Molecular analysis of β -lactamases from four species of *Streptomyces*: comparison of amino acid sequences with those of other β -lactamases

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Genes encoding extracellular β -lactamases of *Streptomyces badius*, *Streptomyces cacaoi*, *Streptomyces fradiae* and *Streptomyces lavendulae* were cloned and mapped in *Streptomyces lividans*. DNA sequence analysis of the β -lactamase genes revealed a high overall G + C content, ranging from 71 to 75 mol %, with a G + C content of 95 mol % at the third position of the codons for all four genes. The primary structure of the β -lactamases including their signal peptides was deduced. The four β -lactamases exhibited homology to each other and to class A β -lactamases from other bacterial genera. We suggest that *Streptomyces* β -lactamases are representatives of a superfamily of genes, from which class A β -lactamases of Gram-negative bacteria may have evolved.

Introduction

Gene structure analysis and the regulation of gene expression in the Gram-positive mycelial bacterium *Streptomyces* are of widespread interest. *Streptomyces* has a complex growth cycle with multiple morphological stages, making it useful for developmental studies. Bacteria from this genus also produce a large number of antibiotics and enzymes of which many are industrially important.

β -Lactamases (EC 3.5.2.6) of chromosomal or plasmid origin have been found in a large number of Gram-positive and Gram-negative bacteria (Citri, 1971). Three evolutionarily distinct β -lactamase groups, classes A, B and C, have been proposed (Ambler, 1980; Jaurin & Grundström, 1981). Examples of class A β -lactamases (penicillinases) are those from *Staphylococcus aureus* (Ambler, 1975), *Bacillus licheniformis* (Yamamoto & Lampen, 1976), *B. cereus* type I enzyme (Thatcher, 1975) and the plasmid-encoded TEM β -lactamases (Ambler & Scott, 1978; Sutcliffe, 1978). The Zn^{2+} -requiring *B. cereus* type II enzyme (Hussain *et al.*, 1985) belongs to class B; class C enzymes comprise the chromosomally determined cephalosporinases from Gram-negative bacteria such as *Escherichia coli* (Jaurin & Grundström, 1981) and *Pseudomonas aeruginosa* (Knott-Hunziker *et al.*, 1982). Recently, a class D group of oxacillin-hydrolysing β -lactamases has been proposed, including

the OXA-2 β -lactamase of *Salmonella typhimurium* (Dale *et al.*, 1985) and the PSE-2 enzyme of *P. aeruginosa* (Huovinen *et al.*, 1988).

The majority of *Streptomyces* strains produce extracellular β -lactamases (Ogawara, 1981). From an evolutionary aspect, it would be of interest to elucidate the molecular structure of *Streptomyces* β -lactamases, since they possibly represent ancestors of other β -lactamases (Ogawara, 1981). The primary structures of the *S. albus* G β -lactamase (Dehottay *et al.*, 1987) and of an *S. cacaoi* β -lactamase (Lenzini *et al.*, 1988) indicate that these enzymes are class A β -lactamases.

To study transcription, secretion and evolutionary aspects of extracellular enzymes in *Streptomyces*, we have undertaken a molecular analysis of the extracellular β -lactamases of this genus. The genes for the β -lactamases of *S. badius*, *S. cacaoi* and *S. fradiae* have been cloned previously in *S. lividans* (Jaurin *et al.*, 1988a) and the DNA sequence encoding the *S. cacaoi* enzyme has been established (Forsman *et al.*, 1989). Here we report the primary structure of β -lactamases of *S. badius*, *S. fradiae* and *S. lavendulae*, and show that *Streptomyces* β -lactamases are representatives of a superfamily of genes from Gram-positive bacteria, from which it is possible that class A β -lactamases of Gram-negative bacteria have evolved.

Methods

Bacterial strains, plasmids, media and culture conditions. *S. lividans* 1326 (Lomovskaya *et al.*, 1972) was used as the host for all *Streptomyces*

† This paper is dedicated to the memory of Dr BengtÅke Jaurin who died on 18 January 1990.

Abbreviation: ORF, open reading frame.

