 type, which is relatively more active against cephalosporins (see Table 1.2). The sequence differences between the two proteins were known to be few (Thatcher 1975, see Table 1.3) and it was suggested (Dubnau & Pollock 1965) that the difference in specificity is due to only one or to a group of closely linked substitutions. The idea was therefore that sequencing of the β-lactamase gene of *B.licheniformis* 6346/c and comparing it with the sequence of the 749/c penicillinase (Meadway 1969) would have comfirmed the small number of differences, of which possibly only one was responsible for the change in activity. All the others were predicted not to influence the specificity of the enzyme.

For these reasons, the system looked particularly suitable for a protein engineering project. With the new tools available to molecular biologists it is now possible to introduce any change one likes in any protein (provided that the gene has been cloned and sequenced) or even construct absolutely new polypeptides. In fact this approach for studying proteins is constantly frustrated by our only partial understanding of the rules governing protein folding and of the relationship linking protein structure and function. Even when a detailed X-ray structure of the protein is available, it is still very difficult to predict what effects an amino acid substitution would have on the structure of a protein or to identify which residues once introduced are likely to have an effect on the activity of an enzyme which is not its complete inactivation.

B.licheniformis offers us two naturally occurring variants of β-lactamase where an interesting difference in activity is already prepared for us to study. What I intended to do was to find out what caused it.

FIGURE 4.1 Comparison of the 6346 and 749 β-lactamases protein sequences

```
      1                                                  50
6346  MKLWFSTLKL KKVAAVLLFS CVALAGCGSN HSNASHSAEK DEKTEMKDDF
749                 A                 AN  QT   QP    N

      51                                                 100
6346  AKLEEQFDAK LGIFALDTGT NRTVTYRPDE RFAFASTIKA LTVGVLLQQK
749                               A
                                 (59)

      101                                                150
6346  SIEDLNQRIT YTRDDLVNYN PITEKHVDTG MTLKELADAS LRYSDNTAQN
749                                                     A
                                                       (133)

      151                                                200
6346  LILKQIGGPE SLKKELRKIG DEVTNPERFE PELNEVNPGE TQDTSTARAL
749

      201                                                250
6346  ATSLQAFALE DKLPSEKREL LIDWMKRNTT GDALIRAGVP EGWEVADKTG
749   V   R                                          D
     (187) (191)                                   (227)

      251                                                300
6346  AGSYGTRNDI AIIWPPKGDP VVLAVLSSRD KKDAKYDDKL IAEATKVVVK
749    A                                                  M
      (238)                                             (287)

      301
6346  ALNMESK
749       NG
        (293)(294)
```

The 6346 beta-lactamase deduced sequence is shown in full; only the positions at which differences were found are marked for the 749 enzyme. In parenthesis the no. of each substitution in Ambler notation.

4.2 A COMPARISON OF THE 6346 AND 749 β-LACTAMASES

The comparison of the gene sequence of the *B.licheniformis* 6346/c β-lactamase with that of the 749/c strain (Section 3.6) highlighted the presence of 53 nucleotide substitutions which give rise to 17 amino acid changes between the 749 protein (Meadway 1969) and the deduced sequence of the 6346 β-lactamase (Figure 4.1).

This number of substitutions is higher than that forecasted previously by Thatcher (1975); he had in fact identified four changes: Gln191 to Arg, Val287 to

Met, Glu293 to Asn and Ser294 to Gly. One other amino acid substitution near the amino terminus of the protein had to be postulated in order to explain some genetic experiments carried out by Sherratt (1969).

In *B.licheniformis* cultures the β-lactamase protein is normally present in at least four forms: the full-length precursor inside the cell, a shorter lipoprotein form, anchored to the cell membrane through a cysteine residue carrying a diglyceride thioether (Nielsen et al. 1981) and two secreted forms of molecular weight of 30500 and 29500 (Lampen et al. 1980).

During the normal procedure of purification of the enzyme, it is the secreted forms which are isolated with a prevalence of the smaller protein. Thatcher, who was working on the small exocellular species, could obvously not detect eight of the amino acid substitutions, because they are located at the amino terminus of the preprotein in the signal peptide and in the other fragments that are lost during the processing of the enzyme. His experiments were based on the comparison of the electrophoretic behaviour of peptides obtained by protease digestion of the 6346 and 749 proteins and sequencing of those 6346 peptides that migrated differently from the corresponding 749 derived ones. Consequently, of the remaining nine differences he could not detect those changes that are electrophoretically silent, that is Thr59 to Ala, Thr133 to Ala, Ala187 to Val, Glu227 to Asp and Gly238 to Ala.

4.3 DESIGN OF MUTATIONS

In order to find out which of the amino acid changes had an influence on the specificity of the enzymes, 749-like residues were substituted at the corresponding position on the 6346 β-lactamase and the effect of the changes subsequently estimated.

On the basis of the findings of Thatcher (1975), the task seemed not unduly daunting with four, maybe five substitutions to be considered. But the sequencing of the β-lactamase gene of *B.licheniformis* 6346/c revealed a number of changes three times higher than that expected. So some educated guesses about which of these residues were most likely to have any influence on the enzyme activity were needed in order to give priority to the more interesting changes.

Eight mutations located in those parts of the preprotein that are shed during the processing to mature secreted enzyme are highly unlikely to play any significant role in the function of the protein. These changes will not be discussed any further.

In the absence of a detailed crystal structure, it was very difficult to decide on a priority list amongst the remaining nine changes, apart maybe for the exclusion of the two substitutions located at the carboxy terminus of the protein, Glu293 to Asn and Ser294 to Gly. In fact these changes are part of a group of five residues which are present at the carboxy terminus of *B.licheniformis* β-lactamases but do not appear in any of the other members of the Class A enzymes. Their direct involvment in the catalytic activity of the enzyme is therefore highly unlikely, but this argument is not sufficient to rule out entirely their possible effect on the specificity of the *B.licheniformis* penicillinases.

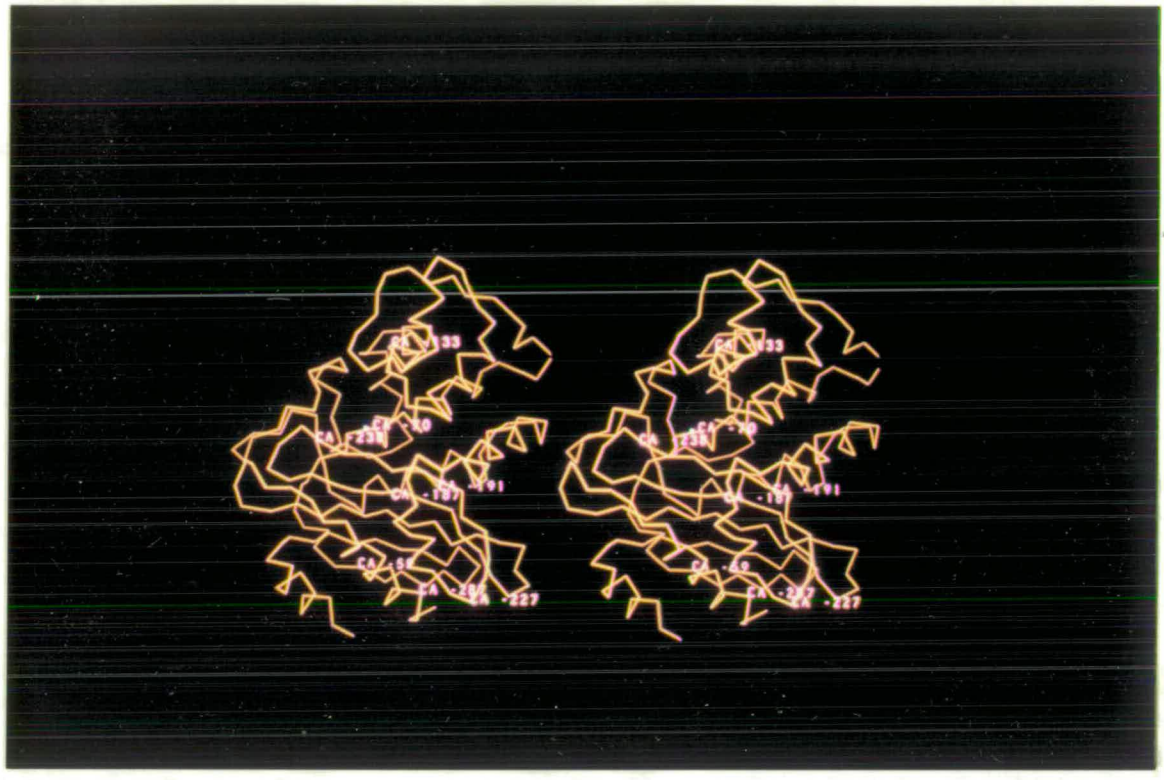
It was therefore with great pleasure that we welcomed the publication of the crystal structure of the *Staphylococcus aureus* PC1 β-lactamase at 2.5A resolution (Herzberg & Moult 1987). When the coordinates of the α-carbon atoms became available, a simple superimposition of the 6346 amino acid sequence onto the diagram of the three dimentional structure of the *St.aureus* enzyme offered a good picture of the position of the substitutions on the molecule (Figure 4.2).

One of the changes, Val287 to Met, is situated in the carboxy terminal helix, which is supposed to extend for a further five residues in the *B.licheniformis* enzymes, among them the above mentioned Glu293 to Asn and Ser294 to Gly substitutions. This region is very far from the active site cleft.

All the other changes are scattered throughout the molecule. The Ala187 to Val and Gln191 to Arg substitutions are exactly one turn apart on the same helix (α8) on the opposite face of the molecule with respect to the active site. Two more substitutions can be found spatially near the carboxy terminus (Glu227 to Asp and Thr59 to Ala). The Thr133 to Ala change is also part of an α-helix (α5) and it is exposed to the surface. The last and nearest change to the active site is Gly238 to Ala.

It was decided to first of all test the importance of the three carboxy terminal differences by introducing a stop codon in position 284 where a Lysine

FIGURE 4.2 Three dimensional structure of *St.aureus* PC1 β-lactamase



The alpha-carbon positions corresponding to the amino acid substitutions found between B.licheniformis 6346 and 749 exocellular beta-lactamases are marked by their relative number (Ambler notation). Glu293 to Asn and Ser294 to Gly substitutions, located in the longer carboxy-terminus of the B.licheniformis enzymes, are not reported.

is found both in 749 and 6346. This mutation would shorten the protein by eleven residues, hopefully without the complete disruption of the α-helical structure of the carboxy terminus. Such a major change could in fact induce distortions in the shape of the molecule and indirectly interfere with the activity of the enzyme.

All the other changes were to be investigated by introducing the 749-like residues in the 6346 protein one by one, starting with the nearest to the active site depression. To date two of them, Gly238 to Ala and Thr59 to Ala, have been introduced.

Another mutant, Asn170 to Met, was designed in order to assess a model of mechanism of action proposed by Herzberg and Moult (1987) as a consequence of the newly produced X-ray structure of the *St.aureus* β-lactamase. This mutation will be described in detail below (Section 5.4).

4.4 DESIGN OF OLIGONUCLEOTIDES

In order to increase the efficiency of mutation and to avoid unexpected unpleasant surprises, oligonucleotides used in SDM experiments must be able to introduce the wanted change without causing any other modification in the DNA sequence. A number of factors were considered when designing the oligonucleotides that were successively used during the course of this work.

Number of mismatches

The number of mismatches needed to direct a certain mutation was always kept to the minimum. For example wanting to change Gly238 to Ala, codon GGA could have been mutated to GCC, GCT, GCA or GCG, all coding for Ala. The oligonucleotide that was actually used was designed to change GGA to GCA because this involved only one mismatch, and therefore produced a more stable hybrid between oligomer and wild-type template.

Position of mismatch

As a rule at least eight matching nucleotides were kept at each side of the mismatch, in order to prevent displacement of the primer from the template by the 3′ to 5′ exonuclease activity of the *E.coli* polymerase I (Klenow fragment) used for the experiments and from any contaminating 5′ to 3′ activity. As far as possible the two regions flanking the mismatch were designed to be the same length. The central position of the mismatch is in fact important when the oligomer is used as a probe to screen for mutant clones. When the mismatch is located near the middle of the molecule, the difference in melting temperature of the two possible hybrids (perfectly matched and mismatched) is maximized, thus facilitating the screening procedure.

Length of the oligomer

An average length of 15 to 20 nucleotides was chosen, because oligonucleotides in this range are less likely to recognize spurious targets and prime DNA synthesis at room temperature and above.

Spurious priming

The sequence of each oligonucleotide was checked for possible priming at more sites than that intended by conducting a computer analysis with the aid of the program FIND. The sequences of pAD19R, insert and M13 were searched for sites with 75% or more identity to the oligomer used. Under these conditions no matches were ever found for any of

the oligonucleotides designed and it is unlikely that any site with a number of matches below this figure would be stable enough to compete successfully with the main target.

Secondary structure

The possible most likely secondary structure of each oligonucleotide was predicted with the program FOLD and its free energy calculated following the rules of Tinoco (Tinoco *et al.* 1973). None of the predicted structures for the oligomers designed (Table 4.1) had a value of free energy below zero and therefore was not likely to obstruct the formation of the primer-template hybrid.

The sequence of the oligonucleotides synthesized and their relevant features are listed in Table 4.1. Of the six oligomers, the top four were used to direct the relative mutations, number 5, designed to change Thr133 to Ala, was used as a sequencing primer. The last oligomer has no mismatches and binds to a site just downstream of the gene between the stop codon and the terminator sequence. It was intended for the sequencing of the 3′ end of the gene.

All the oligonucleotides with the exception of number 4 hybridize to the same strand of pSR81, which is that produced as a single strand upon superinfection with helper phage.

TABLE 4.1 Important features in the design of oligonucleotides

N°	OLIGONUCLEOTIDE[a]	MUTATION DIRECTED	POSITION IN INSERT	ΔG Kcal/mole
1	GCCGATACGCTACCGTT	T59 to A	465-449	+0.9
2	GATTCACTTCCATTAACTCCGG	N170 to M	795-774	+1.3
3	CATATGATGCCGCTCCA	G238 to A	996-980	+2.0
4	AGGCAACATAAGTGGTA	K284 to Stop	1111-1127	+2.0
5	TCTGTGCCGCATTGTCA	T133 to A	681-665	+2.4
6	CAAGTGACTTTATGT	Sequencing	1209-1195	+2.4

a Mismatched nucleotides are underlined

4.5 SYNTHESIS AND TESTING OF THE OLIGONUCLETIDES

The oligonucleotides used for this work were synthesized by the OSWEL DNA SERVICE of the University of Edinburgh on a DNA synthesizer made by Applied Biosystem with the phosphoramidite method and were not subjected to any further purification.

Before use in the SDM experiments, to test that the oligomers were actually binding to the template DNA uniquely and at the correct position, the template made for mutagenesis and the oligonucleotide were used in a sequencing reaction, which is a stringent test for the quality of both template and primer. All the oligonucleotides performed well, giving a clean sequence with very low background, but the optimal concentration of primer to be used in each sequencing reaction had to be determined in each case. Depending on the oligomer, the amount of primer used for the sequencing of one clone varied between 0.2 and 10 pmoles.

4.6 CONSTRUCTION OF THE MUTANTS

The phosphorothioate method of mutagenesis developed by Eckstein and co-workers (Taylor et al. 1985a and 1985b, Nakamaye & Eckstein 1986) was used for constructing all the mutants, with the exception of Lys284 to Stop. This is a very elegant method, which gives a very high efficiency of mutagenesis, yielding up to 95% mutant clones carrying the mutation on both strands. The mutants can therefore be characterized directly by sequence analysis, avoiding the time-consuming screening and purification of the clones.

The Eckstein method for oligonucleotide-directed *in vitro* mutagenesis marketed as a kit by Amersham was used for introducing mutations in the 6346 β-lactamase gene cloned in pSR81 (Section 2.3). Because this plasmid contains the origin of replication of the bacteriophage f1, single stranded template DNA can be made by superinfecting cells harbouring the plasmid with M13K07, an helper phage that preferentially packages the DNA derived from the plasmid.

Before use on the 6346 gene, the kit was tested on the template and oligonucleotide provided by the firm as an internal control of the activity of the enzymes. The oligo directs a nonsense to sense mutation in the β-galactosidase gene of a *lacZ*⁻ M13mp8. Mutant plaques can be easily recognized because the mutation restores β-galactosidase activity, resulting in a white to blue

phenotypic change. Using this test, the performance of the kit was very satisfactory, with yields of mutant plaques of 92%.

However, when employed for the construction of mutants in the 6346 β-lactamase gene, the system did not keep up to its promises, yielding mutants at a frequency of 5 to 15% (see Section 6.13.1). Most probably this drop in efficiency is to be blamed on the presence of the helper phage DNA mixed with the template. In fact, although the quantity of phage DNA packaged is less than that of the template, its presence makes the exact determination of the template DNA concentration quite impossible, resulting in the use of a suboptimal ratio of template to oligonucleotide molecules in the reaction mixture. Phage DNA molecules are also likely to interfere with the reactions, competing with the template molecules for the enzymes.

