Cloning and Sequence Determination of Six *Staphylococcus aureus* β -Lactamases and Their Expression in *Escherichia coli* and *Staphylococcus aureus*

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The plasmid-encoded β -lactamase genes of six strains of *Staphylococcus aureus* were cloned and shown to be expressed in *Escherichia coli*. The cloned genes were re-introduced into *S. aureus* via a shuttle vector, and expressed β -lactamase. However, clones containing only the small amount of DNA found necessary for expression of ampicillin resistance in *E. coli* did not express β -lactamase in *S. aureus*. Much larger pieces of DNA from the original plasmid were necessary to obtain expression in *S. aureus*. Some of the six strains of *S. aureus* synthesized β -lactamase constitutively and some released only a small proportion of the enzyme into the medium. Both these characteristics were maintained in the clones so it is concluded that they are features either of the gene itself or of the surrounding DNA. The cloned genes were sequenced and the putative amino acid sequences of the β -lactamases were compared. There are several differences between the sequences and in particular one change in the N-terminal region, at a position believed to be especially important for export of proteins from the cell, is thought to have a key effect on whether or not the enzyme is found in the medium.

INTRODUCTION

Resistance of *Staphylococcus aureus* to β -lactam antibiotics is an important clinical problem. This resistance is commonly conferred by production of a β -lactamase, an enzyme which cleaves the β -lactam ring, rendering the molecule inactive as an antibiotic. In *S. aureus* this enzyme is often encoded on a plasmid of approximately 30 kb which frequently carries genes conferring resistance to ions such as cadmium, lead, antimony, mercury, arsenate and arsenite. Genes conferring resistance to antibiotics are frequently found on plasmids. One reason for this may be that the genes are more 'mobile' than those of the chromosome; plasmids can be transferred between strains by methods such as conjugation (Forbes & Schaberg, 1983), transduction (Shalita *et al.*, 1980) and phage-mediated transfer (Lacey, 1980). The β -lactamase gene has also been described as part of a transposon (Asheshov, 1966; Shalita *et al.*, 1980; Lyon & Skurray, 1987).

A classification of β -lactamases into classes I–V was proposed by Richmond & Sykes (1973). It is based mainly on enzymes from Gram-negative bacteria, and β -lactamases of Gram-positive organisms, including *S. aureus*, do not fit particularly well into this system. Ambler (1980) proposed a classification of β -lactamases based on amino acid sequence. At that time the amino acid sequence was known of five β -lactamases (*S. aureus*, *Bacillus cereus* 569H, *Bacillus licheniformis* and *Escherichia coli* RTE and pBR322; see Ambler, 1979) which were clearly homologous and so were classified together as class A. Further studies have shown that these enzymes, grouped together by sequence homology, are similar mechanistically, in structure and often in their location, for example, membrane bound or extracellular.

Abbreviation: CBAP, 2-(2'-carboxyphenyl)-benzoyl-6-aminopenicillanic acid.

The β -lactamases of *S. aureus* have been classified immunologically into types A–D, by reaction with antisera raised against a purified extracellular staphylococcal β -lactamase (Richmond, 1965*a*). This serological typing puts the enzymes into groups which show different kinetic characteristics. The β -lactamases of types A and C show high hydrolytic activity, which is increased on interaction with antibody. These are commonly found in strains which are frequently isolated from hospitals (Dyke & Richmond, 1967) while strains producing the less hydrolytically active type B are far less frequently isolated from hospitals. The more recently described D-type β -lactamase (Rosdahl, 1973) shows fairly high hydrolytic activity and some increase when treated with antisera. It is commonly found associated with resistance to fusidic acid (Lacey & Grinsted, 1972). These different β -lactamases are believed to be closely related and differences in reaction with antibody and the rate of hydrolysis of β -lactams are thought to be the result of small changes in amino acid sequence (Richmond, 1965*a*).

The expression of β -lactamase in *S. aureus* is either constitutive or inducible by β -lactams and their analogues. The molecular mechanism of induction is not known although expression was concluded to be negatively controlled by a repressor protein (Richmond, 1965*b*). On transduction of the β -lactamase plasmid to another strain the expression of the gene, i.e. constitutive or inducible, is unchanged, indicating that the control of expression is plasmid-encoded (K. G. H. Dyke, unpublished results). The region responsible for control of β -lactamase expression has been mapped adjacent to the β -lactamase structural gene (Novick & Richmond, 1965).

Strains of *S. aureus* expressing β -lactamase isolated from hospitals during 1960–1970 could be divided into two groups: those which encoded a β -lactamase found almost completely associated with the cell and those in which a high percentage of the enzyme activity was found in the medium in which the cells were growing. The latter were frequently isolated from hospitals, i.e. were endemic, while those in which the majority of enzyme activity was cell associated were not (Dyke *et al.*, 1970). Some of the β -lactamase-encoding plasmids were transduced into another strain of *S. aureus* and the percentage of activity in the media was the same in both strains, suggesting that whether the β -lactamase activity is cell associated or found in the medium is a property of the plasmid rather than the chromosomal ‘background’ of the strain (K. G. H. Dyke, unpublished results).