DNA cloning work. For preparation of chromosomal DNA, *S. lavendulae* DSM 2014 was used. *E. coli* strains DH1 (Hanahan, 1983) and JM103 (Yanisch-Perron *et al.*, 1985) were used as hosts for all *E. coli* DNA cloning work. *Streptomyces* plasmids pJAS01 (Jaurin & Cohen, 1984) and pIJ487 (Ward *et al.*, 1986) and the *E. coli* plasmid pACYC184 (Chang & Cohen, 1978) have been described elsewhere. Plasmid pIJ4642 was kindly provided by Dr T. Kieser (John Innes Institute, Norwich, UK).

E. coli was cultivated at 37 °C in LB medium and on LB agar plates (Bertani, 1951). *Streptomyces* was grown at 30 °C in YEME medium (Bibb *et al.*, 1977) and R2YE medium (Thompson *et al.*, 1980) and on agar plates of these media; the liquid cultures were grown aerobically in a New Brunswick Scientific orbital shaker at 200 r.p.m. *S. lividans* containing pJAS01 or pIJ487 and its derivatives was grown on plates containing 50 µg thiostrepton ml⁻¹, and in liquid cultures containing 20 µg thiostrepton ml⁻¹. *E. coli* containing pACYC184 or pIJ4642 and their derivatives was grown in the presence of chloramphenicol (25 µg ml⁻¹). All standard manipulations of DNA were, if not otherwise stated, done as described by Maniatis *et al.* (1982).

Chemicals and enzymes. All chemicals were of the highest grade commercially available. Restriction endonucleases and DNA-modifying enzymes were purchased from Boehringer Mannheim and Pharmacia. All isotopes were purchased from Amersham. Nitrocefin was obtained from BBL. Nucleotide reagents for DNA sequencing were purchased from Pharmacia. Thiostrepton was a gift of E. J. Squibb & Sons, New Brunswick, NJ, USA. Agaroses were purchased from FMC Corporation, and oligonucleotides were obtained from Symbicom (Umeå, Sweden).

Subcloning of the *S. fradiae* β -lactamase gene. The *S. fradiae* DSM 40063 β -lactamase gene was previously cloned on a ~4000 bp DNA fragment (Jaurin *et al.*, 1988a); before attempting to sequence the gene it was subcloned further. Plasmid pBF-1 containing pACYC184 DNA (Jaurin *et al.*, 1988a) was digested with *Sph*I, religated, and introduced into *E. coli* DH1. The resulting plasmid (Fig. 1a), denoted pBF-1-184, was isolated and purified.

To subclone the ~4000 bp DNA fragment encoding β -lactamase activity in *S. lividans* (Jaurin *et al.*, 1988a and Fig. 1a), pBF-1-184 was digested either with *Bam*HI and *Sph*I or *Xba*I and *Ssp*I (Fig. 1a). Exonuclease III and S1-nuclease treatments at various times generated deletions from both directions, i.e. starting from *Bam*HI or *Xba*I, respectively, as described by Henikoff (1984). The ends were made blunt by Klenow treatment and the molecules were circularized using T4-DNA ligase, whereafter competent *E. coli* DH1 cells were transformed (Hanahan, 1983) with material from each time point. Chloramphenicol-resistant colonies were picked-out for each successive time interval and analysed by restriction enzyme digestion for the location of deletion breakpoints. A selected set of nested deletions from both directions were cut with *Eco*RI and ligated to *Eco*RI-digested pIJ487 (Ward *et al.*, 1986). The ligation mixes were introduced into protoplasts of *S. lividans* by transformation, as described by Bibb *et al.* (1978), except that the protoplasts were spread as a 0.7% R2YE agar overlay on R2YE plates. After regeneration for 3 d at 30 °C, the cells were replicated onto R2YE plates containing 50 µg thiostrepton ml⁻¹. After growth for a further 3 d, the thiostrepton-resistant colonies for each time point were screened directly for β -lactamase production by dropping nitrocefin, a chromogenic cephalosporin (O'Callaghan, 1973), onto the plates. The smallest insert expressing β -lactamase was ~2000 bp and originated by deletion from the *Bam*HI site. This ~2000 bp fragment was further subcloned by utilizing the *Sal*I site located ~400 bp from the left-end of the insert (Fig. 1a). The ~1680 bp *Sal*I fragment of pBF-1-184- Δ 1 (Fig. 1a) was cloned into the *Xho*I site of pJAS01 in the host *S. lividans*. The resulting nitrocefin-positive recombinants confirmed that the complete β -lactamase gene of *S. fradiae* was included on the ~1680 bp *Sal*I fragment.