4.7 PLASMID MUTAGENESIS

The gapped-duplex approach to the construction of oligonucleotide-directed mutations (Kramer et al. 1984) has originally been developed for use with single stranded templates, but, with minor modifications, it can be applied also to double stranded molecules (Morinaga et al. 1984).

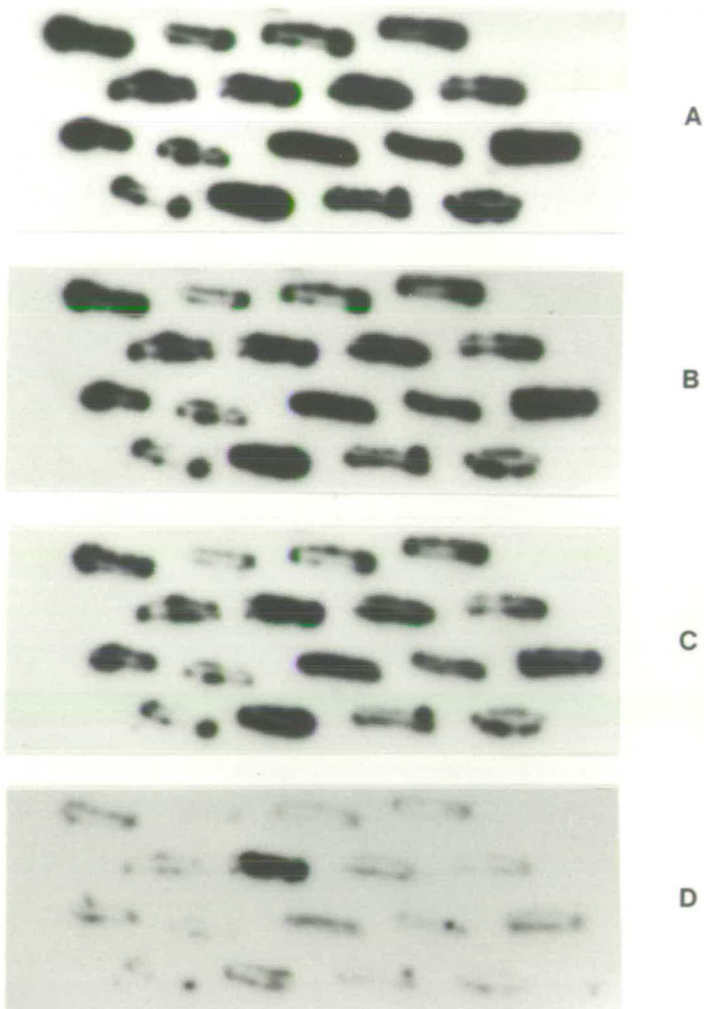
The Lys284 to Stop mutation was constructed according to this method, using as template CsCl purified pSR81 double stranded DNA, as described in Section 6.13.2. The experiment did produce the wanted mutation, but with an efficiency of only 1% and therefore was not used for the construction of any of the other mutants.

4.8 SCREENING OF THE MUTANTS

If the frequency at which mutations are produced is reliably greater than 50%, the sequencing of a few putative clones would easily lead to the identification of the mutant. Because the efficiency of mutant production was constantly lower than 50%, mutant clones had to be identified by screening.

Figure 4.3 shows the results of a typical screening experiment. After hybridisation with the labelled mutagenic primer, colonies carrying the mutant clone are recognized because of the strong signal emitted after the discriminating temperature has been reached. This is defined as the temperature at which the wild type DNA and the oligonucleotide, forming an imperfect duplex,

FIGURE 4.3 Example of mutants screening: mutant Lys284 to Stop.



A Room temperature wash.
B Wash at 43°C.
C Wash at 46°C.
D Wash at 48°C (Tm).

The colony on the bottom right hand corner of each photograph harbours the wild type pSR81 plasmid and was introduced as a negative control.

TABLE 4.2 Comparison of Tm and Td

N°	LENGTH	OLIGONUCLEOTIDE	Tm	Td
1	17	GCCGATACGCTACCGTT	54	52
2	22	GATTCACTTCCATTAACTCCGG	64	54
3	17	CATATGATGCCGCTCCA	52	55
4	17	AGGCAACATAAGTGGTA	48	48

should dissociate, while the perfectly matched mutant should still hybridize to the probe. The melting temperature (Tm) of the duplex is usually considered a good estimate and it is calculated as

$$Tm = 2(A+T) + 4(G+C)\ ^{\circ}C$$

where A refers to the number of adenine residues, T the number of thymine residues, G the number of guanine and C the number of cytosine residues in the oligonucleotide. In fact, from the experience derived from the making of these mutants, the Tm should be used very carefully and only as an indication, especially when the oligomer is longer than 17 nucleotides.

In Table 4.2 the calculated Tm and the temperature (Td) at which actual discrimination between wild type and mutants was achieved are reported. Only in one case, with oligo number 4 which was used to direct the Lys284 to Stop mutation, Tm and Td coincided. With the other 17-mers the difference was not remarkable, but in the case of the longer oligonucleotide used to change Asn170 to Met the deviation was considerable, Td being 10°C lower than Tm.

Once a mutant had been identified, the cells harbouring the clone were streaked out, single colonies picked for template preparation and sequenced across the region of mutation using an appropriate primer for each case. The hybridisation results were confirmed in every occasion.

The mutant genes that coded for β-lactamases with activity properties different from those of the wild type (see Chapter 5) were completely sequenced to ensure that no other change had occurred in the DNA; those having activity similar to the parent enzyme were assumed not to have undergone any other change.

CHAPTER 5

ANALYSIS OF MUTANTS

5.1 INTRODUCTION

As described in Chapter 4, three mutants of the β-lactamase from *B.licheniformis* 6346/c were made in order to study the relationship between structure of the enzyme and its substrate specificity. A preliminary analysis of these "specificity mutants" (Thr59 to Ala, Gly238 to Ala and Lys284 to Stop) concerning their expression in *E.coli* and their activity against a range of substrates is presented here.

The reasons for the design and the outcome of the preliminary analysis of a fourth mutant (Asn170 to Met) are reported in the second part of this Chapter.

5.2 EXPRESSION IN *E.coli*

The expression of the 6346 wild type protein was studied in *E.coli* NM522 (Gough & Murray 1983) harbouring the plasmid pSR81 (see Section 2.3). The mutant genes were also carried by the same plasmid and the protein expressed in *E.coli* TG1 (Gibson 1984). Because the only known genotypic difference between the two strains used is in the F' factor they carry (F'*tra* D36 in TG1) they were considered interchangeable.

5.2.1 Levels of resistance conferred by the mutants

All the mutants were found to confer resistance to the host cells at the normal concentration of ampicillin used for marker selection (40μg/ml). To test the enzyme activity of the mutants relative to that of the wild type enzyme, 1ml aliquots of overnight cultures of cells harbouring the relevant plasmids were sonicated and the activity of the extracts against penicillin G assayed spectrophotometrically (Section 6.17.2). The results of the experiments are shown below in Table 5.1. The marked decrease in activity caused by the Asn170 to Met mutation was expected and will be discussed below. The activity reduction of the Thr59 to Ala mutant was on the other hand puzzling, firstly because this type of change is considered a fairly conservative one and secondly because the position of the residue in the three-dimensional structure of the protein (Figure 1.6) is far from the active site pocket and therefore unlikely to influence directly the activity of the enzyme.

TABLE 5.1 Activity of 6346 mutants against penicillin G

STRAIN	BETA-LACTAMASE	ACTIVITY[a]
NM522	wild type	100
TG1	Gly238 to Ala	100
TG1	Thr59 to Ala	42
TG1	Lys284 to Stop	59
TG1	Asn170 to Met	7
TG1	-	2
NM522	-	2

a Relative to the wild type.

The value corresponding to the Lys284 to Stop mutation is reported here and will be discussed below in Section 5.2.4.

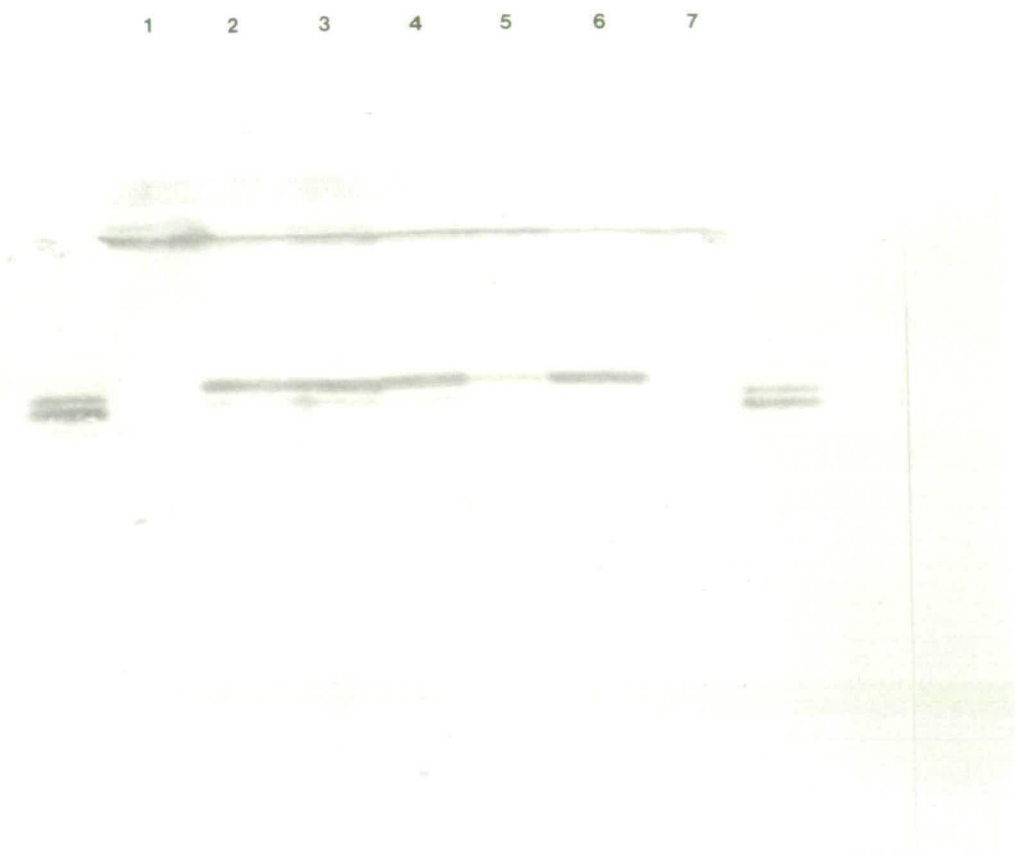
5.2.2 Quantitation of the protein

The different levels of activity found in the mutants could be due to either a genuine alteration of the activity of the enzyme or to the presence in the cells of different amounts of protein. In order to check the possibility that a reduced level of activity was linked to poor expression or instability, it was decided to compare the amount of protein produced by the mutant genes with that of the wild type 6346 β-lactamase.

The wild type protein produced by *E.coli* had shown reactivity to rabbit anti-β-lactamase antibodies similar to that of the secreted enzyme produced by *B.licheniformis* 6346/c, against which the antibodies had originally been raised. This serum was therefore used for the detection of the proteins in Western blots.

Whole extracts of cells containing the mutants and wild type proteins were prepared by simply resuspending cells from 200µl aliquots of overnight cultures in 40µl of loading buffer and boiling for 10 minutes. The sample was then run on an SDS polyacrylamide gel and the separated proteins transferred onto nitrocellulose filter by Western blotting (Section 6.18). After reaction

FIGURE 5.1 Western blot analysis of β-lactamase 6346 mutants.



TRACK	STRAIN	β-LACTAMASE
1	NM522	–
2	NM522	6346 wild type
3	TG1	Gly238 to Ala
4	TG1	Asn170 to Met
5	TG1	Thr59 to Ala
6	TG1	Lys284 to Stop
7	TG1	–

Protein extracts of E.coli cells carrying pSR81 were run on SDS polyacrylamide gel, Western blotted and reacted with anti-6346 β-lactamase antibodies. In the extreme right and left tracks β-lactamase isolated from B.licheniformis 6346/c was used as a control.

with the antibody, high molecular weight cross-reacting material as well as β-lactamase was visible. The amount of this material is equal for all the samples applied to the gel, thus ensuring that no big difference in sample loading had taken place (Figure 5.1). The amount of β-lactamase appears to be the same for the wild type and all the mutants, including the Asn170 to Met, with the only exception of the protein carrying the Thr59 to Ala substitution. We can therefore conclude that except for this last mutation, no changes in β-lactamase expression had taken place for any of the mutants.

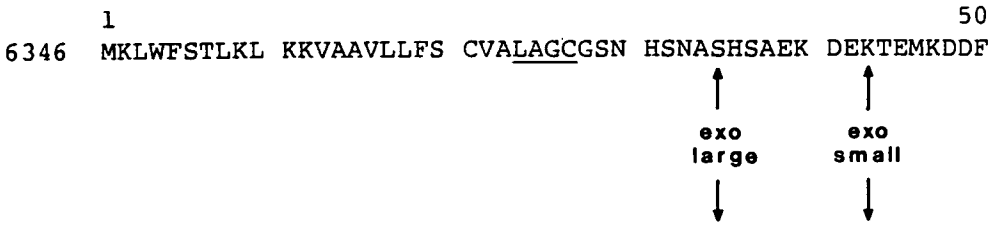
The drop in activity in the case of the Thr59 to Ala mutation could be due either to a lowered protein expression level or to protein instability. Mutant instability is not a new phenomenon in β-lactamases. In the TEM1 enzyme, substitution of Thr71 with any of the other amino acid residues does not normally alter the stability of the protein, but when the disulphide bridge between Cys77 and Cys123 is not present, any change in position 71 results in unstable proteins (Richards, J.H., Fourth β-lactamase workshop, Holy Island, 20th-22nd April 1988). Only a comparison of the messenger RNAs produced by the Thr59 to Ala mutant could tell if expression is altered, but because no changes that could justify a reduced transcription rate or mRNA stability have been introduced in any of the signals preceeding the coding sequence of the gene, and in view of what just said about the TEM enzyme, we favour the possibility of protein instability caused by the introduced change.

5.2.3 Processing of *B.licheniformis* β-lactamase in *E.coli*

The Western blot also showed that the size of the wild type protein expressed in *E.coli* is larger than that of the β-lactamase secreted by *B.licheniformis* 6346/c used as a control. The marker β-lactamase has been prepared as described in Section 6.16.1 from a batch of *B.licheniformis* 6346/c cells grown to logarithmic phase. In the cell supernatant two forms of the enzyme are found with different molecular weight: the amino terminal residue of the smaller form (exo-small) is Lys at position 43 (actual numbering, Fig. 5.2), while the exo-large form starts eight residues upstream at Ser35. The molecular weight of the exo-small enzyme has been calculated to be about 29500 (Lampen et al. 1980).

The larger size of the 6346 wild type enzyme produced in *E.coli* is consistent with the findings of Lai and co-workers (1981) for the expression of

FIGURE 5.2 The amino terminal signal peptide of *B.licheniformis* β-lactamase



The recognition sequence of the glyceryl transferase is underlined. The 749 beta-lactamase cloned in E.coli is known to be anchored to the periplasmic membrane through a glyceryl residue covalently linked to the protein at the cysteine residue contained in this recognition sequence (Lai et al. 1981). The same tetrapeptide is found in the 6346 beta-lactamase. The amino terminal residues of the secreted forms of the enzyme as processed in B.licheniformis, exo-large and exo-small, are also indicated.

the cloned *B.licheniformis* 749 β-lactamase. They demonstrated that in *E.coli* the protein is retained in the periplasm and it is anchored to the outer cell membrane by a glyceride residue covalently linked to Cys27. Despite the numerous amino acid differences found in this region between the 749 and 6346 preproteins, the tetrapeptide Leu-Ala-Gly-Cys, thought to represent the recognition site for the glyceryl transferase, is still present in the 6346 preprotein. It is therefore reasonable to assume that the 6346 β-lactamase is processed in *E.coli* in exactly the same way as the 749 enzyme.

5.2.4 The Lys284 to Stop mutation

The Western blot also revealed some problems with the Lys284 to Stop mutation. The oligonucleotide designed for the construction of this mutant directed a A to T substitution, thus converting the AAA codon for lysine to the ochre stop codon TAA. The resulting β-lactamase would therefore be truncated at position 284, eleven residues upstream the normal carboxy terminus. As it is, the protein expressed in TG1 on SDS polyacrylamide gels does not appear to migrate differently from the 6346 β-lactamase produced by the same strain, seemingly indicating that the two proteins have the same molecular weight.

A reversion of the mutation was ruled out by newly sequencing the mutation region which still showed the presence of the ochre stop codon corresponding to position 284 of the protein sequence. Several explanations could be found for the anomalous behaviour of this protein. TG1 is a *supE* strain, that is it carries a mutation in the anticodon of $tRNA_2^{Gln}$ from CAG to UAG, so that it will insert a glutamine residue at some amber stop codons.