Gram-positive organisms, including *S. aureus*, have a simple peptidoglycan cell wall and do not possess an outer membrane bound periplasmic space. In Gram-positive organisms once a protein has been exported from the cell and released from the membrane, it is found in the medium in which the cells are growing. The β -lactamases of the Gram-positive organisms *B. licheniformis*, *B. cereus* and *S. aureus* have been shown to occur in a membrane-bound form (Nielsen & Lampen, 1982). Each β -lactamase contains an N-terminal extension, missing from the mature extracellular protein, which contains the cysteine residue by which the protein is attached to the membrane. The membrane attachment is by a glyceride thioether modification, identical to that of several outer-membrane proteins of the Gram-negative *E. coli* (Nielsen & Lampen, 1982). The extracellular form is thought to be a cleavage product of this membrane-bound form.

In this study, six strains of *S. aureus* containing a β -lactamase-expressing plasmid were investigated. The strains express β -lactamases of different immunological groups, are constitutively or inducibly expressed and have a characteristic proportion of the enzyme released to the medium in which the cells are growing. The β -lactamase genes were cloned in *E. coli* and the DNA sequenced to compare the amino acid sequences of the different β -lactamases. Restriction fragments of the staphylococcal plasmids were cloned in *S. aureus*, using *S. aureus*/*E. coli* shuttle vectors, to investigate the control of expression and the proportion of β -lactamase in the medium of isolated fragments of DNA, as distinct from the entire staphylococcal plasmids.

METHODS

Bacterial strains and plasmids. The β -lactamase-producing strains of *S. aureus* used are shown in Table 1. The *S. aureus* strains RN4220 (Kreiswirth *et al.*, 1983) and 8325N (Novick & Roth, 1968) were used as the recipients in *S. aureus* transformation experiments. The *S. aureus* plasmid pCW59 (Wilson *et al.*, 1981) was used to clone directly in *S. aureus*. It carries determinants for resistance to tetracycline and chloramphenicol, with a single *Bgl*II site

Table 1. *Strains of Staphylococcus aureus*

The following are used to show phenotypic resistance or sensitivity; Asa, arsenite; Bi, bismuth; Cd, cadmium; Em, erythromycin; Fa, fusidic acid; Hg, mercury; Neo, neomycin; Om, organomercurials; Pb, lead; Pc, benzylpenicillin; Sb, antimony; Tc, tetracycline. Immunological typing is according to Richmond (1965*a*). A low proportion of β-lactamase in the supernatants is < 10%, high is > 10%.

Strain	Source and date isolated	Antibiotic/heavy metal resistance	Plasmid	Plasmid size (kb)	Immunological type	Proportion of β-lactamase activity in supernatant	Reference
PC1	EMS mutant of 524SC	Pc ^r /Asa ^r Asi ^r Sb ^r Hg ^r Bi ^r Pb ^r Cd ^r Om ^r	pPC1	31.8	A	High	Novick (1963)
S1*	EMS mutant of PS80.d [p19789blaI blaZ]	Pc ^r /Asa ^r Cd ^r Hg ^r	pS1	27.5	A	High	Johnston & Dyke (1971)
1071	St Bartholomew's Hospital London, 1963	Tc ^r Pc ^r	p11071	26.5	A	Low	Dyke & Richmond (1967) Dyke <i>et al.</i> (1970)
3796	Kings College Hospital London, 1963	Pc ^r	p13796	26.5	C	Low	
3804	Kings College Hospital London, 1963	Pc ^r /Cd ^{s/r} †Hg ^r	p113804	31.6	C	High	
FAR4	Isolated from a patient in Bristol	Pc ^r Str ^r EM ^r Neo ^r Fa ^r /Cd ^r Asa ^r Hg ^{s/r} ‡	pUB101	21.2	D	High	Lacey & Grinstead (1972)

* S1 is PS80.d [p19789blaI blaZ seg1].
† Reported as Cd^r; Shalita *et al.* (1980).
‡ Reported as Hg^r; Shalita *et al.* (1980).

within the latter determinant. The *S. aureus* plasmid pE194 (Horinouchi & Weisblum, 1982) was used to construct the *S. aureus*/*E. coli* shuttle vectors. This plasmid expresses resistance to erythromycin in both *E. coli* and *S. aureus*.