(a) *S. fradiae* β -lactamase gene

(b) *S. lavendulae* β -lactamase gene

Fig. 1. (a) Subcloning of *S. fradiae* β -lactamase gene. Plasmid pBF-1-184, containing a ~4000 bp DNA insert encoding β -lactamase activity, was digested with *Bam*HI and *Sph*I or *Ssp*I and *Xba*I, respectively, and subjected to exonuclease III digestion (wavy arrows). From the resulting deletion derivatives of pBF-1-184, the smallest clone mediating β -lactamase activity, denoted pBF-1-184- Δ 1, was isolated. The ~2000 bp DNA insert of pBF-1-184- Δ 1 was further subcloned by digestion with *Sal*I, generating a ~1680 bp DNA fragment, that was ligated to *Xho*I-cleaved pJAS01. (See Methods for details.) (b) Cloning and subcloning of *S. lavendulae* β -lactamase gene. The gene was initially cloned on a ~4300 bp *Bam*HI fragment and further subcloned to a ~1400 bp *Bam*HI-*Sau*3A fragment. However, circumstantial evidence showed that the complete gene was not included on this subcloned fragment. Therefore, the β -lactamase gene of *S. lavendulae* was recloned from a *Sal*I genomic library. A ~3200 bp *Sal*I fragment, carrying the complete gene, was identified and cloned in pJAS01. (See Methods for details.) The filled boxes represent the location of β -lactamase genes of *S. fradiae* and *S. lavendulae*, respectively. Open boxes and double lines indicate chromosomal DNA. The restriction enzymes have been abbreviated as follows: B, *Bam*HI; Sa, *Sal*I; S, *Sau*3A; Sp, *Sph*I; Ss, *Ssp*I; X, *Xba*I. Only relevant restriction sites are shown.

Cloning of the *S. lavendulae* β -lactamase gene. Chromosomal DNA of *S. lavendulae*, prepared as described by Hopwood *et al.* (1985), was digested with *Bam*HI and ligated to *Bam*HI-cleaved pJAS01. After introduction into *S. lividans*, as described above, about 12000 thiostrepton-resistant colonies were obtained. The recombinants were screened for β -lactamase production with nitrocefin. Plasmid DNA from two β -lactamase positive clones was purified and characterized by restriction enzyme mapping; both contained an identical ~4300 bp *Bam*HI insert. These plasmids were transformed into *S. lividans* to

demonstrate that the β -lactamase production obtained was mediated by plasmid DNA and not due to some rearrangement or mutation in the host.

Subcloning of the *S. lavendulae* β -lactamase gene. To subclone the *S. lavendulae* β -lactamase gene, the ~4300 bp *Bam*HI fragment was purified from an agarose gel by electroelution and partially digested with *Sau*3A. The DNA was separated by electrophoresis on a 1.2% preparative GTG agarose gel. DNA molecules ranging from ~600–4000 bp were isolated by electroelution. These *Sau*3A-digested DNA molecules were ligated to *Bam*HI-cleaved pJAS01. The ligation mix was introduced into *S. lividans* by protoplast transformation. Plasmid DNA from several β -lactamase-positive clones was purified and analysed. The plasmid with the smallest insert size contained a ~1400 bp *Bam*HI–*Sau*3A DNA fragment (Fig. 1*b*). However, it was later shown by DNA sequencing that the ~1400 bp *Bam*HI–*Sau*3A fragment did not encode the complete gene. Therefore, chromosomal DNA of *S. lavendulae* was digested with various restriction enzymes and Southern blot hybridization analysis was done with the ~1400 bp *Bam*HI–*Sau*3A fragment as a probe. A ~3200 bp chromosomal *Sal*I fragment containing the β -lactamase gene was identified and mapped (Fig. 1*b*). *Sal*I-digested chromosomal DNA of *S. lavendulae* was separated by electrophoresis on a 1% preparative GTG agarose gel. DNA molecules ranging from ~2000–6000 bp were isolated by electroelution. This *Sal*I-digested fractionated chromosomal DNA was ligated into *Xho*I-cleaved pIJ4642. The ligation mixes were introduced by transformation (Hanahan, 1983) into *E. coli* DH1. More than 90% of all transformants contained *Sal*I inserts of chromosomal origin. This genomic library was subjected to colony hybridization screening with the ~1400 bp *Bam*HI–*Sau*3A fragment as a probe. Of 1500 colonies, one showed positive hybridization. Analysis of the plasmid of this clone, denoted p β L-FLK-4642, by restriction-enzyme mapping confirmed that the ~3200 bp *Sal*I fragment had been cloned. Plasmid DNA of p β L-FLK-4642 was linearized with *Bgl*II and ligated to *Bam*HI-digested pJAS01. The recombinant DNA molecules were introduced into protoplasts of *S. lividans* by transformation. After regeneration, the resulting thiostrepton-resistant colonies produced a red zone within 15 s of addition of nitrocefin.