The stop codon of the wild type 6346 β-lactamase and of the Lys284 to Stop mutant are presented below in Table 5.2.

It has been noted before that the $tRNA_2^{Gln}$ can mutate to have a UAA anticodon and therefore suppress ochre stop codons (Inokuchi et al 1979). If such a mutation had occurred in the strain used here, the normal stop codon at position 1155-1157 would also be expected to be partially suppressed in both wild type and mutants. However no other stop codons can be found in frame between this position and the terminator sequence and apart from the main β-lactamase band, no other evident cross-reacting protein band of greater molecular weight appears in the cells carrying the plasmid compared to the pattern of bands given by the host cell alone, suggesting that no suppression giving rise to a β-lactamase of higher molecular weight than normal has taken place at the normal stop codon.

It is also possible that the mutant protein is actually truncated as expected, but runs anomalously on the SDS polyacrylamide gel, migrating in a similar way to the full length protein. Checking these possibilities would imply the sequencing of the carboxy terminus of the Lys284 to Stop mutant protein, an

TABLE 5.2 Stop codons in wild type 6346 and Lys284 to Stop mutant β-lactamases

BETA-LACTAMASE	1119-1121	1155-1157[a]
6346 wild type	AAA	UAA
Lys284 to Stop	UAA	UAA

a Position of codons is numbered as in Figure 3.2.

exercise that requires pure protein in much greater amounts than those currently available. The behaviour of this enzyme however seems to indicate that it is actually distinct from the wild type. Its activity profile is exactly the same as that of the wild type 6346 β-lactamase, but it seems to be less active towards penicillin G than the wild type (Table 5.1) and its sensitivity to iodine is greatly reduced: while the 6346 enzyme is completely inactivated after a 30 minutes treatment, over the same time span the mutant β-lactamase is still 90% active (see below Section 5.3.2).

5.3 PRELIMINARY STUDIES OF ENZYMATIC ACTIVITY

It was already mentioned in Section 1.7 that the most interesting differences between the 749 and 6346 β-lactamases were those concerning their activity against a range of β-lactams, or substrate profile. Generally speaking, the 6346 enzyme is a much better cephalosporinase than the 749 and both the relative rate of hydrolysis and dissociation constants are altered by probably only one of the amino acid changes found between the two enzymes.

5.3.1 Purification of the enzymes

Wild type enzymes were isolated from the *B.licheniformis* 749/c and 6346/c strains as described in Section 6.16.1. The cloned 6346 β-lactamase and the mutants derived from it were partially purified from periplasmic extracts of *E.coli* as follows. Cells from one liter batches of overnight cultures of *E.coli* harbouring the relevant pSR81 plasmid were collected by centrifugation (15 minutes, 6000g) and resuspended in 20ml of ice cold 30mM NaCl, 10mM Tris-HCl pH7.5. The cells were then pelleted by centrifugation (15 minutes, 12000g), resuspended in 20ml of isotonic sucrose solution (20% w/v sucrose in 1mM EDTA, 30mM Tris-HCl pH7.3) and left shaking for 5 minutes at room temperature. After pelleting again, the cells were osmotically shocked by resuspension in 20ml of cold distilled water and left shaking for 5 minutes at 0°c. The cell debris was separated from the soluble periplasmic fraction by centrifugation (15 minutes, 12000g). Most of the proteins left in the supernatant were precipitated by slow addition of ammonium sulphate to 85% saturation at room temperature. The solution was left to equilibrate for 1 hour, then the precipitated protein was separated from the supernatant by centrifugation (30 minutes, 27000g). Under these condition practically all the β-lactamase liberated during the cold osmotic shock was still in the supernatant. The ammonium sulphate was removed from

the sample by extensively dialysing the enzyme solution against 50mM sodium succinate pH4.6. The sample was finally loaded onto a 1ml FPLC S column preequilibrated with 50mM sodium succinate pH4.6. The column was washed with the buffer until no more material absorbing at 280nm was detected in the effluent, then the β-lactamase was eluted with a gradient of NaCl (0 to 300mM in 50mM sodium succinate pH4.6). Fractions of 1ml volume were collected and assayed for β-lactamase activity with nitrocefin. In each case, aliquots of the fraction corresponding to the peak of activity (estimated concentration 3-5 µg/ml) were later used for the activity analysis.

5.3.2 Sensitivity to iodine

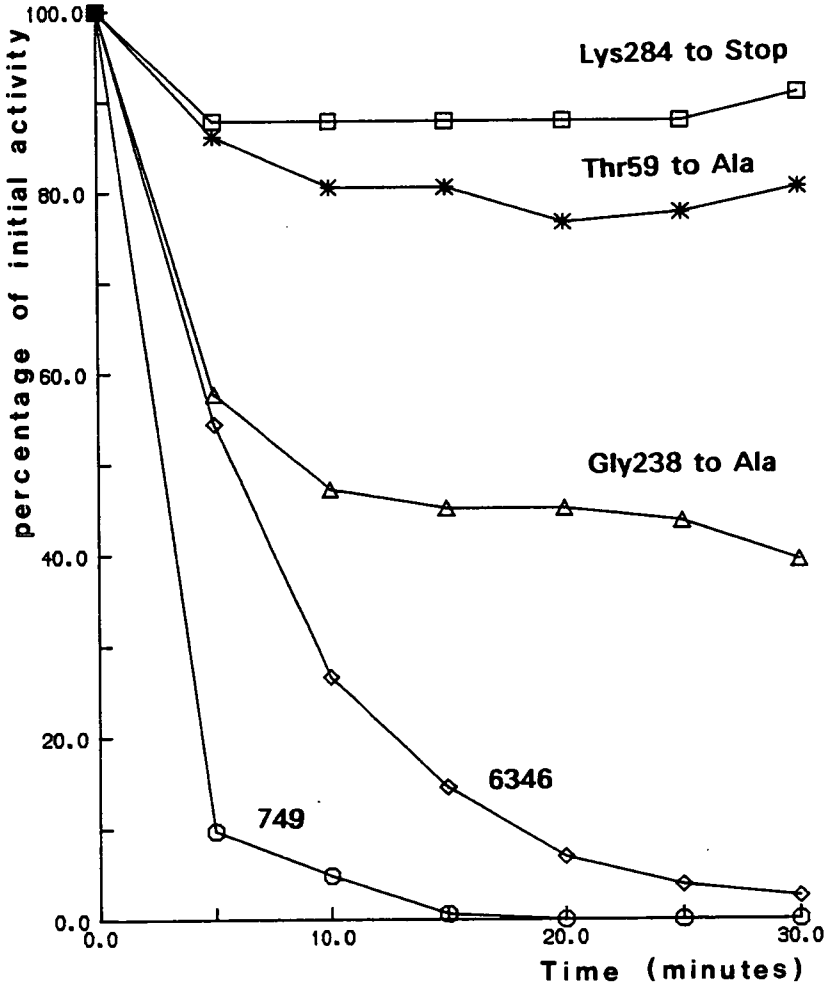
The 749 and 6346 β-lactamases have been reported to differ markedly in their sensitivity to iodine. The 6346 enzyme activity does not decrease greatly after 30 minutes of treatment with iodine, while over the same period of time the 749 β-lactamase shows a drop in activity of more than 50% (Pollock 1965b).

The sensitivity to iodine of the 6346 mutant proteins was tested here and compared to that of the wild type enzyme and of the 749 β-lactamase. During the experiment, the enzymes were treated with I_2 at a final concentration of 20mM in 20mM potassium phosphate buffer pH7.0. At 5 minutes intervals, samples were taken, the iodine neutralized by addition of one tenth of the volume of 200mM sodium thiosulphate and their activity assayed spectrophotometrically. The chromogenic substrate nitrocefin was used for the assays, because its degradation could be followed at 486nm, while most other substrates must be observed in the u.v. region of the spectrum, where any change would be masked by the absorption due to the iodine. The results of these experiments are summarized in Figure 5.3.

It is interesting to notice that contrary to previous observations, both wild type enzymes were found to lose completely their activity during the 30 minutes treatment. The 6346 β-lactamase had a much slower decrease of activity, reaching complete inactivation after about 30 minutes of treatment; the 749 enzyme, on the other hand, showed a sudden drop to about 10% of the original activity in the first 5 minutes of incubation and was not detectable after 15 minutes. Repetition of the experiments on the wild type enzymes showed good reproducibility.

Somewhat surprisingly, three of the 6346 mutants were noted to present a higher degree of resistance to iodine treatment than both the wild type enzymes. Even small changes in the primary sequence of the protein, in this case the Gly238 to Ala and the Thr59 to Ala substitutions, seem to influence the sensitivity of tyrosine residues to iodine in a complex way, probably involving small but important conformational changes in the enzyme. At this stage it is therefore not possible to distinguish between the contributions of the different mutations to the distinct sensitivity to iodine showed by the 749 and 6346 β-lactamases.

FIGURE 5.3 Iodine inactivation of *B.licheniformis* 6346/c β-lactamase mutants



5.3.3 Spectrophotometric assays

The reactions of the wild type and mutant enzymes with a range of β-lactam substrates were followed spectrophotometrically at the wavelengths reported below (Table 5.3).

For each assay 5µl of a 35mg/ml substrate solution were first mixed with the buffer (100 mM sodium phosphate buffer pH 6.8, 3 ml final volume) in a quartz cuvette, then the enzyme was added, mixed well and the progress of the reaction recorded by a Perkin-Elmer spectrophotometer connected to a computer. The recorded data were fitted to the Michaelis-Menten equation with the help of kinetic data analysis programs (Coulson, unpublished) and the V_{max} and K_m calculated. All the reaction progress curves were found to fit well the equation, with the exception of those relative to the hydrolysis of cephalosporin C. With this substrate the progress curves were S shaped, suggesting a complex reaction pathway. Values relative to cephalosporin C have therefore not been reported.

The values of V_{max} and K_m of the wild type 6346 and 749 β-lactamases and of the 6346 mutant enzymes are presented in Tables 5.4 and 5.5. In both tables the values for the wild type enzymes isolated from *B.licheniformis* are found in the first two columns. In the third column the K_m and V_{max} of the 6346 wild type β-lactamase cloned in *E.coli* are reported as a control of the possible influence that the different processing of the protein might have on the activity.

TABLE 5.3 Wavelength at which reactions were followed

SUBSTRATE	λ	Δε (µM)
Penicillin G	232	0.94
6-APA	223	1.2
Ampicillin	235	0.67
Cephaloridine	260	20.83
Cephalosporin C	260	10.43
Cephalosporin PAC	260	8.28

TABLE 5.4 K_m (μM) of mutants derived from 6346/c β-lactamase

SUBSTRATE	749[a]	6346[a]	6346 cloned	Thr59 to Ala	Gly238 to Ala	Lys284 to Stop
Penicillin G	87.5 (4.14)	25.7 (1.06)	40.4 (5.85)	17.6 (2.55)	107.4 (3.19)	24.7 (1.59)
6-APA	31.3 (2.0)	<8	<8	8.05 (1.83)	18.6 (5.4)	8.3 (2.12)
Ampicillin	93.5 (10.59)	35.3 (4.03)	17.4 (2.38)	28.8 (5.22)	60.7 (7.16)	31.2 (1.94)
Cephaloridine	69.6 (0.48)	66.7 (3.36)	55.2 (2.88)	63.8 (2.40)	99.3 (4.32)	85.9 (1.92)
Cephal. PAC	47.9 (2.41)	67.4 (0.72)	46.7 (4.22)	53.5 (1.08)	54.9 (7.85)	52.7 0.60)

a Purified from B.licheniformis strains 749/c and 6346/c.

b In parenthesis the range of the measurements.

TABLE 5.5 V_{max} of mutants derived from 6346 β-lactamase[a]

SUBSTRATE	749[b]	6346[b]	6346 cloned	Thr59 to Ala	Gly238 to Ala	Lys284 to Stop
Penicillin G	100	100	100	100	100	100
6-APA	9.3	19.5	5.6	14.7	5.9	13.2
Ampicillin	59.7	82.6	65.6	89.8	62.2	85.4
Cephaloridine	5.6	46.9	53.3	46.3	16.4	42.2
Cephal. PAC	2.3	32.2	26.3	14.2	2.3	14.5

a Rate relative to penicillin G.

b Purified from B.licheniformis strains 749/c and 6346/c.

It must be noted that, because of the small amount of enzyme available for the cloned enzymes, the measurements could not be repeated, so that the error margin of the affinity constant values are relatively high. The differences in K_m for penicillin G between 749 and 6346 β-lactamases is clearly detectable; as reported before (Pollock 1965b) the affinity constant of the 6346 enzyme for this substrate (25.7μM) is considerably lower than the K_m of 749 (87.5μM). The substrate profiles determined here for the two enzymes also confirmed that 6346 is a much better cephalosporinase than 749; in fact, while the relative rate of hydrolysis of 749 for cephalosporins is only 2-5%, the 6346 β-lactamase breaks down cephalosporin PAC at 32% of the rate at which it hydrolyses penicillin G and destroys cephaloridine at a relative rate of 47%. The substrate profile of the enzyme produced by the 6346 gene cloned in *E.coli* agrees with that of the β-lactamase isolated from *B.licheniformis* 6346/c, confirming that the part of the leader peptide that is lost during maturation of the enzyme in Bacillus but is still present in the *E.coli* produced protein does not interfere with the activity of the enzyme itself. The affinity constants of the *E.coli* and Bacillus enzymes present a greater variation, that can be attributed to the fact that the measurements relative to the cloned 6346 could not be repeated for lack of material.

The Lys284 to Stop mutation, already discussed in Section 5.2.4, does not influence the activity of the enzyme at all and the affinity constants as well as the relative rates of hydrolysis resemble closely those of the wild type 6346 β-lactamase.

The substitution of Thr59 with Ala, although possibly reducing the stability of the enzyme (Section 5.2.2), does not interfere with its activity parameters. Both the affinity constants and the substrate profile of this mutant are very close to those of the parental 6346 enzyme for all the substrates tested.

The situation is very different when Ala replaces Gly238. The activity profile of this 6346 mutant changes drastically, coming to resemble very closely that of the 749 β-lactamase. The relative rate of hydrolysis of cephalosporins decreases from 45-50% to 16% for cephaloridine and from 25-30% to 2% for cephalosporin PAC. The affinity constants for cephalosporins are similar in the two wild type enzymes (about 50μM) and do not appear to be significantly altered by the introduction of the Gly238 to Ala substitution. However, differences in K_m are noticeable for the penicillins. The affinity constant for penicillin G rises four fold in this mutant with respect to the 6346 and the other

mutant enzymes. An increase in K_m is also appreciable in the case of ampicillin and of 6-aminopenicillanic acid, for which it rises about two fold.

5.3.4 Discussion

The data just presented are in agreement with the results of the *in vivo* recombination experiments of Dubnau and Pollock (1965). Enzymes derived by the recombination of an inactive 749 gene with an active 6346 gene could present either of the parental specificity profiles (although the 749 type was much less frequent) but it was not possible to isolate mutant β-lactamases with intermediate enzymatic characteristics (Dubnau & Pollock 1965). It was predicted that the type of activity was most likely determined by only one amino acid position.

It is now clear that the residue in position 238 influences greatly the activity of the *B.licheniformis* β-lactamases. When Gly occupies this position, the enzyme is of the 6346 type, presenting a reduced affinity for penicillin G and relatively high activity against cephalosporins. If residue 238 is an Ala, the rate of hydrolysis of cephalosporins drops considerably, while the K_m for penicillin G increases. This change seems to be necessary and sufficient for the determination of the β-lactamase type in *B.licheniformis*.

Although for the sake of completeness it would be desirable to check the possible influence on activity of all the other changes found in the secreted proteins, we can quite confidently predict that no other substitution is involved in the determination of the activity profile of the *B.licheniformis* β-lactamases. It has already been mentioned that the Thr59 to Ala substitution is located at the other side of the protein molecule with respect to the active site, so that it is no surprise that it exercises no influence on the activity of the enzyme. All the other changes found between the 6346 and 749 β-lactamases are located mainly on the surface of the protein at a distance from the substrate-binding cleft, except for the Gly238 to Ala substitution.

The residue in position 238 is situated at the end of the β3 strand, which forms one side of the active site depression and could conceivably make contacts with the side chain of the substrate. In absence of the atom coordinates of the residues surrounding the active site, it is not possible at this stage to envisage more clearly the role of residue 238. In carboxypeptidase R61 the β3 strand runs antiparallel to the side chain of the substrate at a distance

that would allow hydrogen-bonding (Knox et al. 1987). If the same type of arrangement is assumed for β-lactamases, a change from Gly to Ala at the last position of this β-strand could induce slight changes in the torsion of the strand itself, thus altering the interactions with the substrate side chain. On the other hand, because no evident contact with other parts of the substrate molecule is detectable,it is still not explained why a change in position 238 should determine the relative preference for one β-lactam nucleus with respect to the other one.

In all Class A β-lactamases, except those of *Streptomyces cacaoi* and *St.aureus* which in this region present a single amino acid insertion, position 238 (position 274 in Figure 1.4) is occupied by a Gly, like the 6346 enzyme. It would be informative to introduce the 749 like Ala in one of these other enzymes, for example the TEM enzyme, and study the influence on specificity that such change would have in a non-bacillary β-lactamase.