E. coli strain MC1061 (Casadaban & Cohen, 1980) was used as a recipient for transformation and JM107 (Yanisch-Perron *et al.*, 1985) was used for transformations involving *lacZ'* expression of the vector for selection of recombinants. The plasmid pRW33 (Mézes *et al.*, 1983) was used to clone in *E. coli*. This plasmid carries determinants encoding resistance to chloramphenicol and tetracycline, with a single *EcoRI* site in the former and single *HindIII*, *BamHI*, *SalI* and *HincII* sites within, or affecting expression of the latter. Plasmids pUC18 and pUC19 (Yanisch-Perron *et al.*, 1985) and pJS62 were used with pE194 in the construction of shuttle vectors. Plasmid pJS62 (a gift from J. Sutherland) was constructed by cloning a gene conferring to chloramphenicol into the *ScaI* site of the ampicillin-resistance-conferring gene of a modified pUC19. The resulting plasmid is *Cm^r* and *Ap^s*. It contains the unique polylinker cloning sites, but the insert introduces a second *EcoRI* site (J. Sutherland, personal communication).

Media and reagents. For *S. aureus*, CY medium (Novick, 1963) was used for growth of cultures in liquid. CY solidified with 2% (w/v) agar was used as solid medium, supplemented as appropriate with chloramphenicol (10 µg ml⁻¹), erythromycin (20 µg ml⁻¹), tetracycline (5 µg ml⁻¹), starch (0.4%, w/v) and 2-(2'-carboxyphenyl)-benzoyl-6-aminopenicillanic acid (CBAP) (5 µg ml⁻¹).

For *E. coli*, LB medium (Maniatis *et al.*, 1982) was used, and for plates, this was solidified with 2% agar. The following additions were used in the plates when appropriate: ampicillin (50 µg ml⁻¹), tetracycline (12.5 µg ml⁻¹), chloramphenicol (10 µg ml⁻¹) and erythromycin (100 µg ml⁻¹).

Growth was at 37 °C under aerobic conditions, except for experiments involving *S. aureus* strain S1 which was grown aerobically at 30 °C.

Restriction enzymes were from Amersham, Bethesda Research Laboratories (BRL) or New England Biolabs. DNA ligase (from bacteriophage T4) was obtained from Boehringer Corporation Ltd. (BCL) and Klenow fragment from Pharmacia. All enzymes were used according to the manufacturer's instructions. CBAP was obtained from Sigma and nitrocefin from Glaxo-Allenburys Research, Greenford, UK.

Preparation of plasmid DNA and recombinant DNA techniques. The method of Novick & Bouanchaud (1971) was used to purify plasmid DNA from *S. aureus* for restriction enzyme analysis and cloning. Transformation of 8325N and RN4220 protoplasts was as described by Lindberg (1981). *E. coli* DNA was prepared by the alkaline lysis method, described by Maniatis *et al.* (1982). Transformation of *E. coli* MC1061 was carried out using the method of Casadaban & Cohen (1980) and that of JM107 according to the method described in the Amersham M13 Cloning and Sequencing Handbook.

Electrophoresis. Plasmid DNA and restriction-enzyme-digested DNA were electrophoresed through gels of 1% (w/v) agarose in TBE buffer (500 mM-Tris, 500 mM-boric acid, 100 mM-EDTA) containing 500 µg ethidium bromide l⁻¹. The DNA bands were visualized on a UV transilluminator and photographed.

DNA sequencing. The clones for sequencing were made by cloning in M13mp18 and M13mp19 (Messing *et al.*, 1977), or in plasmids pUC18, pUC19 and pJS62. The sequencing was done by the dideoxynucleotide chain termination method of Sanger *et al.* (1977). For M13 clones this was as described in the Amersham M13 Cloning and Sequencing Handbook and universal and 'far' primer (Pharmacia), and an oligonucleotide (5'-TTCAAACAGTCCACATGCC-3'), complementary to part of the β -lactamase gene (near the 5' end), were used as primers. Sequencing primer was annealed to the single-stranded M13 template DNA by heating the annealing mixture at 100 °C for 3 min and then allowing at least 15 min for it to cool to room temperature (New England Biolabs Sequencing Manual). For sequencing double-stranded plasmid clones the method of Korneluk *et al.* (1985) was used. In addition to the other primers, reverse primer (BCL) was also used to sequence plasmid DNA. Sequencing reactions were done with [α -³⁵S]dATP (Amersham) at 37 °C. All nucleotides were from BCL.

Purification of oligonucleotide. The oligonucleotide was obtained from the Dyson-Perrins Laboratory, Oxford. The oligonucleotide solution was heated overnight at 55 °C and then cooled on ice. The solution was dried down under vacuum and resuspended in sterile water. To assess the purity of the oligonucleotide preparation it was labelled using [γ -³²P]ATP (Amersham) and T4 polynucleotide kinase (Amersham) as described by Carter *et al.* (1985). A solution of 10 pmol µl⁻¹ in 1 mM-Tris/HCl pH 7.5 containing 0.1 mM-EDTA was used as a primer in sequencing reactions.