DNA sequence analysis. The β -lactamase gene from *S. badius* DSM 40139 was cloned on a ~1300 bp fragment to plasmid pJAS01 (Jaurin *et al.*, 1988*a*). The resulting plasmid, denoted p β B-1, was cut with *Sph*I/*Bgl*II and the fragment carrying the β -lactamase gene was isolated and purified from a preparative agarose gel. The fragment was first cloned into *Sph*I/*Bam*HI-digested pACYC184, followed by insertion into M13 phages mp18 and mp19 (Norrander *et al.*, 1983) by utilizing the *Hind*III site on pACYC184 and the *Pst*I site derived from pJAS01 and the corresponding sites in M13 mp18 and mp19. Also, a number of subfragments of the *Hind*III–*Pst*I fragment were cloned into M13 phages mp18 and mp19. These subfragment clonings in the M13 phages were a combination of partial *Sau*3A and directed clonings that together comprised almost the entire β -lactamase gene. To fill in gaps, sequence specific oligonucleotides were used as primers.

To sequence the *S. fradiae* β -lactamase gene, the ~1680 bp *Sal*I fragment shown in Fig. 1(*a*) was cloned, in both orientations, into *Sal*I-digested M13 mp19. These constructs were digested with *Sac*I/*Bam*HI, followed by Exonuclease III and S1-nuclease digestion (Henikoff, 1984). After Klenow treatment, religation and transformation into *E. coli* JM103, templates were prepared from 100–150 bp successive deletions from both orientations. DNA sequence analysis was done as above with M13 universal primer throughout.

To sequence the *S. lavendulae* β -lactamase gene, various subfragments of the ~1400 bp *Bam*HI–*Sau*3A fragment (Fig. 1*b*) were cloned into M13 mp18 and mp19. DNA sequence analysis was done using M13 universal primer. The ~3200 bp *Sal*I fragment was cloned into M13 mp19, and an oligonucleotide hybridizing close to the *Bam*HI site

was used for sequencing upstream the gene. On the basis of the resulting sequence, another oligonucleotide was synthesized and used to sequence the other strand downstream.

DNA sequence analysis was done using the dideoxy method (Sanger *et al.*, 1977) using deoxyadenosine 5'- α -[³⁵S]thiotriphosphate as label. Both strands were sequenced at least once in every position.

The termination-reaction mixtures were routinely incubated at 37 °C with Klenow fragment and the terminated products were separated on wedge-shaped 6% sequencing gels (Sanger *et al.*, 1977). Where compressions were a problem, the sequencing reactions were incubated at 50 °C with Klenow fragment and the terminated DNA products were separated on wedge-shaped sequencing gels containing 50% formamide.

Computer analysis. The Geneus sequence-analysis system as well as the programs developed by R. Staden (see Harr *et al.*, 1986) were utilized to assemble and analyse the sequences. The software package PC/Gene, developed by IntelliGenetics, was also used. For multiple amino acid alignments the IntelliGenetics program 'GENALIGN' was used; this program was developed by Dr Hugo Martinez (Sobel & Martinez, 1986).