5.4 THE Asn170 to Met MUTATION

A detailed mechanism of action was proposed for the *St.Aureus* β-lactamase based on the modelling of an ampicillin molecule into the active site of the enzyme (Herzberg & Moult 1987) (see Section 1.5.1). It was suggested that the acylated intermediate of the reaction of β-lactamases with β-lactam substrates is hydrolysed by a molecule of water bound to the enzyme. This molecule would sit in a small depression in the active site cleft, hydrogen bonded to the side chains of Asn170 and Glu166. The polarisation of the water molecule due to the proximity of Glu166 would make it a better nucleophile, capable of hydrolysing the acyl-bond, thus releasing the penicilloic (or cephalosporoic) acid.

The Asn170 to Met mutant was designed to test this hypothesis. It was thought that the substitution of Asn with the bulkier and less hydrophilic Met would disrupt the hydrogen bonding with the water molecule and also make the binding sterically impossible. The consequent absence of the bound water molecule would make the hydrolysis of the acyl-enzyme more difficult, having to rely on the solvent molecules which would be sterically hampered from reaching the acyl-bond. The acyl-enzyme would be much longer lived than in the wild type enzyme and could therefore be "trapped".

5.4.1 Analysis of the Asn170 to Met mutant

Partially purified enzyme was prepared from *E.coli* cells carrying the mutant gene as described above for the other mutants (Section 5.3.1). The enzyme was produced in similar amounts to the wild type β-lactamase (see Figure 5.1) and its activity against penicillin G was reduced by about twentyfold (Table 5.1).

Because of the small amount of enzyme purified it was not possible to carry out a kinetic burst experiment (Hartley & Kilby 1954). So it was decided to qualitatively study the reaction of this enzyme with β-lactam substrates with experiments based on the rationale that if the acyl-enzyme intermediate is longer lived than the wild type one it should be possible to detect it. Two approaches were taken.

In the first experiment an affinity column was synthesized by coupling 6-aminopenicillanic acid to activated Sepharose (6-aminohexanoic acid-activated-Sepharose-4B). If the acyl-intermediate of the mutant enzyme is long lived, then the enzyme sample should either be blocked onto the column or its elution should be retarded with respect to the wild type enzyme. On the other hand mutant enzyme pre-treated with for example penicillin G would already be in the acylated state and therefore would not interact with the 6-aminopenicillanic acid of the affinity column. The column (0.8x1.5 cm) was equilibrated with 0.1M sodium phosphate buffer pH 6.8, and the sample eluted with the same buffer. No difference was noted between the wild type 6346 enzyme and the mutant β-lactamase, either pre-treated or in the absence of penicillin G.

In the second experiment, mutant and wild type enzyme (about 500ng) were mixed with one hundredfold molar excess of [phenyl-4(n)-^{3}H] benzylpenicillin (^{3}H-penicillin G). The sample was immediately applied to a Sephadex G25 column (0.8x11 cm) pre-equilibrated with 0.1M sodium phosphate buffer pH 6.8 and 200µl fractions collected. The elution pattern of the radioactively labelled β-lactam noted by taking 100µl sample of each fraction, mixing it with 4ml scintillation fluid (333ml Triton X100, 667ml toluene, 4.0g 2,5-diphenyl-oxazole, 0.1g 1,4-di-2-(4-methyl-5-phenyloxazolyl)benzene per litre) and counting in a Beckman LS7000 Liquid Scintillation System machine. If the mutant enzyme is actually blocked in its acylated state a small part of the substrate should be found linked to the protein fractions, separated from the bulk

of the free substrate. As expected, when wild type 6346 β-lactamase and ^{3}H-penicillin G were mixed and applied to the column, only one radioactivity peak, corresponding to the processed β-lactam substrate, was found. The same elution pattern was also noted for the Asn170 to Met mutant, where again only one radioactivity peak was detected.

5.4.2 Discussion

With the experiments described above it was not possible to detect a stable or long lived acyl-enzyme as it was expected on the basis of the reaction mechanism proposed by Herzberg & Moult (1987). It is suspected however that in the affinity column experiment the 6-aminopenicillanic acid conjugate could have being sterically inaccessible to the enzyme, thus making the acylation reaction impossible or anyway very difficult. Because there is no easy way to check this eventuality, it would be desirable to repeat the experiment with a different affinity column conjugate, maybe a cephalosporin attached to the activated Sepharose through its 3′ side chain. The β-lactam would therefore be presented to the enzyme in a different orientation from that of the 6-aminopenicillanic acid (or other penicillins) which must be linked to the Sepharose through its 7′ reactive group. In any case before proceeding any further in the analysis of this mutant, it would be imperative to isolate greater quantities of purified enzyme, which would make proper measurements of the kinetics of reaction with β-lactams possible.

CHAPTER 6

MATERIALS AND METHODS

6.1 CHEMICALS

Chemicals used for the experiments described in this thesis were purchased from:

- BDH LTD., Pool, Dorset
- FSA LABORATORIES SUPPLIES, Loughborough Leics
- MAY & BAKER LTD., Dagenham, England
- SIGMA CHEMICAL COMPANY, Pool, Dorset
- BOEHRINGER CORPORATION (LONDON) LTD., Lewes, Sussex

Agarose for routine DNA analysis was obtained from MILES SCIENTIFIC. In experiments where better separation of DNA fragments was needed such as Southern blots, fragment purification and restriction analysis, low electroendoosmosis (EEO) agarose bought from FMC CORPORATION, Marine Colloids div., Rockland, USA was used.

Rolls of nitrocellulose filter were purchased from ANDERMAN & COMPANY LTD., Kingston-upon-Thames, Surrey.

Nitrocefin was a gift from GLAXO GROUP RESARCH LTD.

Radioactively labelled nucleotides were obtained from AMERSHAM INTERNATIONAL PLC., Amersham, Buckingamshire.

Oligonucleotides used for priming and for the site-directed mutagenesis (SDM) experiments were synthesised on a Applied Biosystem DNA synthesizer with the phosphoramidite method by the OSWEL DNA SERVICE of the University of Edinburgh.

6.2 ENZYMES

Restriction enzymes and most of the DNA modifying enzymes were obtained from:

- AMERSHAM INTERNATIONAL PLC., Amersham, Buckingamshire
- BOEHRINGER CORPORATION (LONDON) LTD., Lewes, Sussex
- NEW ENGLAND BIOLABS, CP Laboratories, Bishop's Stortford, Herts

6.3 BACTERIAL STRAINS

Bacillus licheniformis strains 749/c and 6346/c (Dubnau & Pollock 1965) were obtained from Miss J. Fleming, this department. *Escherichia coli* strains: see Table 6.1

TABLE 6.1 Strains of *Escherichia coli* K12

STRAIN	GENOTYPE[a,b]	REFERENCE[c]	SOURCE
C600	*supE44, tonA21, thr1, leuB6, thi1, lacY1*	1	N.E.Murray
ED8654	*supE44, supF, hsdR514* M^+ S^+, *met*$^-$, *trpR*	2	N.E.Murray
NM522	Δ(*lac-proAB*), *supE, thi, hsdD5,* [F' *proAB*, *lacI*^{q}, *lacZ* ΔM15]	3	N.E.Murray
TG1	Δ(*lac-proAB*), *supE, thi, hsdD5,* [F' *traD36, proAB, lac*^{q}, *lacZ* ΔM15]	4	Amersham
JM109	*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1,* λ^-, Δ(*lac-proAB*), [F' *traD36, proAB, lacI*^{q}, *lacZ* ΔM15]	5	A.Robinson

a Genotype symbols according to Bachmann (1983).

b Genotype nomenclature according to Demerec et al. (1966); Novick et al. (1976).

c 1. Appleyard 1954; 2. Borck et al. 1976; 3. Gough & Murray 1983; 4. Gibson 1984; 5. Yanisch-Perron et al. 1985.

6.4 PLASMIDS AND BACTERIOPHAGES

See Tables 6.2 and 6.3.

6.5 MEDIA AND ANTIBIOTICS

Dried media were purchased from OXOID LTD, Basingstoke, Hants., England and from DIFCO LABORATORIES, Michigan, USA.

Unless otherwise stated the following quantities are per liter of media.

L-BROTH Difco Bacto-tryptone 10g, Difco Bacto yeast extract 5g, NaCl 5g, adjusted to pH7.2.

TABLE 6.2 Plasmids

PLASMID	RELEVANT MARKERS	REFERENCE[a]	SOURCE
pACYC184	Cm^r Tet^r	1	N.E.Murray
pBR322	Amp^r Tet^r	2	N.E.Murray
pAT153	Amp^r Tet^r	3	N.E.Murray
pTZ19R	Amp^r *lacZ*	4	D.J.Finnegan
pACYCbla	Amp^r Tet^r	-	this work
pAD19R	Tet^r *lacZ*	-	this work
pSR81	Tet^r Amp^r	-	this work

a 1. Chang & Cohen 1978; 2. Bolivar et al. 1977; 3. Twigg & Sherratt 1980; 4. Mead et al. 1986.

TABLE 6.3 Bacteriophages

PHAGE	DESCRIPTION	REFERENCE[a]	SOURCE
λNM1149	immunity insertion vector with single sites for *Eco* RI and *Hind* III	1	N.E.Murray
M13mp18	*lacZ*, used for sequencing	2	N.E.Murray
M13K07	Km^r, helper phage for pTZ19R and derived plasmids	3	D.J.Finnegan
λpen	λNM1149 recombinant carrying 749 β-lactamase gene	4	W.Brammar
λcI857	used as DNA size marker in restriction experiments	laboratory stock	N.E.Murray
λ1149pen	λNM1149 recombinant carrying 6346 β-lactamase gene	-	this work

a 1. Murray et al. 1977; 2. Yanisch-Perron et al. 1985; 3. Mead et al. 1986; 4. Brammar et al. 1980.

2xTY BROTH Difco Bacto-tryptone 16g, Difco Bacto yeast extract 10g, NaCl 5g.

SOB BROTH Difco Bacto-tryptone 20g, Difco Bacto yeast extract 5g, NaCl 0.6g, KCl 0.5g. Just prior to use add 10ml of a stock solution consisting of 1M $MgCl_2$ and 1M $MgSO_4$ sterilized by filtration.

CH/S BROTH (Pollock 1965) 1% w:v Difco casaminoacids, 0.02M potassium phosphate buffer pH7.2, 0.1% v:v Pollock's salts ($MgSO_4$-$7H_2O$ 25g, $FeSO_4$-$7H_2O$ 100mg, $ZnSO_4$-$7H_2O$ 100mg, $MnSO_4$-$4H_2O$ 10mg, $CuSO_4$-$5H_2O$ 1mg, $Na_2Cr_2O_7$ 0.2mg, HCl 1ml dissolved in 100ml H_2O)

NUTRIENT BROTH Oxoid nutrient broth 25g, adjusted to pH7.2.

L PLATES Difco agar 15g, NaCl 5g in L-broth.

BBL PLATES Baltimore Biological Laboratories trypticase 10g, NaCl 5g, Difco agar 10g.

BBL TOP Baltimore Biological Laboratories trypticase 10g, NaCl 5g, Difco agar 6.5g.

MINIMAL MEDIUM PLATES Difco agar 15g, 10ml 20% glucose, 250µl 0.5% w:v thiamine, 200ml 5x Spizizen salts.

5x SPIZIZEN SALTS (Anagnostopoulos & Spizizen 1961) K_2HPO_4-$3H_2O$ 91.5g, KH_2PO_4 30g, $(NH_4)_2SO_4$ 10g, trisodium citrate-$2H_2O$ 5g, $MgSO_4$-$7H_2O$ 1g, adjusted to pH7.2.

PHAGE BUFFER KH_2PO_4 3g, Na_2HPO_4 7g, NaCl 5g, 0.1M $MgSO_4$ 10ml, 0.01M $CaCl_2$ 10ml, 1% w:v gelatin 1ml.

All the antibiotics were obtained from the Royal Infirmary, Edinburgh. Stock solutions were prepared as follows and stored at -20^0C.

Tetracycline	10mg/ml	in 50% v:v ethanol
Kanamycin	10mg/ml	in water
Ampicillin	100mg/ml	in water
Chloramphenicol	10mg/ml	in water

6.6 STOCK SOLUTIONS

TE 10mM Tris-HCl, 1mM EDTA, adjusted to pH8.0 with concentrated HCl

SEQUENCING TE 10mM Tris-HCl, 0.1mM EDTA, adjusted to pH8.0 with concentrated HCl

TM 100mM Tris-HCl, 50mM $MgCl_2$,adjusted to pH8.5 with concentrated HCl

BCIG 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside 25mg/ml in dimethylformamide

IPTG isopropyl-β-D-thiogalactopyranoside 25mg/ml in water

PEG 20% w:v PEG 6000, 2.5M NaCl, made daily

FORMAMIDE DYES 98% v:v deionized formamide, 0.1% w:v bromophenol blue, 0.1% w:v xylene cyanol, 10mM EDTA

10x TBE 890mM Tris, 890mM boric acid, 25mM EDTA

40% ACRYLAMIDE STOCK 38% w:v acrylamide, 2% w:v bis-acrylamide. Stir with Amberlite MB-1 (20g/l), filter through Whatman filter paper.

0.5x TBE GEL MIX 40% acrylamide stock 150ml, 10xTBE 50ml, urea 460g

5x TBE GEL MIX 40% acrylamide stock 150ml, 10xTBE 500ml, urea 460g, bromophenol blue 50mg

6x AGAROSE LOADING BUFFER 40% w:v sucrose, 0.25% w:v bromophenol blue

DMSO Dimethylsulphoxide from a new bottle was dispensed into plastic universal bottles under N_2 and stored at -70°C. For daily use small aliquots were dispensed in microfuge tubes and stored at -70°C.

TFB 10mM KMES, 100mM KCl, 45mM $MnCl_2$, 10mM $CaCl_2$, 3mM $HACoCl_3$, filter sterilized

20x SSC 3M NaCl, 0.3M trisodium citrate

20x SET 3M NaCl, 20mM EDTA, 0.4M Tris-HCl pH7.8

PHENOL All the phenol used for the experiments described in this thesis was redistilled under N_2 atmosphere. The redistilled liquid was collected into 400ml of 1M Tris-HCl pH7.5 buffer containing 1.5g 8-hydroxyquinoline (quantities relative to 5Kg of starting phenol) and left to stir for about 30 minutes. The mixture was stored in plastic bottles in the dark at -20°C.

6.7 HANDLING OF BACTERIAL STRAINS

For long term storage of *E.coli* strains, 5ml of L-broth were inoculated with a single colony and shaken overnight at 37°C. The antibiotic resistance was checked when appropriate. Aliquots of the culture (1.5ml) were spun down in microfuge tubes and the cell pellet resuspended in 1 ml 10 mM $MgSO_4$. This cell suspension was then mixed with 2 ml of 80% v:v glycerol and stored at -20°C.

For daily use a drop of the glycerol stock was streaked on an L-broth agar plate supplemented with the appropriate antibiotic when necessary. Under these conditions bacteria remained viable for about a month.

B.licheniformis strains were kept as spores at 4°C. The viability of the spores does not decrease over many years, which makes them very convenient for long term storage. To induce sporulation a single colony was used to inoculate 100-200 ml of nutrient Oxoid broth in a 2l conical flask. The culture was left to shake at 37°C for 5 to 7 days; the sporulation process was followed by examining a thin film of culture under a microscope. The cells and spores were pelleted in a bench centrifuge and washed in sterile water at least 4 times. This treatment removes all traces of nutrients and also kills by osmotic shock the remaining bacterial cells. The spores were finally resuspended in 5-10ml of sterile distilled water and heated for 1 hour at 60°C in a water bath. They were washed again in water 3 times and finally resuspended in water at a concentration of about $3x10^9$ cfu/ml.

Unless otherwise stated, liquid cultures were started by inoculating 5ml of broth with a single colony or, in the case of *B.licheniformis*, with 100µl of spore suspension and left to grow overnight at 37°C with moderate shaking. This starter was then normally diluted 1:100 in broth and grown in a rotary shaker at 37°C in a conical flask of volume 5 to 10 times bigger than the culture itself. When appropriate, antibiotics were added to obtain the following final concentrations (expressed in µg/ml):

Chloramphenicol	50
Ampicillin	40
Tetracycline	20
Kanamycin	50

6.8 HANDLING OF BACTERIOPHAGES

6.8.1 Storage of Bacteriophage λ

Single plaques of bacteriophage λ from a fresh plate containing 10^5-10^6 pfu were picked using a sterile pasteur pipette and mixed with 500µl of phage buffer. After addition of one drop of chloroform this stock can be stored for 1-2 months at 4°C.

6.8.2 Plating bacteriophage λ

The appropriate phage dilution (100µl) was used to infect 200µl of an overnight culture of host cells to which 30µl 100mM $MgSO_4$ had been added. The culture was left at room temperature for 15-20 minutes, then mixed with BBL top agar (3ml), molten and left to cool to about 45°C, and poured on a BBL plate. The plate was inverted and incubated at 37°C overnight.