Assay of β -lactamase activity. An overnight culture of *S. aureus* was diluted 1 in 100 in fresh CY broth and grown aerobically, following the OD₆₇₅ as a measure of growth. After 1 h, if required, CBAP was added to a final concentration of 7.5 µM. Samples were taken by removing 1 ml of culture, adding 150 µl 8-hydroxyquinoline and microfuging for 5 min. The supernatant was decanted to a fresh tube and the pellet resuspended in 1 ml CY and 150 µl 8-hydroxyquinoline. The supernatant and pellet fractions were assayed for β -lactamase activity at 37 °C, in a cuvette containing 0.1 mg nitrocefin in 0.05 M-phosphate buffer pH 7.0 to which the sample was added (usually 10–50 µl). The A₄₈₂ was followed and the activity of each sample determined in µmol product produced min⁻¹ (mg dry weight of cells)⁻¹.

***S. aureus* β -lactamase plate test.** The test used was a modification of the method of Dyke *et al.* (1966). Colonies

grown on starch plates (either with or without CBAP) were covered by a solution of penicillin G (0.5 g), 0.1 M-phosphate buffer pH 7.0 (20 ml) and stock iodine solution (5 ml). The solution was poured off the plate and the colonies producing β -lactamase were identified by their white haloes, which appeared after approximately 30 s incubation at 25 °C.

RESULTS

Cloning in *E. coli*

The β -lactamase gene was cloned from purified *S. aureus* plasmid DNA, prepared from the strains shown in Table 1, using the vector pRW33. The β -lactamase-encoding recombinant plasmids were selected on plates containing ampicillin. A comparison of the largest β -lactamase-

Fig. 1. Comparison of fragments from staphylococcal plasmids that contain the β -lactamase gene. The names in parentheses correspond to the names of the clones, containing the fragment shown, in *E. coli*. A dashed vertical line shows the position of a restriction site in one orientation in the invertible region and a dashed horizontal line indicates an incomplete map for *Hind*III. An arrow indicates the position and orientation of the β -lactamase gene.

Fig. 2. The smallest fragments cloned from the staphylococcal plasmids conferring resistance to ampicillin in *E. coli* and the strategy used to sequence the β -lactamase genes. The arrows indicate the start and the extent of the sequences obtained from these clones.

producing fragments is shown in Fig. 1. The fragment of DNA containing the gene was made smaller by sub-cloning and a comparison of the smallest Ap^r clones obtained is shown in Fig. 2.

Sequencing the β -lactamase genes

The genes were sequenced using the strategy outlined for each in Fig. 2. The genes of pPC1, pS1, pI1071 and pUB101 were sequenced completely and their nucleotide sequence, together with the partial nucleotide sequence of pII3804 and pI3796 genes, is shown in Fig. 3. The amino acid sequence of the proteins is compared in Fig. 4.

*Cloning the β -lactamase genes in *S. aureus**

The β -lactamase genes were cloned in *S. aureus* using two shuttle vectors for transfer of DNA between *E. coli* and *S. aureus*: pAE704 and pAE705 (Fig. 5).

Table 2. β -Lactamase activity of staphylococcal plasmids and clones

Plasmid	Construction of plasmid	Orientation of insert*	With inducer		Without inducer	
			Total activity†	Percentage in medium	Total activity†	Percentage in medium
pPC1			289	28	249	18
pAE206‡	pAE201 <i>HincII</i> <i>PvuII</i> /pAE704 <i>SmaI</i> <i>PvuII</i>		<i>Does not transform S. aureus</i>			
pAE207‡	pAE201 <i>HincII</i> <i>PvuII</i> /pAE705 <i>SmaI</i> <i>PvuII</i>	—	41	18	39	22
pS1			607	11	6	<1
pAE306‡	pAE301 <i>HincII</i> <i>PvuII</i> /pAE704 <i>PvuII</i>	—	986	55	7	<1
pAE307‡	pAE301 <i>HincII</i> <i>PvuII</i> /pAE705 <i>SmaI</i> <i>PvuII</i>	—	242	20	5	<1
pI1071			124	<1	3	<1
pAE110	pI1071 <i>PvuII</i> <i>BclI</i> /pCW59 <i>BglII</i> <i>HaeIII</i>		77	8	2	<1
pAE115	pAE101 <i>HincII</i> /pAE704 <i>SmaI</i>	+	347	<1	6	<1
pAE116	pAE101 <i>HincII</i> /pAE704 <i>SmaI</i>	—	385	<1	5	<1
pAE117	pAE101 <i>HincII</i> /pAE705 <i>SmaI</i>	—	33	<1	5	<1
pAE118	pAE101 <i>HincII</i> /pAE705 <i>SmaI</i>	+	254	<1	5	<1
pI3796			142	<1	4	<1
pAE504	pAE501 <i>HincII</i> /pAE704 <i>SmaI</i>	+	310	<1	8	<1
pAE505	pAE501 <i>HincII</i> /pAE704 <i>SmaI</i>	—	454	1	9	<1
pAE506	pAE501 <i>HincII</i> /pAE705 <i>SmaI</i>	—	18	<1	6	<1
pI13804			77	23	<1	<1
pAE404	pAE401 <i>EcoRI</i> /pAE704 <i>EcoRI</i>	+	133	28	3	<1

* Orientation of insert is described in the text.