Results and Discussion

DNA sequence analysis of *Streptomyces* β -lactamases

DNA fragments encoding extracellular β -lactamases of *S. badius*, *S. cacaoi* and *S. fradiae* had been cloned previously (Jaurin *et al.*, 1988*a*), and the β -lactamase genes of *S. badius* and *S. cacaoi* subcloned on DNA fragments of ~1300 and ~1250 bp, respectively (Jaurin *et al.*, 1988*a*). The subcloning of the *S. fradiae* β -lactamase gene on a ~1680 bp DNA fragment is described in Methods and Fig. 1(*a*); the cloning and subcloning of a β -lactamase gene of *S. lavendulae* is described in Methods and Fig. 1(*b*). Initially, β -lactamase activity of *S. lavendulae* was mapped to a ~1400 bp DNA fragment. Later, we found that this fragment did not encode the whole structural gene; the complete gene was therefore recloned from a genomic library of *S. lavendulae* (see Methods and Fig. 1*b*).

The DNA fragments encoding the four β -lactamase genes were subjected to DNA sequence analysis. We have presented the sequence of the *S. cacaoi* DSM 40057 gene previously (Forsman *et al.*, 1989), but for comparative purposes it is included here. The high (73 mol%) G+C content of *Streptomyces* (Benigni *et al.*, 1975) results in a highly biased codon usage with a remarkable predominance (more than 90 mol%) of G or C in the third and most degenerate position of the translated codons (Bibb *et al.*, 1984). Therefore, the DNA sequences were analysed for open reading frames (ORFs) with such a codon preference at the third position. For each β -lactamase gene, only one ORF was found that could fulfil this criterion. When ORFs were translated from the first ATG or GTG codon, the primary sequence of *S. badius* and *S. cacaoi* (Fig. 2), and *S. fradiae* and *S. lavendulae* (Fig. 3) β -lactamases was deduced and found

to consist of 313, 314, 306 and 305 amino acids, respectively.

The G+C content of the β -lactamase genes of *S. badius*, *S. cacaoi*, *S. fradiae* and *S. lavendulae* was 70.7, 70.7, 75.3 and 74.6 mol%, respectively, values which are in good agreement with the overall 73 mol% G+C content of *Streptomyces* DNA (Benigni *et al.*, 1975). The codon preference for G or C at the third position for the four genes was also analysed: it was 94.5 mol% for *S. badius* and *S. cacaoi*, 95.6 mol% for *S. fradiae* and 94.0 mol% for *S. lavendulae*. The codon distribution showed a good correlation with a compilation of codon usages of *Streptomyces* genes (Hopwood *et al.*, 1986). There was no pronounced preference for C over G at third positions when synonymous codons allow such a choice, as was described for the α -amylase gene of *S. limosus* (Long *et al.*, 1987). The absence of leucine TTA codons in the four β -lactamase genes should be noted; it has recently been suggested that the tRNA recognizing this codon is only expressed late in development (Chater *et al.*, 1988). Accordingly, genes that are expressed early should lack this codon. This is in agreement with the observation that β -lactamases of *S. badius*, *S. cacaoi* and *S. fradiae* are expressed relatively early in growth (Jaurin *et al.*, 1988a). However, the fact that the *ampC* β -lactamase gene of *E. coli*, which has seven TTA codons, is also expressed relatively early in growth when introduced into *S. lividans* (Forsman & Jaurin, 1987), is intriguing.

DNA sequences just upstream of the coding regions of the β -lactamases are given in Fig. 4. When these putative ribosome-binding sites were compared to the sequence close to the 3'-end of 16S rRNA of *S. lividans* (Bibb & Cohen, 1982), a significant degree of complementarity was observed. As shown in Fig. 4, a four- to six-base-long homology to the complementary sequence of the 3'-end

of 16S rRNA of *S. lividans* was found. These homologous sequence stretches were located seven to eight bases upstream of the respective initiation codon. Many *Streptomyces* genes do not possess a high degree of complementarity to the 3'-end of 16S rRNA (Hopwood *et al.*, 1986). However, the genes representing the β -lactamase gene family indigenous to *Streptomyces* (this paper; Dehottay *et al.*, 1987; M. V. Lenzini & J. Dusart, personal communication) all show a high degree of complementarity to the ribosomal sequence.