6.8.3 Plate lysates

Concentrated phage stocks were obtained by plating about $5x10^5$ pfu on a fresh moist plate. The growth was followed and normally stopped after 6-7 hours when small, just confluent plaques were visible. To harvest, the plate was covered with 3-4ml of L-broth and left at 4°C overnight for the phage to diffuse into the liquid. The broth was then pipetted off and the cells eliminated by adding a drop of chloroform and spinning in a bench centrifuge for 10 minutes. Normally titers 10^9-10^{10} pfu/ml were obtained with this method.

6.8.4 Bacteriophage M13 storage

Bacteriophage M13 was usually stored as frozen single stranded DNA prepared as from Section 6.9.6. When fresh bacteriophage was needed, 2µl of DNA were used to transform 200µl of competent *E.coli* NM522 or TG1 cells (Section 6.11). Typically at least 100-200 plaques were obtained from one single transformation.

6.8.5 Plating of bacteriophage M13

Bacteriophage M13 was plated on lawns of *E.coli* NM522 or TG1 cells by mixing 100µl of the appropriate phage dilution with 200µl of a fresh overnight culture of plating cells and 3ml of BBL top agar molten and cooled to about 45°C and plated on minimal medium plates. The phages were left to grow on the inverted plate at 37°C overnight. When a blue to white selection of recombinant phages was required, 30µl of the β-galactosidase inducer solution (IPTG) and 30µl of the coloured substrate (BCIG) were added to each plate.

6.9 DNA ISOLATION

6.9.1 Plasmid DNA purification: 1ml scale

Small scale plasmid DNA purification was used mainly for recombinant analysis after cloning experiments. Single colonies of putative recombinants were grown overnight at 37°C with shaking. This culture (1.5ml) was then treated as follows (Ish-Horowicz & Burke 1981):

1. The cells were harvested by spinning in a bench centrifuge for 5 minutes and resuspended in 100µl lysis solution (50mM glucose, 20mM Tris-HCl pH8.0, 10mM EDTA, 0.1% w:v lysozyme added just prior to use) and incubated for 5 minutes at room temperature.

2. Alkaline SDS solution (200µl 0.2N NaOH, 1% w:v SDS, made weekly) were added, mixed gently by inverting the tube a few times and left on ice. After 5 minutes 150µl precooled 3M potassium acetate pH4.8 were added, mixed gently and the tube was left for 5 minutes more on ice. The precipitated protein, SDS and chromosomal DNA were then removed by centrifugation for 10 minutes. The supernatant was transferred to a clean tube and spun again in order to make sure that no precipitate was carried over to the next step.

3. The nucleic acid was then ethanol precipitated, the pellet washed with 70% v:v ethanol, vacuum dried and resuspended in 30µl TE. Typically 8µl portions were used for each restriction reaction and any remaining solution was stored at -20°C.

6.9.2 Plasmid DNA purification: large scale

This is a scaled up version of the method described above for small scale isolation of plasmid DNA including a final density gradient centrifugation

step.

1. An overnight culture (2ml) of cells harbouring the plasmid was used to inoculate 200ml of L-broth supplemented with the appropriate antibiotic for plasmid selection and shaken overnight at 37°C in 2l conical flask.

2. The cells were harvested by centrifugation for 10 minutes at 10000g and the pellet resuspended in 7ml of lysis solution (50mM glucose, 20mM Tris-HCl pH8.0, 10mM EDTA, 0.1% w:v lysozyme added just prior to use). After 5 minutes incubation on ice, 14ml 0.2M NaOH, 1% w:v SDS was added, mixed gently and left on ice for 10 minutes. Lastly 10.5ml of 3M potassium acetate pH4.8 was added and after 5 minutes on ice the tangle of proteins, SDS and chromosomal DNA precipitated by centrifugation for 30 minutes at 16000g.

3. The supernatant was carefully collected through a funnel fitted with a plug of silanised glass wool prewashed with ethanol and air dried which prevents contamination by the precipitate. The plasmid DNA was precipitated by addition of 50ml of propan-2-ol and incubation on ice for at least 30 minutes. The DNA was pelleted by centrifugation for 15 minutes at 16000g, washed with 10ml 70% v:v ethanol and vacuum dried for about 30 minutes.

4. The pellet was then redissolved in 9.4ml TE, 0.6ml ethidium bromide (10mg/ml) and 9.5g of CsCl was added. An 11.5ml polyallomer Sorvall tube (crimp seal) was filled with the mixture and when necessary the weight adjusted by small additions of 0.95g/ml of CsCl in TE. The closed-circular supercoiled plasmid DNA band was separated from the RNA (bottom pellet) and the nicked and linear plasmid DNA (top band) after a 60 hour spin in a 50Ti fixed angle rotor at 38000rpm at 18°C.

5. After separation the plasmid band was visualised under u.v. light and removed with a sterile plastic syringe. The ethidium bromide was extracted by adding to the DNA solution equal volumes of propan-2-ol saturated with TE and NaCl until both organic and aqueous layers were colourless. Two volumes of water were then added to the aqueous phase in order to lower the concentration of salt present in solution. The DNA was ethanol precipitated and resuspended in 500µl of TE.

6. Any remaining contaminating protein was eliminated by phenol extraction and a final ehtanol precipitation. After it was vacuum dried the DNA pellet was resuspended in 100-500µl of TE and stored at -20°C.

6.9.3 Preparation of bacterial chromosomal DNA

Chromosomal DNA of bacteria was prepared following an adaptation of

the method of Marmur (1961).

1. A small (5ml) overnight culture of the cells was used to inoculate 500ml of L-broth and left to grow at 37°C with vigorous shaking overnight.

2. The cells were harvested by centrifugation for 15 minutes at 16000g and resuspended in 25ml of sucrose solution (25% w:v sucrose in 50mM Tris-HCl pH8.0, 250mM EDTA pH8.0) to the cells, which were left on ice until lysis occurred, normally within 5-10 minutes.

3. Half a volume of 0.25M EDTA pH8.0 was added and the cells left on ice for 5 minutes.

4. One volume of Triton solution (2% Triton X-100, 62.5mM EDTA, 50mM Tris-HCl pH8.0) was added and the mixture left on ice for 20 minutes.

5. One fifth of the volume of 5M $NaClO_4$ and one volume of CIA (chloroform:isoamylalcohol 24:1) were added.

6. The mixture was spun in a bench centrifuge for 5 minutes, the aqueous top layer transferred to a small beaker and 2 volumes of ethanol added; the precipitated DNA was wound around a glass rod and dissolved in 0.1xSSC. Once dissolved the solution was made up to 1xSSC by adding one tenth of the volume of 10xSSC and shaken for 15 minutes with an equal volume of CIA. The procedure for protein extraction described in this point was repeated until little or no protein was left on the interface. The DNA was finally resuspended in a small volume (i.e. 2ml) of 0.1xSSC.

7. Ribonuclease solution (20mg/ml in 0.15M NaCl pH5.0, preheated for 10 minutes at 80°C and stored at -20°C) was added to a final enzyme concentration of 50µg/ml and left to react for 30 minutes at 37°C.

8. Finally the DNA solution was phenol extracted twice and dialysed extensively against 10mM Tris-HCl pH8.0.

6.9.4 Large scale preparation of bacteriophage λ DNA

1. A fresh overnight culture of host cells (10ml) was diluted into 200ml of L-broth supplemented 10mM Mg^{2+} in a 2l flask and shaken at 37°C. The growth of the culture was followed at OD_{650}. When it reached about 0.5, 4-6x10^{10} phages were added and shaking continued. After rising to about 2, the OD_{650} dropped to about 0.5 when the cells lysed. At this point 0.5ml of chloroform was added and the culture shaken for a further 10 minutes.

2. NaCl (8g) and DNAse and RNAse to 1µg/ml final concentration were added and the culture was left to stand for 1 hour at

room temperature. The cell debris was eliminated by centrifuging the culture for 10 minutes at 16000g.

3. To the supernatant containing the phage 20g of PEG 6000 was added, slowly dissolved and left standing overnight at 4°C.

4. The following day the phage precipitate was collected by centrifugation for 10 minutes at 16000g. The supernatant was discarded, while the phage pellet was resuspended in 5ml of phage buffer by gently shaking the centrifuge bottles at 4°C for 2 hours.

5. The phage suspension was then layered onto a CsCl step gradient in a 14ml polyallomer tube. The gradient, proceeding from bottom to top of the tube, was formed by 1.5ml of 1.7g/ml, 2ml of 1.5g/ml and 2ml of 1.3g/ml solutions of CsCl in phage buffer.

6. After a 2 hour spin at 38000rpm in a 6x250 rotor (swing-out buckets), the phage band was visible to the naked eye and could be collected with a syringe by side puncture.

7. The sample was dialysed extensively against TE in order to eliminate the CsCl and phenol extracted. The sample was finally dialysed again against TE. This procedure routinely yielded a few hundred micrograms of DNA, depending on the phage used.

6.9.5 Bacteriophage M13: RF DNA purification

1. A fresh single plaque of bacteriophage was used to infect 1.5ml of a culture of *E.coli* host cells grown in L-broth to OD_{650}=0.3. The infected cells were grown in a large (20ml) glass test tube at 37°C with vigorous shaking for approximately 4 hours.

2. The culture was then transferred to a microfuge tube and spun for 5 minutes. The titer of phage in the supernatant was checked. This mini-lysate was usually found to contain about $1x10^{11}$pfu/ml.

3. A culture of host cells grown in L-broth to OD_{650}=0.2 was infected with the mini-lysate at a final concentration of $1x10^{9}$pfu/ml and left to grow overnight at 37°C in a 500ml flask with very good aeration.

4. The following day the cells were pelleted by centrifuging for 10 minutes at 10000g and the supernatant titered again.

5. Finally a 500ml culture of *E.coli* host cells grown in L-broth to OD_{650}=0.1 was infected with the phage at a final concentration of $1x10^{10}$pfu/ml. The infected cells were grown for 2 hours, after which time most of the phage was expected

to be in its replicative double stranded form (RF) inside the host cells.

6. The isolation of the RF of the bacteriophage followed the same procedure as for plasmid isolation (see Section 6.9.2).

6.9.6 Bacteriophage M13: single stranded DNA purification

1. A fresh single plaque of the bacteriophage was toothpicked into 1.5ml of 1:100 dilution of overnight culture of *E.coli* host cells in a 20ml sterile glass test tube. The infected cells were grown for 4.5-5.5 hours at 37°C with very vigorous shaking.

2. The culture was transferred to a microfuge tube and spun for 5 minutes in a bench centrifuge. The supernatant was transferred to a clean tube and the process repeated again in order to eliminate any remaining cells.

3. To the supernatant 150µl of PEG (20% w:v PEG 6000, 2.5M NaCl made daily) was added, the tube inverted a few times and then left to stand for 10 minutes at room temperature. The phage was precipitated by centrifugation for 10 minutes. The supernatant was carefully sucked off with a drawn out pasteur pipette attached to a vacuum pump. Routinely the tube was spun again briefly and all residual PEG solution sucked off.

4. The phage pellet was resuspended in 100µl sequencing TE, phenol extracted and ethanol precipitated. The final DNA pellet was left to dry under vacuum, resuspended in 30µl of sequencing TE and stored at -20°C. Typically 8µl of this solution was used for one sequencing reaction (see Section 6.12.2).

6.9.7 pTZ19R derived plasmids: single stranded DNA

1. pTZ19R transformed host cells (NM522 or TG1) were grown to OD_{650}=0.5-0.6 in 2xTY media supplemented with 0.001% thiamine and the appropriate antibiotic. Cells from this culture (2ml) were infected with M13K07 helper phage at a final concentration of $2x10^9$pfu/ml (m.o.i.=10) and shaken vigorously for 1 hour at 37°C. Infected cells (800µl) were used to inoculate 20ml 2xTY media supplemented with kanamycin to 70µg/ml and grown overnight at 37°C with very good aeration.

2. The following day all the cells were removed by centrifuging the culture twice for 10 minutes each time at 12000g. In a 30ml Corex tube 4ml of PEG solution (20% w:v PEG 6000, 2.5M NaCl, made daily) were added to the supernatant, the tube inverted a few times and left to rest for 15 minutes at room temperature.

3. The phage was precipitated by centrifugation at 27000g for 15 minutes, the PEG solution carefully removed and the phage pellet resuspended in 400µl sequencing TE.

4. The coat proteins were eliminated by phenol extraction, the DNA was ethanol precipitated, dried, resuspended in 30µl sequencing TE and stored at -20°C. Routinely 8µl of this DNA solution were used for each sequencing reaction.

When single stranded DNA was needed for SDM experiments, a cleaner preparation was needed. One extra PEG precipitation was introduced in the above procedure as follows: the first phage pellet was resuspended in 1ml sequencing TE, 200µl PEG solution were added, the tube left at room temperature for 15 minutes and spun for 10 minutes in a microcentrifuge. The second phage pellet was then resuspended in 100µl sequencing TE and treated as above at point 4.

6.10 DNA HANDLING

6.10.1 Phenol extraction

Redistilled phenol was used to remove contaminating proteins from DNA solutions (Maniatis *et al*. 1982). As a rule one volume of phenol was added to the DNA in a Corex or plastic microfuge tube, depending on the volume, and mixed gently by rolling or inverting the tube several times. The organic layer was separated from the aqueous by low speed centrifugation for 5 minutes and discarded. The procedure was repeated two or three times until both layers were clear and no protein was visible in the interface. The phenol treatment was followed by one extraction with one volume of phenol:CIA 1:1 mixture (CIA=chloroform:isoamylalcohol 24:1) and finally by one extraction with one volume of CIA to remove residual phenol.

6.10.2 Precipitation of DNA

Phenol extraction of DNA solutions was normally followed by ethanol precipitation to further clean and to concentrate the DNA. This method was also used when a quick change of buffer was required (Maniatis *et al*. 1982).

Before precipitating, the DNA solution (normally in TE) was supplemented with ions by adding one tenth volume of 3M potassium acetate pH4.8. Cold

ethanol (-20°C 2 volumes) was added to the DNA mixed by inverting the tube and incubated either for 1 minute in a dry ice/methanol bath or for 15 minutes at -70°C. The precipitated DNA was collected by centrifuging for 5 minutes at 12000g and, after discarding the supernatant, washed with cold 70% v:v ethanol. After spinning again for 3 minutes the supernatant was discarded and the pellet vacuum dried and resuspended in the appropriate buffer.

TABLE 6.4 Buffer conditions used for restriction digests

LOW SALT	MEDIUM SALT	HIGH SALT
Acc II	*Acc* I	*Bam* HI
Alu I	*Ava* I	*Bgl* II
Dra I	*Ava* II	*Eco* RV
Hha I	*Eco* RI	*Sal* I
Kpn I	*Hae* II	*Sau* 3AI
Pvu II	*Hae* III	*Sca* I
Sac I	*Hinc* II	*Ssp* I
Sma I[a]	*Hind* III	*Stu* I
	Nae I	*Xba* I
	Pst I[a]	*Xho* I
	Ppu MI	

a KCl was used instead of NaCl for SmaI and ammonium sulphate for PstI. For composition of buffers see text.

6.10.3 Restriction analysis

Routinely 0.5-1µg of DNA was cut with the restriction enzyme in a small final volume (10-20µl) with 5 units of enzyme in the appropriate restriction buffer. Incubation of the reaction was normally for 2 hours at 37°C except for *Sma* I which was incubated at 25°C. Depending on the conditions for optimum activity required by the enzyme one of three buffers containing low, medium or high salt concentration was used (Table 6.4). The buffers were stored as stock

solutions of 10x strength at -20°C. The compositions of the three buffers were:

- **Low salt** 100mM Tris-HCl pH7.4 100mM $MgSO_4$, 20mM NaCl, 1mM DTT
- **Medium salt** 100mM Tris-HCl pH7.4, 100mM $MgSO_4$, 50mM NaCl, 1mM DTT
- **High salt** 500mM Tris-HCl pH7.4, 100mM $MgSO_4$, 100mM NaCl

When double digests were needed, the enzyme requiring the lower salt concentration was left to react first. After 2 hours the higher salt concentration buffer and the second enzyme were added and incubation resumed.

6.10.4 Filling-in sticky ends

When blunt ended DNA fragments were needed but the DNA could conveniently be restricted only with an enzyme producing 3' recessed ends (sticky ends), the sticky ended fragments were made blunt ended by a filling-in reaction (Maniatis *et al*. 1982).

1. After restriction the DNA (1µg) was phenol extracted and ethanol precipitated to remove any trace of the enzyme.
2. The DNA pellet was resuspended in 2µl 10xNT buffer (500mM Tris-HCl pH7.2, 100mM $MgSO_4$, 1mM DTT, 500µg/ml bovine serum albumin), 1µl each 2mM dNTPs as needed depending on the sequence of the overhang, 5 units polymerase I Klenow fragment and water to 20µl final volume. The reaction was incubated for 30 minutes at room temperature.
3. Before proceeding to any following manipulation of the DNA (for example ligation), the enzyme was denatured by incubation of the reaction mixture at 65°C for 10 minutes followed by phenol extraction and ethanol precipitation.