† Total activity is expressed as μmol product produced min^{-1} (mg dry wt of culture) $^{-1}$ at 37 °C.

‡ Construction of this plasmid is described in the text.

The isolated β -lactamase structural gene of pI1071 (cloned as a 1.1 kb *HindIII*–*HincII* fragment in pAE703), and that of pUB101 (cloned as a 1.3 kb *HindIII* fragment in pAE703), although conferring resistance to ampicillin in *E. coli*, were not expressed in *S. aureus*. However, when larger fragments of the staphylococcal plasmids were cloned in *E. coli* and then transferred to *S. aureus*, β -lactamase was expressed. The clones constructed are shown in Table 2.

The plasmids pAE115, pAE116, pAE117, pAE118, pAE504, pAE505 and pAE506 were constructed by cloning a fragment containing the β -lactamase gene into the *SmaI* site of the vector (Fig. 5). The orientation is defined as '+' if the *HindIII* site of the polylinker of the vector is nearer to the 3' end of the β -lactamase gene, and '–' if the *HindIII* site is nearer to the 5' end of the gene.

In the construction of pAE206, the vector was cut with *PvuII* and *SmaI*; pAE201 was cut with *HincII* and *PvuII*. The *HincII*–*PvuII* β -lactamase-containing fragment of pAE201 is ligated to the following vector fragments: the *PvuII*–*SmaI* fragment of pAE704 containing the erythromycin resistance determinant and the *PvuII* fragment encoding the *E. coli* origin of replication. In pAE206, which failed to transform *S. aureus*, the small *PvuII*–*SmaI* fragment from the vector, containing the polylinker, is missing. This fragment is also missing from plasmids pAE207 and pAE307. The orientation of the insert in both these plasmids is described as '–', by analogy with the other clones, although the *HindIII* site of the polylinker, used to define the orientation, is lost.

Plasmid pAE306 has the insert cloned into the *PvuII* site (A) of vector pAE704 (Fig. 5).

One plasmid expressing β -lactamase in *S. aureus* was constructed by cloning directly in *S. aureus*. Plasmid pAE110 was made by cloning a *BclI*–*PvuII* fragment of pI1071 in the staphylococcal plasmid pCW59.

Estimation was made of the β -lactamase activity of the parent staphylococcal plasmid and of the clones, in the supernatant and associated with the cell, grown in the presence and absence of inducer (Table 2).

						60
1		TTAA	TAACCTTAGCC	ATTTC AACAC	CTTCTTTCAA	ATATTTTATAA
2	HindIII					
3	CTTACTACGC	TCGTTA--G	--T-----	---TG---	-----	-----
4		-----G	--T-----	---TG---	-----	-----
5	-----A-	--A-----G	--T-----	---TG---	-----	-----
6		-----A-	--T-----	---TG---	-----	T-----
		-35		-10	*	120
1	TAAACTATTG	ACACCGATAT	TACAATTGTA	ATATTATTGA	TTTATAAAAA	TTACAACGTG
2						
3	-----A-	-----	-----	-----T-	-----	-----T--
4	-----A-	-----	-----	-----T-	-----	-----T--
5	-----A-	-----ATA	-----	-----T-	-----G-	-----
6	-----A-	-----A	-----	-----A-	-----G-	-----
		S+D				180
1	AATATCGGAG	GGTTTATTTT	GAAAAAGTTA	ATATTTTTAA	TTGTAATTGC	TTTAGTTTAA
2						
3	-----T-	-----	-----	-----	-----C-	-----
4	-----T-	-----	-----*	-----	-----C-	-----
5	-----	-----	-----	-----	-----G-	-----
6	-----T-	-----	-----	-----	-----C-	-----
						240
1	AGTGCATGTA	ATTCAAACAG	TTCACATGCC	AAAGAGTTAA	ATGATTTAGA	AAAAAAATAT
2						
3	-----	-----	-----C-	-----A-	-----	-----
4	-----	-----	-----C-	-----A-	-----	-----
5	-----	-----	-----	-----	-----	-----
6	-----	-----	-----	-----	-----	-----
						300
1	AATGCTCATA	TTGGTGTTTA	TGCTTTAGAT	ACTAAAAGTG	GTAAGGAAGT	AAAATTTAAT
2						
3	-----	-----	-----	-----	-----	-----
4	-----	-----	-----	-----	-----	-----
5	-----	-----	-----	-----	-----	-----
6	-----	-----	-----	-----	-----	-----
			TaqI			360
1	TCAGATAAGA	GATTTCCTA	TGCTTCAACT	TCAAAAGCGA	TAAATAGTGC	TATTTTGTTA
2						
3	-----	-----	-----G-	-----	-----	-----
4	-----	-----	-----G-	-----	-----	-----
5	-----	-----	-----G-	-----	-----	-----
6	-----	-----	-----G-	-----	-----	-----
		RsaI		RsaI		420
1	GAACAAGTAC	CTTATAATAA	GTAAATAAAA	AAAGTACATA	TTAACAAAGA	TGATATAGTT
2						
3	-----A-	-----	-----	-----A-	-----	-----
4	-----A-	-----	-----	-----A-	-----	-----
5	-----	-----	-----	-----	-----	-----
6	-----	-----	-----	-----A-	-----	-----
				EcoRV		480
1	GCTTATTCTC	CTATTTTAGA	AAAATATGTA	GGAAAAGATA	TCACTTTAAA	AGCACTTATT
2						
3	-----	-----	-----	-----	-----	-----A-
4	-----	-----	-----	-----	-----	-----
5	-----	-----	-----	-----C-	-----	-----
6	-----	-----	-----	-----	-----	-----A-
						540
1	GAGGCTTCAA	TGACATATAG	TGATAATACA	GCAACAATA	AAATTATAAA	AGAAATCGGT
2						
3	-----	-----	-----	-----	-----	-----
4	-----	-----	-----	-----	-----	-----
5	-----	-----	-----	-----	-----	-----
6	-----	-----G-	-----	-----	-----	-----T--
		MboI				600
1	GGAATCAAAA	AAGTTAAACA	ACGTCTAAAA	GAAGTAGGAG	ATAAAGTAAC	AAATCCAGTT
2						
3	--G-----	-----	-----	-----	-----	-----
4	-----	-----	-----	-----	-----	-----
5	-----	-----	-----	-----	-----	-----
6	-----	-----	-----	-----	-----	-----
						660
1	AGATATGAGA	TAGAATTAAA	TTACTATTCA	CCAAAGAGCA	AAAAAGATAC	TTCAACACCT
2						
3	-----	-----	-----	-----	-----	-----
4	-----	-----	-----	-----	-----	-----
5	-----	-----	-----	-----	-----	-----
6	-----	-----	-----	-----	-----	-----