6.10.5 Electrophoresis on agarose gels

Analysis of DNA restriction fragments was carried out on 0.7% w:v agarose 1xTBE gels in an apparatus in which the gels were totally submerged (constructed in this laboratory from perspex).

To pour the gel, agarose was added to the buffer and dissolved by boiling in a microwave oven. The cleared solution was left to cool to about 50°C and poured on a slab forming plate fitted with a well forming comb. After the

gel had set, the comb was removed and the gel submerged in 1xTBE buffer to which the fluorescent dye ethidium bromide had been added to a final concentration of 0.5µg/ml.

DNA samples were mixed with one third volume of loading buffer (6x loading buffer: 40% w:v sucrose, 0.25% w:v bromophenol blue, stored at 4°C), pipetted in the sample wells and run into the gel at high voltage. When the blue dye had migrated into the gel, the voltage was lowered to about 70-100 volts for big (150-200ml) gels and 40-50 volts for minigels (25ml).

When separation was completed the gel was removed from the apparatus, viewed under u.v. light and when necessary photographed using Ilford FP4 film through a red filter (Kodak Wratten filter no.9).

6.10.6 Recovery of DNA from agarose gels

When purified DNA fragments were required, the restricted sample was run as above on 0.7% w:v low EEO agarose gels. On this type of agarose DNA fragments migrated as sharper bands, ensuring separation of fragments of similar size.

The gel was viewed under u.v. light and the target fragment band excised from it with a razor blade, making sure that the minimum of surrounding agarose was included. The gel slice was placed in sterile dialysis tubing together with a small volume (0.5-1ml) of 1xTBE making sure not to trap any air bubbles, placed on a submerged gel apparatus and covered with the same buffer. The DNA was electrophoresed out of the gel onto one side of the tubing at 200 volts for 1 hour, then into the buffer by inverting the current for 1 minute.

The buffer surrounding the gel slice was carefully collected, the slice and the tubing rinsed with a small volume of clean buffer which was also saved. The DNA solution was phenol extracted, ethanol precipitated and resuspended in the appropriate buffer. Typically about 50% DNA recovery was obtained.

6.10.7 DNA ligation

For ligation the restricted DNA was normally diluted to about 100ng/µl. The reaction was carried out in a 10µl volume containing the vector and insert DNAs, 1µl 10mM ATP, 1µl 100mM DTT, 1µl 10x ligation buffer (500mM Tris-HCl

TABLE 6.5 Ligation reactions and controls

VECTOR	INSERT	LIGASE	DTT	ATP	BUFFER	WATER	REMARKS
-	1	-	1	1	1	6	cutting control
1	-	-	1	1	1	6	cutting control
1	-	1	1	1	1	5	ligation control
1	0.1	1	1	1	1	5	series of ligations
1	1	1	1	1	1	4	
1	5	1	1	1	1	-	

a All volumes are given in microlitres. Total volume of each reaction cocktail always equal to 10 microliters. For composition of stock solutions see text.

pH7.5, 100mM $MgCl_2$, 2mM spermidine) and T4 ligase. Typically 0.01 units of ligase were used for sticky ended ligation in a 10µl reaction mix, while blunt ended reactions required 1 unit of the enzyme. In both cases the ligation mixture was incubated overnight at 15°C. A series of controls were set up alongside a number of ligation reactions where different ratios of vector to insert DNA were used (Table 6.5). Half of each reaction mixture was used to transform an appropriate *E.coli* host strain (Section 6.11).

6.11 RECOVERY OF RECOMBINANTS

Recombinant DNA molecules (plasmid or phage) were rescued by transforming with the ligation mixture appropriate host cells treated to make them competent and plating them on selective plates.

6.11.1 Preparation of competent *E.coli* cells

The method used throughout this work to make competent *E.coli* cells was that devised by Hanahan (1983). Fresh competent cells were made every time they were needed and used immediately.

1. Typically 50ml of L-broth were inoculated with a single

colony of the host strain taken from a fresh plate and shaken at 37°C until the OD_{650} was 0.6-0.7. The culture was transferred immediately to chilled sterile universal bottles and left on ice for 15 minutes.

2. The cells were collected by centrifugation in a bench centrifuge at 4°C for 5 minutes and, after removing carefully all the supernatant, resuspended in 25ml of cold TFB buffer (see below) and left for 5 minutes on ice.

3. After spinning for 5 minutes at 4°C, the supernatant was removed and the cells resuspended in 4ml of cold TFB buffer. Immediately 280µl DMSO was added and mixed well with the cells. After 5 minutes on ice, 280µl DTT was added, the culture swirled to mix it well and left on ice for 10 minutes. Finally 280µl DMSO was mixed with the cells and after 5 minutes more on ice 200µl aliquots of the competent cells were dispensed into chilled sterile glass test tubes, ready to use.

Solutions used

TFB: 10mM KMES, 100mM KCl, 45mM $MnCl_2$, 10mM $CaCl_2$, 3mM $HACoCl_3$, filter sterilised and stored at 4°C

KMES: 1M MES adjusted to pH6.2 with KOH, filter sterilised and stored at -20°C

DMSO: dimethylsulphoxide bottled in plastic microfuge tubes under N_2

DTT: 2.25M DTT, 40mM potassium acetate pH6.0, filter sterilised, stored at -20°C in 500µl aliquots.

6.11.2 Transformation and transfection

For transformation the DNA solution (1-10µl containing 10-50ng of vector DNA) was mixed with 200µl of competent cells in a chilled sterile glass test tube, left on ice for 30 minutes, then heat-shocked for 90 seconds at 42°C and immediately cooled in ice. L-broth (800µl) was added to each tube and the cells left to develop resistance at 37°C for 1 hour before aliquots (50-200µl) were spread on selective L-plates. The plates were inverted and incubated overnight at 37°C.

For transfection the same procedure was used, but after heat-shocking and chilling, 3ml of molten BBL top agar and 200µl of plating cells (usually 200µl

of an overnight culture of the same strain used for transformation) were added to the cells. The mixture was poured on a dry plate (BBL for bacteriophage λ, minimal medium for M13) and incubated at 37°C overnight.

6.12 DNA SEQUENCING

6.12.1 Shotgun cloning

The shotgunning strategy for DNA sequencing (Messing *et al*. 1981, Deininger 1983) requires that the fragment of interest is randomly cut into smaller segments, which are then cloned in a suitable single stranded vector and sequenced. The steps followed for the shotgunning of *B.licheniformis* 6346/c β-lactamase are outline here.

1. The fragment of DNA to be sequenced was excised from pACYCbla using *Eco* RI and purified as described in Section 6.10.6.

2. The fragment mixture (5-10μg) was self-ligated in a 30μl volume of ligation mixture (see Section 6.10.7) for 3 hours at 15°C.

3. The mixture of circularised and linear fragments was sonicated in an ELECTROSONIC H60-2 apparatus (Headland, London). The sample was given two bursts of 30 seconds each and spun to the bottom of the tube in between treatments.

4. End repair of the fragments was carried out by adding 2μl 0.25mM dNTPs (Chase mix, see below) and 20 units polymerase I Klenow fragment and incubating the mixture at 15°C overnight.

5. To the reaction cocktail 6μl agarose loading buffer were added. The sample was applied to a 1% w:v low EEO agarose gel with well size of 1cm and run in a minigel at 40mA until the bromophenol blue had entered the gel for about 2cm. The gel was viewed under u.v. light and a slot was cut at the lower limit of the required size fragments by comparing with an adjacent track where pBR322 which had been cut with *Sau* 3AI was run (size markers: 1.65Kb, 0.65Kb, 0.35Kb and smaller). The trough was filled with 1xTBE and electrophoresis resumed. Every 45 seconds the buffer in the slot was collected and replaced by new buffer. This process was repeated until the upper size of wanted fragments had reached the trough. In this way fragments of size between 300 and 700 bases were separated from the mixture. The electroeluted DNA was then purified by phenol extraction, ethanol precipitated and finally resuspended in 20μl TE.

6. The vector was prepared by digesting M13mp18 double stranded DNA with *Sma* I.

7. Aliquots of M13mp18 cut vector (200ng) were ligated with the end repaired purified fragments in 10µl reaction mixtures as described in Section 6.10.7.

8. *E.coli* NM522 host cells were made competent by the method of Hanahan (see Section 6.11), transformed with the ligation mixtures and plated out on minimal medium indicator plates.

9. White single plaques were picked and used for making templates (see Section 6.9.6). The clones were stored as single stranded DNA at -20°C until needed. When fresh phage or more single stranded DNA was required, 2µl of template were used to transform 200µl of competent *E.coli* NM522, yielding 100-200 fresh plaques.

6.12.2 Sequencing reactions

The dideoxy chain termination sequencing procedure devised by Sanger and co-workers (1977) with minor changes was followed. A brief description of the method is given here.

In a microfuge tube 8µl of template DNA were mixed with 1µl of primer and 1µl TM (100mM Tris-HCl pH8.5, 10mM $MgCl_2$); 0.2pmoles of universal sequencing primer (17-mer from NEB) or of reverse sequencing primer were used for each sequencing reaction, but the concentration of primer had to be determined for the purpose-made oligonucleotides. For annealing the sample was heated at 70°C for 3 minutes, incubated for 30 minutes at 37°C and chilled immediately on ice. Primed templates were either used immediately or stored overnight frozen at -20°C.

The sequencing reactions were carried out in siliconised microtitre plates with U-shaped wells as follows.

1. Primed template (2µl) was dispensed in each of 4 wells marked T, A, G, C.

2. The deoxy-dideoxynucleotide mixes (2µl, see Table 6.6) were aliquoted in each of the appropriate wells with a Hamilton repetitive syringe fitted with a luer tip.

3. For four clones, a "Klenow solution" was prepared by mixing 3.6µl 0.1M Tris-HCl pH8.5, 3.6µl 0.1M DTT, 24µl water, 2.4µl [^{35}S]-dATP (8µCi/µl, 650Ci/mmol) and 6 units of polymerase I Klenow fragment. This solution was made at the last moment and kept on ice until used.

4. The Klenow mix was dispensed to the wells in 2µl aliquots and the solutions mixed by tapping gently the microtitre plate onto the bench. The reaction was left to proceed for 20-25 minutes at room temperature.

5. Chase mix (0.25mM dATP, 0.25mM dCTP, 0.25mM dGTP, 0.25mM dTTP) was added in 2µl aliquots to each well and combined to the rest of the reaction mixture. Incubation at room temperature was resumed for a further 20 minutes.

6. The reaction mixes were then either stored overnight at -20°C or used immediately. Just prior to electrophoresis 2µl formamide dyes (98% v:v deionized formamide, 0.1% w:v bromophenol blue, 0.1% w:v xylene cyanol, 10mM EDTA) were added to each well and the samples denatured by floating the microtitre plate on an 80°C water bath for 15 minutes and transferring it quickly on ice.

TABLE 6.6 Composition of deoxy-dideoxynucleotides mixes

STOCK SOLUTION	T MIX	C MIX	G MIX	A MIX
0.5mM dTTP	25	500	500	500
0.5mM dCTP	500	25	500	500
0.5mM dGTP	500	500	25	500
10mM ddTTP	50	-	-	-
10mM ddCTP	-	8	-	-
10mM ddGTP	-	-	16	-
10mM ddATP	-	-	-	1
sequencing TE	1000	1000	1000	500

a All volumes are given in microlitres.

6.12.3 Plasmid sequencing

In some cases, for example when the minus strand of pTZ19R derived plasmids had to be read, plasmid sequencing was carried out on CsCl purified DNA (see Section 6.9.2) as follows.

1. A volume of DNA solution corresponding to 2µg of plasmid

was dried down under vacuum, resuspended in 40µl denaturing buffer (0.2M NaOH, 0.2mM EDTA pH8.0) and allowed to stand at room temperature for 5 minutes. To neutralise the sample, 4µl 2M ammonium acetate pH4.5 was added and the DNA precipitated by adding 2 volumes of cold ethanol. After 5 minutes at -70°C, the precipitated DNA was collected by spinning the tube in a bench centrifuge for 10 minutes at room temperature, the pellet washed with 1ml 70% v:v ethanol and vacuum dried.

2. The denatured DNA was resuspended in 1µl oligonucleotide primer (2.5pmol/µl), 2µl [^{35}S]-dATP (8µCi/µl, 650Ci/mmol), 1.5µl 10x annealing buffer (70mM Tris-HCl pH7.5, 70mM $MgCl_2$, 300mM NaCl, 100mM DTT, 1mM EDTA pH8.0) and water to 15µl and incubated for 15 minutes at 37°C for the annealing reaction to take place.

3. To the annealed DNA 1µl of polymerase I Klenow fragment (2 units/µl) were added and 4µl of this mixture dispensed to each of four labelled microfuge tubes. To each tube 2µl of the relative deoxy-dideoxynucleotide mix (see Table 6.6) was added and the tubes spun for 30 seconds. After incubating for 20 minutes at 37°C, 2µl of Chase mix were added to each tube, mixed by spinning for 30 seconds and incubation resumed for 20 minutes more.

4. The sample was dried under vacuum, resuspended in 4µl formamide dyes and denatured by incubating for 3 minutes in a boiling water bath and immediate cooling on ice.

6.12.4 Electrophoresis of DNA on polyacrylamide gels

Pouring the gel

Routinely 20x40cm glass plates, one rectangular and one notched at the end, were used for running sequencing gels. Plates were cleaned thoroughly with water and detergent, rinsed well and dried. Just before use, they were first wiped with ethanol, then with chloroform and the notched plate was siliconized. The plates were taped together on all sides except the notched top side with 2.5cm wide yellow Selotape, separated by 1cm wide plasticard spacers.

Normally 10xTBE gradient gels were used for DNA sequence analysis. For each gel a 5x gel solution (7ml 5xTBE gel mix, 7µl TEMED, 15µl AMPS) and 0.5x gel solution (30ml 0.5xTBE gel mix, 35 µl TEMED, 70µl AMPS) were prepared in two small beakers. In a 10ml pipette, 3ml 0.5xgel solution were taken up, followed by all the 5x gel solution and a gradient was formed by sucking up the pipette 2-3 air bubbles. With the plate held diagonally, the solution was poured

down one edge, the plates lowered to stop the flow and the rest of the 0.5x gel solution poured down along the same edge using a prefilled 20ml plastic syringe. A edge former was introduced to rest on top of the solution about 0.5cm under the top border of the notched plate. The gel was left lying on the bench to set for 30 minutes or overnight.

If longer runs of DNA samples were required, for example when trying to extend readings of particular clones, non-gradient gels were prepared by using 40ml 0.5x gel solution (40ml 0.5xTBE gel mix, 50µl TEMED, 100µl AMPS). The solution was poured into the taped plates with a 20ml plastic syringe.

Solutions used

0.5x TBE gel mix: 150ml 40% acrylamide stock, 50ml 10x TBE, 460g urea, water to 1l

5x TBE gel mix: 150ml 40% acrylamide stock, 500ml 10x TBE, 460g urea, water to 1l

10x TBE: 890mM boric acid, 890mM Tris, 25mM EDTA

40% acrylamide stock: 38% w:v acrylamide, 2% w:v bis-acrylamide, stirred with 20g/l amberlite MB-1 and filtered through Whatman filter paper

AMPS: 25% w:v ammonium persulphate, prepared fresh each day

Running the gel

After clamping the gel onto a vertical electrophoresis apparatus (constructed in this laboratory from perspex), the buffer tanks were filled with 1xTBE and checked for leakages; the upper edge former was removed and substituted by a shark-tooth comb (from BRL). Normally a 3mm point-to-point comb was used, but when long runs were required this comb was substituted by the wider 6mm comb.

Denatured DNA samples were applied to the wells with a drawn-out micropipette and run at 40W constant power until the bromophenol blue marker dye reached the bottom of the gel (or at a set time after that for long runs).

When the run was completed, the notched plate was gently levered off

and the gel, resting on the other plate, fixed by submerging it in 1l 10% v:v methanol, 10% v:v acetic acid for 15 minutes. The gel was transferred onto 3MM Whatman filter paper, covered with Saran wrap and dried under vacuum at 80°C.

The dried gel was autoradiographed by placing it in direct contact with X-ray screen film (Cronex 4) in a cassette for autoradiography overnight or longer as required by the intensity of the signal.

6.12.5 Sequence assembly

Gel readings were entered directly into a computer held databank with the aid of a Graph/Bar digitizer (Science Accessories Corporation) controlled by a gel reading program (Coulson, unpublished). Before entering the sequence database each gel reading was checked for homology with the vector M13 sequence by running the program SCREENV (Staden 1982); when homology was detected the program automatically rejected the sequence. DBAUTO (Staden 1982a) was used to assemble automatically the gel readings into a sequence database. Any correction or problem readings were dealt with by using the general database handling program DBUTIL (Staden 1982a).

6.13 SITE-DIRECTED MUTAGENESIS

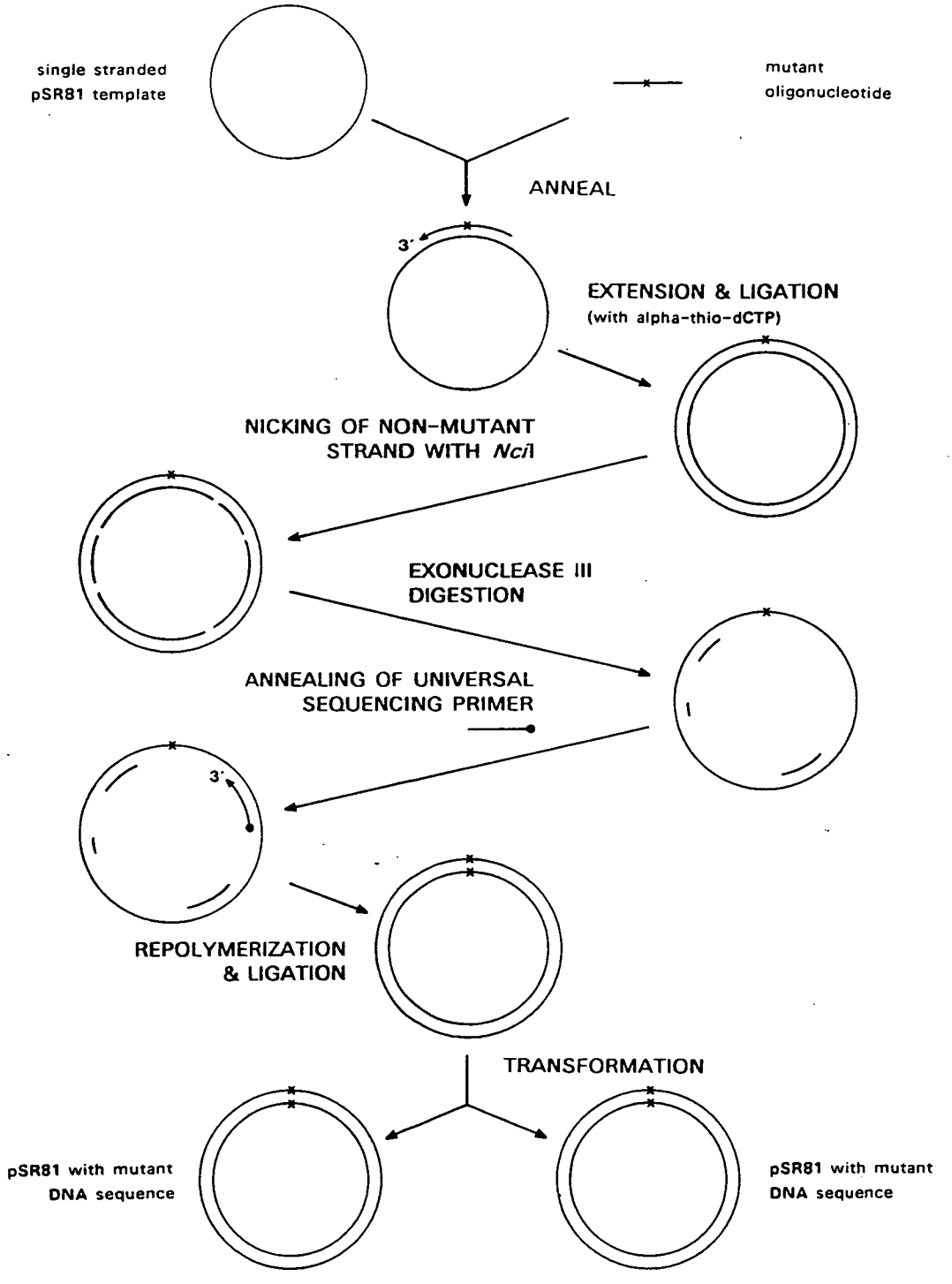
6.13.1 Phosphorothioate method
(Figure 6.1)

The procedure for site-directed mutagenesis devised by Eckstein and co-workers (Taylor *et al*. 1985a, 1985b, Nakamaye & Eckstein 1986) and marketed as a kit by Amersham with minor modifications was used for constructing most of the mutants in this work.

Phosphorylation of oligonucleotides

For each oligonucleotide the following were mixed together in a plastic microfuge tube: 2.5µl oligonucleotide (20nmol/ml), 3µl 10x kinase buffer (1M Tris-HCl pH8.0, 100mM $MgCl_2$, 70mM DTT, 10mM ATP), 25µl water, 2 units T4 polynucleotide kinase. The reaction mixture was incubated at 37°C for 30 minutes and the enzyme denatured by placing in a 70°C water bath for 10 minutes. The stock of phosphorylated oligonucleotide (1.6pmol/µl) was stored at -20°C.

FIGURE 6.1 Site-directed mutagenesis: the phosphorothioate method



Experimental details are described in the text.

Oligonucleotide-directed mutagenesis reaction

1. The phosphorylated mutagenic oligonucleotide (5µl, 1.6pmol/µl) was annealed with 10µl single stranded pSR81 template (about 1µg/µl, see Section 6.9.7) in a microfuge tube in which 7µl "Buffer 1" and 12µl water had been mixed. The tube was capped and incubated at 70°C for 3 minutes, then at 37°C for 30 minutes and finally transferred on ice.

2. The second mutant DNA strand was synthesized by adding to the tube 10µl "$MgCl_2$ solution", 38µl "Nucleotide mix 1" (containing the analogue [αS]-dCTP), 12µl water, 12 units polymerase I Klenow fragment and 12 units T4 DNA ligase. The reaction mixture was incubated overnight at 16°C.

3. To the sample, 170µl water and 30µl 5M NaCl were added and any remaining single stranded DNA removed by passing the sample through a double layer of nitrocellulose filter mounted on a small filtering unit. The filter was washed with 100µl 0.5M NaCl and all the filtrate collected, ethanol precipitated and resuspended in 50µl "Buffer 2".

4. The non-mutant DNA strand was removed by nicking and digestion with exonuclease III. To 10µl of the filtered sample (the other 40µl were stored at -20°C) 65µl "Buffer 3" and 5 units *Nci* I were added and the enzyme left to nick the non-mutant strand for 90 minutes at 37°C. To the reaction mixture the following were added: 12µl 0.5M NaCl, 10µl "Buffer 4", 2µl exonuclease III (25 units/µl). The digestion of the nicked non-mutant strand was allowed to proceed for 30 minutes at 37°C, then the enzymes were inactivated by incubation at 70°C for 15 minutes.

5. Because pSR81 contains many *Nci* I restriction sites, it was difficult to control the digestion reaction so that only that part of the non-mutant strand opposite the mutation was removed, leaving enough DNA for the priming of the repolymerisation reaction. Another primer was therefore annealed to the DNA before proceeding to the next step. Phosphorylated universal sequencing primer (5µl, 1.6pmol/µl) was added to the reaction mixture and left to anneal for 3 minutes at 70°C and for 30 minutes at 37°C.

6. The digested non-mutant strand was repolymerised and ligated by adding to the sample 13µl "Nucleotide mix 2", 5µl "$MgCl_2$ solution", 3 units polymerase I, 2 units T4 DNA ligase and incubating the reaction mix for 3 hours at 16°C. This sample was stored at -20°C until needed.

Transformation

The sample from the SDM experiment (20µl) was used to transform 200µl competent *E.coli* TG1 cells prepared as described above in Section 6.11.

After spreading onto a selective (Tet) L-broth plate, the cells were incubated at 37°C for 16-24 hours.

Analysis of mutant progeny

The Tet^r colonies arising from the transformation of *E.coli* TG1 were replica plated, their DNA fixed onto nitrocellulose filters (see Section 6.14.2) and probed with radioactively labelled mutagenic oligonucleotide. Washes of the filters at progressively higher temperatures and autoradiography led to discrimination between mutant and non-mutant colonies (see Section 6.15.2, Figure 4.3). Routinely mutation efficiencies of 5-15% were obtained with the Eckstein method.

6.13.2 Gapped-duplex method
(Figure 6.2)

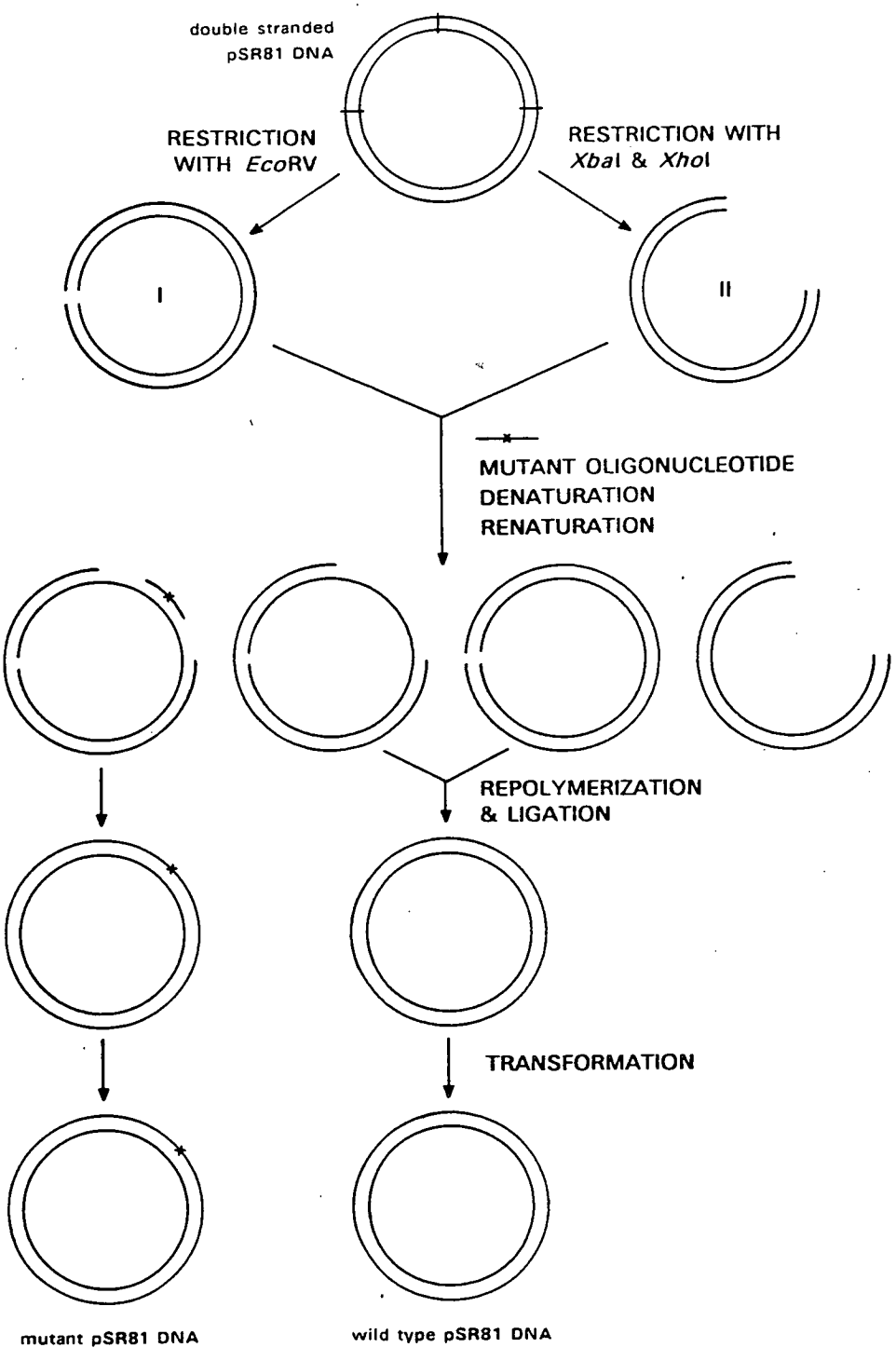
The gapped-duplex method of site-directed mutagenesis devised by Fritz and co-workers (Kramer *et al*. 1984) for use with single stranded DNA, can be successfully applied to plasmids as well (Morinaga *et al*. 1984). One of the mutants constructed for this work, Lys284 to Stop, was made following this method.

1. Double stranded pSR81 DNA prepared by CsCl purification (Section 6.9.2) was restricted with *Xho* I (cutting in the *bla* gene near its 5' end) and *Xba* I (cutting in the polylinker just downstream the *bla* gene). The fragments were separated on a 0.7% w:v low EEO agarose gel and the bigger fragment (7.4Kb) comprising the whole of the plasmid except most of the *bla* gene, recovered from the gel (fragment I) (see Section 6.10.6). Separately more pSR81 DNA was linearized with *Eco* RV, cutting the plasmid at a single site inside the Tet^r gene (fragment II).

2. Fragments I and II (0.2µg each) were mixed in a plastic microfuge tube with 5µl phosphorylated oligonucleotide (1.6pmol/µl), 2µl 10x polymerase-ligase buffer(1M NaCl, 65mM Tris-HCl pH7.5, 80mM $MgCl_2$, 10mM β-mercaptoethanol). The DNA strands were first separated by placing the tube in a boiling water bath for 3 minutes, then allowed to renature slowly and anneal to the oligonucleotide by incubating at 30°C for 30 minutes, at 4°C for 30 minutes and at 0°C for 10 minutes.

3. To the complex mixture of gapped duplex molecules the following were added: 1µl each 10mM dATP, dCTP, dGTP, dTTP, 2µl 10mM ATP, 2µl T4 DNA ligase (1 unit/µl), 0.5µl polymerase I Klenow fragment (5 units/µl). The repair reaction was incubated at 12.5°C overnight.

FIGURE 6.2 Site-directed mutagenesis: the plasmid gapped-duplex method



Experimental details are described in the text.

Competent *E.coli* TG1 cells were transformed with 10µl of the sample and plated on selective (Tet) L-broth plates and screened as above. The efficiency of mutation achieved with this method was 1%.

6.14 HYBRIDIZATION - DNA blotting onto nitrocellulose filters

6.14.1 Southern transfer

To check cloning experiments, usually the recombinant DNA was restricted, run on a low EEO agarose gel and transferred onto nitrocellulose filters where homology with parental DNA could be detected by hybridization. The DNA bands were transferred from the agarose gel to the filter according to the method of Southern (1975).

1. The agarose gel on which the DNA fragments had been separated was immersed in several volumes of 1.5M NaCl, 0.5M NaOH for 1 hour at room temperature with constant shaking in order to denature the DNA. The gel was then neutralised by soaking it in several volumes of 1M Tris-HCl pH8.0, 1.5M NaCl for 1 hour at room temperature with constant shaking.

2. A plexiglass plate bigger than the gel was placed on two rubber bungs inside a tray filled with 10xSSC (20xSSC: 3M NaCl, 0.3M trisodium citrate) and the level of the buffer in the tray raised to about 1cm below the plate. A piece of Whatman 3MM filter paper was wetted in 2xSSC and laid on top of the plate with its overhanging ends dipped into the buffer.

3. The gel was placed on the plate with its original underside uppermost and air bubbles trapped underneath carefully removed. A piece of nitrocellulose filter was cut just bigger than the gel, prewetted in 2xSSC for 2-3 minutes and laid on top of the gel making sure not to trap any air bubbles.

4. Two pieces of Whatman 3MM filter paper, cut to exactly the same size as the gel, were wetted in 2xSSC, placed over the nitrocellulose filter and overlaid with a stack of paper towels cut just smaller than the gel (5-8cm high). The pile of paper was pressed down with another plate and a weight (0.5-1Kg). To prevent evaporation of the tank buffer and short-circuiting of fluid, Saran wrap was stretched between the edges of the gel and the sides of the tray. Usually the transfer was allowed to proceed for at least 24 hours, occasionally replacing the paper towels when wet.

5. When transfer was completed, the paper towels and the two layers of filter paper were discarded, the nitrocellulose filter peeled off the gel and soaked in 6xSSC for 5 minutes at room

temperature. The filter was drained on filter paper and allowed to air dry.

6. To fix the DNA, the filter was placed between two sheets of Whatman 3MM filter paper and baked at 80°C under vacuum for 2 hours.

6.14.2 Colony transfer

Screening of mutants was carried out by replica plating the colonies obtained from the transformation of the SDM DNA samples (see Section 6.13) and fixing their DNA on nitrocellulose filters. Hybridization with the labelled mutagenic oligonucleotide followed (Section 6.15.2). The fixing of DNA from bacterial colonies was according to the method of Grunstein & Hogness (1975) and Hanahan & Meselson (1980) with the omission of the stage of bacterial growth on the filters.

1. Bacterial colonies were replica plated on selective L-broth plates on an asymmetric grid, 50 colonies to a plate, and left to grow overnight at 37°C.

2. A circle of dry nitrocellulose filter was lowered carefully onto the plate using two flat-bladed forceps.

3. When completely wet, the filter was peeled off the plate and laid, colony side up, onto a stack of three sheets of Whatman 3MM paper previously soaked in denaturing solution (1.5M NaCl, 0.5M NaOH). After 3 minutes the filter was transferred to another stack of 3MM paper soaked in neutralising buffer (1.5M NaCl, 1M Tris-HCl pH7.5) and left for 5 minutes. Finally the filter was transferred to paper saturated with 4xSET (20xSET: 3M NaCl, 20mM EDTA, 0.4M Tris-HCl pH7.8) for 5 minutes more and left to air dry on filter paper for 15-30 minutes.