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Fnu4HI 720
1 GCTGCTTTCG GTAAGACTTT AAATAAACTT ATCGCAAATG GAAAATTAAG CAAAGAAAAAC
2 -----
3 -----
4 -----
5 -----
6 -----

780
1 AAAAAATTCT TACTTGATTT AATGTTAAAT AATAAAAGCG GAGATACTTT AATTAAAGAC
2 -----
3 -----
4 -----
5 -----
6 -----

NdeI 840
1 GGTGTTCCAA AAGACTATAA GGTGCTGAT AAAAGTGGTC AAGCAATAAC ATATGCTTCT
2 -----
3 -----T-----G-----G-----
4 -----
5 -----
6 -----T-----G-----

XbaI DdeI HaeIII 900
1 AGAAATGATG TTGCTTTTGT TTATCCTAAG GGCCAATCTG AACCTATTGT TTTAGTCATT
2 -----
3 -----
4 -----
5 -----
6 -----

960
1 TTTACGAATA AAGACAATAA AAGTGATAAG CCAAATGATA AGTTGATAAG TGA AACCGCC
2 -----
3 -----
4 -----
5 -----
6 -----

EcoRV 1020
1 AAGAGTGTA TGAAGGAATT TTAATATTCT AAATGCATAA TAAATACTGA TAACATCTTA
2 -----
3 -----
4 -----
5 -----
6 -----GT-CT GTTGC-AA-T -G-AT-TA--

HincII HincII EcoRV 1080
1 TATTTTGAT TATATTTTGT ATTATCGTTG ACATGTATAA TTTTGATATC
2 -----
3 -----G-----CG-
4 -----
5 -----
6 ATA-AAT-T- A-C-AAAA-G -G-C-TC-GT -TGA ACTATT -CACATATAA ACAATTTAAC

1140
1
2
3
4
5
6 AAGGATGTTA TCACTGTAGC CGTTGGCTAC TATCTAAGAT ATGCATTGAG CTATCGTGAT

1200
1
2
3
4
5
6 ATATCTGAAA TATTAAGAGA ACGTGGTGTA AACGTTTCATC ATTCAAACGA TCTACCGTTG

1252
1
2
3
4
5
6 GGTTCAAGAA TATGCCCCAA TTTTATATCA AATTGGAAG AAAAAGCATA AA

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Fig. 3. The nucleotide sequence of the β -lactamase genes of (1) pPC1, (2) pS1, (3) pI1071, (4) pI3796, (5) pI13804, (6) pUB101. The region coding for the β -lactamase is underlined. Restriction enzyme sites in any of the sequences are shown by a horizontal line above the top printed line. A dash in sequences (2)–(6) means that the nucleotide is the same as the top printed line and a * within the sequence means that a base is missing. The position of the transcriptional and translational initiation signals (S + D – Shine–Dalgarno sequence) are shown. The start of the mRNA is marked by a * above the nucleotide sequence.

Fig. 4. The putative amino acid sequence of the staphylococcal β -lactamases. The sequences are: 1, pPC1 and pS1; 2, pI1071; 3, pI13804 (up to amino acid 118 only); and 4, pUB101. For 2, 3 and 4 unless an amino acid change is indicated the sequence is the same as 1. A vertical line shows the end of the data for pI13804 and (3) indicates that there is no sequence data. A * indicates a silent change in the codon. The vertical arrow indicates the cleavage site at the end of the leader peptide. The mature protein is numbered according to Ambler (1979). The amino acids of the leader peptide are numbered from L1 to L22.

DISCUSSION

Chang & Cohen (1974) cloned the β -lactamase gene from the staphylococcal plasmid pI258 in *E. coli* and showed that it conferred resistance to ampicillin. All six staphylococcal β -lactamase genes used in the present study were expressed in *E. coli* even when the piece cloned from *S. aureus* included only 139 base pairs upstream of the translational start of the β -lactamase. The same piece did not permit expression of the gene when cloned back in *S. aureus* but fragments of DNA which also included upstream sequence sufficient to code for a second protein did permit such expression. This upstream DNA is being investigated to determine whether or not a second protein is required for expression. The constitutive or inducible expression of β -lactamase is determined, at least in part, by the fragment that could be expressed in *S. aureus*. Similarly, the proportion of the enzyme found in the medium is a characteristic of the cloned piece of DNA.

Fig. 5. Construction of ampicillin-sensitive shuttle vectors pAE704 and pAE705. The following are used to show phenotypic resistance (r) or sensitivity (s): Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Tc, tetracycline. The *E. coli* origin of replication is marked *ori*. The *PvuII* site labelled 'A' is referred to in the text. Plasmid pAE701 was constructed in the following way. Plasmid pE194 was cut with *XbaI* and the ends filled in with Klenow, and ligated to pRW33 cut with *EcoRI* and the ends filled in. The resultant Tc^r, Em^r plasmid in *E. coli* was digested with *EcoRI*, ligated and used to transform *S. aureus* to Em^r. The plasmid obtained, pAE701, is pE194 with an additional single *EcoRI* site, outside the region coding for Em^r and the origin of replication.

Comparison of the nucleotide sequence of the six staphylococcal β -lactamase genes (part only of pI3796) and pII3804) is very informative. The transcriptional control sequences at -35 (TTGACA) and -10 (TATTAT) are identical for pI1071, pPC1, pUB101, pI3796, pII3804 and also for pI258 (Wang & Novick, 1987). There are differences in the nucleotide sequences outside this region but the spacing between the transcriptional control signals is the same. There is another possible -10 sequence (TAATAT) three nucleotides before the sequence marked as -10 in Fig. 3. The -10 sequence marked is that described by McLaughlin *et al.* (1981) for the pI258 β -lactamase and the start of the mRNA was shown by them to be the A, seven nucleotides downstream from the -10 . In plasmids pI1071, pPC1 and pI3796 and A is also found at this position, whereas in pUB101 and pII3804 the A is replaced by a G. Purines are usually found at

mRNA initiation sites and, in *E. coli*, A is more common than G (see Glass, 1982). A Shine-Dalgarno sequence (GGAGGG) occurs 22 nucleotides from the mRNA start site.

The nucleotide sequences of the coding region of the β -lactamase genes are highly conserved and all begin at a TTG codon. ATG is the most commonly found initiation codon but there are other cases of TTG as an initiation codon, for example, the sporulation gene *gerA* in *Bacillus subtilis* (Feavers *et al.*, 1985). The open reading frame corresponds to a protein of 281 amino acids and, for pPC1, includes at the C-terminal end the known sequence of the 257 amino acids for the extracellular PC1 β -lactamase (Ambler, 1975). The derived amino acid sequences for the proteins from pPC1, pS1, pI1071 and pUB101, and for part of the protein of pII3804, are shown in Fig. 4 and they are very similar.

Nielsen & Lampen (1982) described the processing of PC1 β -lactamase by a lipoprotein leader peptidase, involving membrane attachment by a thioether modification. The modification occurs at the cysteine at position L17. The target cleavage site of the lipoprotein leader peptidase is reported to be even more highly conserved than that of the leader peptidase and the amino acid sequence before the cysteine (–leucine–serine–alanine–cysteine–) of PC1 β -lactamase fits with the consensus sequence described (Nielsen & Lampen, 1982). This amino acid sequence is present in all the β -lactamase leader sequences examined here so that it is likely that similar processing occurs in these proteins.

The β -lactamases of pPC1, pS1 and pI258 have identical amino acid sequences and so presumably behave indistinguishably. Within the N-terminal region the β -lactamase of pUB101 has only one amino acid change, the conservative substitution of an alanine for a valine at L9 when compared with pS1, and this does not alter the prediction, based on Chou & Fasman (1978*a*, *b*), that this region will form a β -sheet. The substitution of a methionine in the β -lactamase of pII3804 at position L10 for the isoleucine found in the other β -lactamases increases the likelihood of an α -helix formation but a β -sheet is still the predicted structure. The β -lactamase produced from pI1071 is found in only very small amounts in the medium and differs from that of pUB101 at position L20 where a proline substitutes for the serine. This β -lactamase contains the site of thioether modification and therefore it is possible that it is the release of the enzyme from the membrane that is blocked. For PC1 it is assumed that the protein is released from the membrane by cleavage by a peptidase after L22. It is thus a possibility that the presence of proline at L20, three amino acids before the cleavage site, in the β -lactamase of pI1071 is what prevents processing of the protein and hence results in very low amounts of β -lactamase in the medium. This is being investigated further by site-directed mutagenesis at this site.