4. To fix the DNA, the filter was placed between two sheets of Whatman 3MM paper and baked at 80°C under vacuum for 2 hours.

6.15 HYBRIDIZATION - Labelling of DNA probes and hybridization conditions

6.15.1 Double stranded probes

Nick translation

Nick translation (Rigby *et al*. 1977) was used for the preparation of radioactively labelled double stranded DNA probes (plasmid, phage or fragments).

1. In a microfuge tube 0.5-1µg of DNA (in 1-5µl) were mixed with 20µl 1x buffer/cold dNTPs (see below), 1.5µl [α^{35}S]-dCTP (10µCi/µl, 3000Ci/mmol), 1µl DNAse I stock solution and nick formation left to proceed for about 1 minute at room temperature.

2. To the reaction mixture 0.5µl *E.coli* DNA polymerase I (1unit/µl) was added and the tube incubated for 3 hours at 15°C.

3. The unincorporated radioactive nucleotide was separated from the nick-translated DNA by passing the sample through a sterile Sephadex G50 (fine) column (15x0.7cm). The sample was eluted with TE and its progress followed with a minimonitor. The faster migrating peak of radioactivity, corresponding to the labelled DNA, was collected. Incorporation efficiency was checked by spotting a 5µl aliquot on a small glass filter (4F/C), letting it dry and determining the Cerenkov counts. The yield was usually 1-2x10^6cpm (Cerenkov).

Solutions used

1x buffer/cold dNTPs: 100µl 4x buffer, 4µl 2mM dTTP, 2mM dGTP, 2mM dATP, 1µl 1M β-mercaptoethanol, 290µl water

4x buffer: 210mM Tris-HCl pH7.5, 21mM $MgCl_2$, 20µg/ml bovine serum albumin

DNAse I stock solution (2x10^{-5}mg/ml DNAse I in polymerase I buffer): 1µl 2x polymerase dilution buffer, 4µl 1mg/ml DNAse I, 1ml glycerol. Mix 20µl of 2x10^{-3}mg/ml DNAse I solution from above with 1ml 2x polymerase I dilution buffer and 1ml glycerol to get working DNAse I stock solution. Stored at -20°C.

2x polymerase I dilution buffer: 2ml 1mg/ml bovine serum albumin, 100µl 2M ammonium sulphate, 20µl 1M β-mercaptoethanol, 100µl 1M Tris-HCl pH7.5.

Hybridization

Before starting the hybridization the nitrocellulose filter carrying the DNA to be analysed was washed in about 30ml prewarmed hybridization buffer (see below) for 30 minutes at 37°C. The filter was then placed in a sealed plastic bag with the probe (1-2x10^6cpm, denatured by incubating for 10 minutes at 95°C and immediate cooling on ice), 50µl calf thymus sonicated DNA (3.5mg/ml) and 10ml

hybridization buffer. Hybridization was carried out at 37°C overnight.

The following day the radioactive probe was discarded and the filter subjected to a series of washes as follows:

- 2xSSC, 0.1% w:v SDS. About 50ml in the bag at 37°C for 1 hour
- 2xSSC, 0.1% w:v SDS. About 100ml in a box at 37°C for 1 hour, twice
- 1xSSC, 0.1% w:v SDS. About 200ml in a box at room temperature for 1 hour
- 1xSSC. About 100ml at room temperature for 10–15 minutes.

The filter was then blotted dry, covered with Saran wrap and autoradiographed at −70°C with a preflashed film and an intensifying screen.

Solutions used

Hybridization buffer: 50% v:v formamide, 4xSSC, 1x Denhardt solution

20x Denhardt solution: 0.4% w:v bovine serum albumin, 0.4% w:v polyvinylpyrolidine, 0.2% v:v Ficoll, filter sterilized and stored in aliquots at −20°C.

6.15.2 Labelled oligonucleotides (mutant screening)

Kinasing

In a microfuge tube 15pmoles of oligonuceotide (in 1–2µl) were mixed with 3µl 10x kinase buffer (0.5M Tris–HCl pH8.0, 0.1M $MgCl_2$), 3µl [γ^{32}P]–ATP (10µCi/µl, 3000Ci/mmol), 1µl 100mM DTT and water to 30µl final volume. T4 polynucleotide kinase was added (2 units) and the reaction incubated at 37°C for 30 minutes. The labelled oligonucleotide was diluted with 3ml 6xSSC, filtered through a Millex filter (Millipore sterile Millex–GU 0.22µm filter unit) and the filter washed with 1ml 6xSSC. The probe was either used immediately or stored for a few days at −20°C in a plastic sterile Petri dish.

Hybridization

For screening of mutants, the nitrocellulose filters on which DNA of colonies obtained from the transformation of the SDM sample had been fixed

(Section 6.14.2) were prehybridized at 67°C for 2-3 hours in a sealed bag containing 10ml of hybridization buffer (6xSSC, 10x Denhardt, 0.2% w:v SDS). After rinsing in 50ml 6xSSC for 1 minute, the filter was dropped in a sterile Petri dish containing kinased mutagenic oligonucleotide prepared as above, making sure not to trap any air bubble under the filter. Hybridization was carried out overnight at room temperature.

The following day the filter was washed three times in 100ml 6xSSC for 5 minutes each time at room temperature and the excess liquid drained. The filter was covered with Saran wrap and autoradiographed at -70°C with preflashed film and intensifying screen for 1-4 hours. This gave the background grid (see Figure 4.3).

The filter was then washed for 5 minutes in about 50ml 6xSSC prewarmed to a temperature 5°C lower than the calculated Tm (see Section 4.8) and autoradiographed. The washing and autoradiography procedure was repeated at temperatures progressively higher by 2-3°C until discrimination between mutant and wild type colonies was reached. Routinely a colony carrying the wild type gene was introduced in the filter to be screened as a negative control.

6.16 PREPARATION OF BETA-LACTAMASE SAMPLES

6.16.1 β-lactamase isolation from *B.licheniformis*

The preparation of the exocellular β-lactamase of *B.licheniformis* strains 6346/c and 749/c followed a modified version of the procedure given by Yamamoto and Lampen (1976).

1. *B.licheniformis* spores (200µl) were inoculated in 20ml L-broth and grown with shaking at 37°C for about 8 hours. The culture was transferred to two 5l conical flasks containing 1l CH/S medium each and incubation continued overnight. This inoculum was used to start a 45l culture in CH/S broth which was grown in a fermenter for 8 hours to late logarithmic phase.

2. The cells were removed from the culture broth by passing the culture through a continuous centrifuge and the pH of the supernatant lowered to 4.8 by addition of glacial acetic acid.

3. Phosphocellulose P11 (200g dry weight) was pre-swollen as recommended by the manyfacturers, converted to its H^+ form (pH3.5) and added to the cell supernatant. The suspension

was stirred gently overnight.

4. The following day the supernatant was discarded. The cellulose was washed in a beaker twice with about 2l 0.01M KH_2PO_4/NaOH pH5.0 and packed in a 4x100cm column. The β-lactamase was eluted from the column with 0.2M KH_2PO_4/NaOH pH7.6 and 25ml fractions collected.

5. The active fractions were pooled and ammonium sulphate slowly added to 100% saturation at 4°C. The solution was left to equilibrate overnight at 4°C with continuous stirring.

6. The precipitated protein was collected by centrifugation for 20 minutes at 16000g and resuspended in a small volume (10-20ml) of 0.01M ammonium acetate buffer pH8.0. The sample was dialysed overnight at 4°C against the same buffer.

7. The dialysed sample was applied to a 5x90cm Sephacryl S200 column pre-equilibrated with 0.01M ammonium acetate buffer pH8.0 and eluted with the same buffer. The major active fractions (10ml each) were pooled, freeze-dried and the β-lactamase powder stored at 4°C. Routinely 20-50mg of pure β-lactamase was isolated with this method.

6.16.2 β-lactamase isolation from *E.coli*

Cloned 6346 β-lactamase and the mutant proteins derived from it were prepared as crude extracts from *E.coli* cells harbouring the relative plasmid.

1. A periplasmic extract was prepared from a 1l overnight culture of *E.coli* cells collected by centrifugation for 15 minutes at 6000g. The cells were resuspended in 20ml ice cold 30mM NaCl, 10mM Tris-HCl pH7.5, collected by centrifugation (15 minutes, 12000g) and resuspended in 20ml isotonic solution (20% w:v sucrose, 1mM EDTA, 30mM Tris-HCl pH7.3). After shaking for 5 minutes at room temperature, the cells were pelleted again and osmotically shocked by resuspending them in 20ml ice cold distilled water and shaking for 5 minutes at 0°C.

2. The cell debris was removed by centrifugation (15 minutes, 12000g) and ammonium sulphate added to the supernatant to 85% saturation at room temperature. The solution was left to equilibrate for 1 hour at room temperature with constant stirring and the precipitated protein separated from the β-lactamase containing supernatant by centrifuging for 30 minutes at 27000g. The sample was dialysed extensively at 4°C against 50mM sodium succinate pH4.6.

3. The sample was loaded onto a 1ml FPLC S column pre-equilibrated with 50mM sodium succinate pH4.6. After loading, the column was washed with the same buffer until

no more material absorbing at 280nm was detected in the effluent, then the β-lactamase was eluted with a NaCl gradient (0-300mM in sodium succinate pH4.6). Fractions of 1ml volume were collected and assayed. The fraction containing the peak of β-lactamase activity was stored at -20°C. The estimated enzyme concentration in this fraction was about 3-5µg/ml.

6.16.3 Sonicated extracts

Sonicated extracts of *E.coli* cells carrying the bacillary β-lactamase gene were prepared as follows:

1. Cells from an overnight culture (1ml) were collected by centrifugation in a bench centrifuge for 5 minutes and resuspended in 100µl water.

2. The tube was placed on ice and the cell suspension sonicated for 30 seconds at 5µm peak-to-peak. The sonicated extract was normally assayed immediately.

6.17 BETA-LACTAMASE ASSAYS

6.17.1 Qualitative assay with nitrocefin

Nitrocefin is a chromogenic cephalosporin which allows rapid detection of β-lactamase activity. The hydrolysis of its β-lactam bond results in a colour change from yellow to pink which can be detected by the naked eye (O'Callaghan *et al*. 1972).

A working solution was prepared by adding 0.5ml dimethylsulphoxide to 5mg nitrocefin. As soon as the solid was dissolved, 9.5ml 0.1M potassium phosphate buffer pH7.0 was added and the solution mixed well. This stock was stored in the dark at 4°C for up to two weeks.

Nitrocefin was used to detect *bla*$^+$ recombinant plasmids or bacteriophages by flooding the plates with a small volume (2-3ml) of working solution. Usually within 10-15 minutes β-lactamase producing colonies or plaques were surrounded by a pink halo and could be picked and purified by replating, ready for further analysis.

Nitrocefin was also used for detection of β-lactamase activity in column eluates. In a microtitre plate 10-20µl aliquots of the column fractions were

mixed with an equal volume of working solution. Usually fractions containing the peak of activity turned pink within a few seconds.

6.17.2 Spectrophotometric assays

Spectrophotometric assays were carried out at room temperature in a Perkin-Elmer spectrophotometer connected to a computer dedicated to the collection of data.

In a 3ml quartz cuvette the β-lactam substrate (5µl, 35mg/ml) was mixed with buffer (0.1M sodium phosphate buffer pH6.8) and the absorption baseline recorded. The enzyme was added (usually 10-100µl of the appropriate dilution), mixed well and the decay of the substrate recorded at the wavelength appropriate to the substrate used (see Table 5.3).

6.18 WESTERN BLOTS

Western blots (Burnette 1981) were used to semiquantitatively detect β-lactamase in whole cell protein extracts of *E.coli* carrying the cloned gene. Protein samples were run on a SDS polyacrylamide gel and transferred onto nitrocellulose filter. The filter was hybridized with rabbit anti-β-lactamase antibody followed by anti-rabbit IgG antibody conjugated to alkaline phosphatase. Upon soaking of the filter in alkaline phosphatase substrate solution, β-lactamase showed up as dark coloured bands.

6.18.1 Pouring the gel

An SDS-discontinuous buffer system gel at 12.5% polyacrylamide concentration was prepared. Glass plates (16x20cm) were thoroughly cleaned with soapy water, rinsed, dried and wiped with ethanol. They were assembled with two perspex side spacers and taped together.

1. A resolving gel was prepared by mixing in a small beaker 12.5ml acrylamide stock (see below), 3.75ml resolving buffer, 0.3ml 10% w:v SDS, 1.5ml AMPS, 11.95ml water and 15µl TEMED. The mixture was immediately poured down one side of the slab forming plates and the top overlaid with a small amount of water to get a clean interface. The gel was left standing upright on the bench until set.

2. The water lying at the top of the gel was poured off and the stacking gel mixture (1.25ml acrylamide stock, 2.5ml stacking buffer, 100µl 10% w:v SDS, 0.5ml AMPS, 5.65ml water, 8µl

TEMED) was layered on top. A well-forming comb was inserted immediately between the plates and the gel left standing until set.

3. When the gel had set, the tape was discarded and the comb removed. The plates were clamped to an upright electrophoresis apparatus constructed from perspex in this laboratory and upper and lower tanks filled with tank buffer.

Solutions used

Acrylamide stock: 30% w:v acrylamide, 0.8% w:v bis-acrylamide, filtered through Whatman filter paper No.1 and stored in the dark at 4°C for up to one month

Resolving gel buffer: 3M Tris-HCl pH8.8

Stacking gel buffer: 0.5M Tris-HCl pH6.8

AMPS: 1.5% w:v ammonium persulphate

Tank buffer: 25mM Tris, 0.19M glycine, 0.1% w:v SDS (makes pH8.0)

6.18.2 Preparation of the sample and running the gel

Whole protein extracts of *E.coli* cells carrying the *bla* gene were prepared from 200µl of an overnight culture. The cells were collected by centrifugation in a bench centrifuge for 5 minutes, resuspended in 40µl loading buffer (62.5mM Tris-HCl pH6.8, 2% w:v SDS, 5% v:v β-mercaptoethanol, 10% w:v sucrose, 0.25% w:v bromophenol blue) by pipetting up and down several times and boiled for 15 minutes. Pure protein samples, normally used as controls, were diluted in 40µl of the same loading buffer and boiled for 3 minutes.

The samples were loaded on the gel with a Hamilton syringe and usually run overnight at 8-10mA constant current until the blue dye reached the bottom of the gel.

6.18.3 Western blotting

At the end of the electrophoretic run the plates were removed from the tank, separated and the orientation of the gel marked by cutting the top corner

corresponding to lane 1.

A piece of nitrocellulose filter and two pieces of Whatman 3MM filter paper were cut slightly bigger than the gel and wetted in tank buffer (25mM Tris, 0.19M glycine, 20%v:v methanol, 0.1% w:v SDS). The electrophoretic transfer was assembled as follows, making sure that no air bubbles were trapped between layers:

1. foam pad
2. 3MM filter paper
3. gel
4. nitrocellulose filter, marked as gel
5. 3MM filter paper
6. foam pad

The sandwhich was mounted in the blotting tank (Biorad Trans-Blot^{TM} Cell) which was then filled with tank buffer. Transfer was carried out for 4 hours at 50-60 volts, 0.3A.

6.18.4 Antibody reaction and staining

When the transfer was completed, the nitrocellulose filter was peeled off the gel and blocked by soaking in 100ml TS buffer (see below) containing 5% fat free milk powder (Sainsbury's). The filter was left gently rocking for 30-60 minutes, then transferred to a sealed plastc bag containing 20ml 1:1000 dilution of rabbit anti-6346 β-lactamase antibody (prepared in this department by Miss J.Fleming) in TS/milk. The antibody-antigen reaction was left to proceed overnight at 4°C with gentle shaking.

The following day, the filter was washed with four 100ml changes of T-TS over 30 minutes, then reacted with the second antibody by closing it in a plastic bag containing 15ml 1:7500 dilution of alkaline phosphatase conjugate anti-IgG in TS/milk for 2 hours at room temperature. The filter was then washed as above.

For detection, the filter was placed in a box containing 10ml alkaline phosphatase buffer in which 66µl nitro blue tetrazolium and 33µl 5-bromo-4-chloro-3-indolyl-phosphate substrates (Promega Protoblot System)

had been diluted. The box was rocked continuously until the desired development of color was obtained. To stop the reaction, the filter was dropped in another box containing about 100ml of water, then allowed to air dry. The colour remains stable indefinitely.

Solutions used

TS: 0.9% w:v NaCl, 10mM Tris-HCl pH7.4

T-TS: 0.9% w:v NaCl, 10mM Tris-HCl pH7.4, 0.05% v:v Tween-20

Alkaline phosphatase buffer: 0.1M Tris-HCl pH9.5, 0.1M NaCl, 5mM $MgCl_2$.

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