Unlike the export of β -lactamases from Gram-positive organisms, export of β -lactamases from Gram-negative bacteria is reported to be by cleavage of a conventional leader peptide by a leader peptidase. This signal peptidase processing site of an *E. coli* β -lactamase has been extensively investigated (Plückthun & Knowles, 1987) by deletion of amino acids and the results support the idea that the amino acids at the C-terminal end of the leader peptide are important for processing, in particular, the amino acid at the position equivalent to L20 in the staphylococcal β -lactamases. Plückthun & Knowles (1987) also concluded that many different sequences are permitted at the start of the mature protein, so the lysine at position 32 may not affect processing although it is a very different amino acid from the glutamic acid that is found in this position for all the β -lactamases that are found in a high proportion in the medium. This conclusion must be contrasted with that for the coat protein of bacteriophage f1, in which Russell & Model (1981) and Boeke *et al.* (1980) showed that replacement of glutamic acid at this site by leucine or tyrosine slowed down the export of coat proteins.

The nucleotide sequence of the start of pI3796 β -lactamase is shown in Fig. 3. The sequence is identical to that of pI1071 except that in pI3796, following the TTG initiation codon there are only four As rather than five. The autoradiograph from a sequencing gel comparing pPC1, pI1071 and pI3796 is shown in Fig. 6. The loss of a base introduces a 'frameshift' mutation in the third amino acid of the protein and a TAA stop codon follows directly. There does not seem to be another suitable initiation codon nearby, allowing a different translation 'start'. The DNA for sequencing was cloned directly into M13 from a clone, Ap^r in *E. coli*, of pI3796 β -lactamase. If this is the correct sequence of this gene it is hard to explain how a functional β -lactamase is

Fig. 6. 'A' track sequencing gel of plasmids pPC1 (a), pI1071 (b) and pI3796 (c). The position of the Shine-Dalgarno sequence (S+D) and the first amino acid (Met) of the β -lactamase are shown. The position of the base deletion in pI3796 is marked by an arrow.

produced from this DNA. Possible explanations might include: (i) ribosomal 'slippage' to compensate for the loss of a base; (ii) some unique tRNA, coded for in *S. aureus* and *E. coli* where the gene is expressed, allowing the recognition of two rather than three bases in an amino acid codon; (iii) a different codon is recognized as the start signal for translation of the protein; (iv) some aberration in the cloning. The last explanation can to some extent be ruled out because several clones have been sequenced and the region sequenced in both directions using independently made clones.

Within the mature protein sequence there are some differences in the amino acid sequences. The three-dimensional structure of the mature β -lactamase has been determined (Herzberg & Moulton, 1987) and amino acid 121 is within an α -helical region. Since this amino acid is alanine in PC1 and pS1 β -lactamases and glutamic acid in those from pUB101 and pI1071 it would be expected that there would be a significant effect on folding. Glutamic acid is found in this position for class A β -lactamases from *B. licheniformis*, *B. cereus* 569H, *E. coli* pBR322 and *E. coli* RTM (Ambler, 1979) so that it is the pPC1 and pS1 proteins that are unusual. The serine at the active centre of class A proteins is conserved at position 70 for all the enzymes (Knott-Hunziker *et al.*, 1979). Other non-conservative differences occur at positions 226 and 229 but are outside regions thought to form α -helices (Herzberg & Moulton, 1987).

It was originally thought that the immunological classification using antibody raised against PC1 β -lactamase was due to changes in the amino acid sequence of the proteins (Richmond, 1965 *a*). Plasmids pI1071, pS1 and pPC1 produce A-type β -lactamase, pII3804 produces C-type

(Dyke & Richmond, 1967), while that of pUB101 was classified as D-type (Rosdahl, 1973). There are no amino acid changes that can be clearly correlated with this immunological classification. Perhaps other factors, such as the folding of the protein or the presence of other proteins, may contribute to the detected differences.

Downstream of the open reading frame there are no obvious transcription termination signals in any of the plasmids for which the nucleotide sequence has been obtained. It is clear that soon after the end of the gene, homology between the nucleotide sequences diminishes. Plasmids which are known to be closely related, such as pPC1, pS1 and pI258, have identical downstream sequences, including a direct repeat of a sequence found in only one copy in pI1017 (Fig. 3). The sequence of pUB101 is very different from all the other plasmids 14 nucleotides after the translational stop signal (Fig. 3). It still remains a possibility that the β -lactamase structural gene is the first of an operon and therefore the transcriptional stop is a considerable distance downstream.

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