Comparison of amino acid sequences of β -lactamases

Table 1 represents the results of a homology search, using the region method of GENALIGN, among the four β -lactamases reported here, and the *S. albus* G (Dehottay *et al.*, 1987) and *S. cacaoi* (Lenzini *et al.*, 1988) β -lactamases. A homology search was also done with nine non-*Streptomyces* β -lactamases, as described in Table 1. Based on the homology scores, the β -lactamases reported by us could be divided into two groups, with the *S. badius* and *S. cacaoi* enzymes in one group, and the β -lactamases of *S. fradiae* and *S. lavendulae* in the other. This homology was also confirmed at the DNA level by Southern hybridization experiments (Jaurin *et al.*, 1988b), and in an amino acid sequence homology comparison among the four β -lactamase genes using the algorithm of Lipman & Pearson (1985) (data not shown). The *S. albus* G enzyme (Dehottay *et al.*, 1987) showed strong homology to the second group, and interestingly, the *S. cacaoi* β -lactamase described by Lenzini *et al.* (1988) exhibited intermediate homology. However, all six *Streptomyces* β -lactamases showed an overall high homology to the class A β -lactamases in contrast to the β -lactamases of classes B, C and D (Table 1). Also, the three class A β -lactamases of *Bacillus* exhibited clustered homology scores, as did the class A Gram-negative β -lactamases (Table 1).

Obviously, sequence comparison of the *S. cacaoi* β -lactamase analysed here and the one reported by Lenzini *et al.* (1988) revealed significant sequence dissimilarities (Table 1). These dissimilarities could be explained by a difference between the strains used or by the presence of two different β -lactamase genes in the *S. cacaoi* genome. However, fatty acid analysis of the strain used by us, performed by the German Collection of Micro-organisms and Cell Cultures (DSM), showed that the β -lactamase gene cloned by us originates from the original strain *S. cacaoi* ISP 5057.

Active-site serine β -lactamases, to which class A enzymes belong, have been shown to possess the consensus sequence Phe-(X)₃-Ser-(X)₂-Lys, with the serine as the active site residue (De Meester *et al.*, 1987). This consensus sequence was found in all β -lactamases

<i>S. lividans</i>	5' -AGAAAGGAGGTGATCCAG-3'	
<i>S. badius</i>	5' -CGCCAGGAGGTTTCGCC	ATG -3'
<i>S. cacaoi</i>	5' -GACCCGGAGGTTTCGGAAC	ATG -3'
<i>S. fradiae</i>	5' -AGGGAGAAGGGGTCATC	GTG -3'
<i>S. lavendulae</i>	5' -CGTAGGGGAGAGCGCATC	GTG -3'

Fig. 4. Ribosomal binding site alignment. The top line shows the complementary DNA sequence of the 3'-end of *S. lividans* 16S rRNA. The four sequences below this are the regions upstream from the presumed initiation codon (boxed) of the four β -lactamase genes examined. Underlined nucleotides show complementarity to the 3'-end of *S. lividans* 16S rRNA.

Table 1. Homology between amino acid sequences of β -lactamases

References for sequences are as follows: *S. badius*, *S. fradiae* and *S. lavendulae* (this paper); *S. albus* G (Dehottay *et al.*, 1987); *S. cacaioi* (this paper and Lenzini *et al.*, 1988); *E. coli* TEM-1 (no. 11; Sutcliffe, 1978); *K. pneumoniae* (Arakawa *et al.*, 1986); *Staph. aureus* (Wang & Novick, 1987); *B. licheniformis* (Neugebauer *et al.*, 1981); *B. cereus* type I (Wang *et al.*, 1985); *B. cereus* type III (Hussain *et al.*, 1987); *E. coli ampC* (no. 14; Jaurin & Grundström, 1981); *B. cereus* type II (no. 13; Hussain *et al.*, 1985); and *Sal. typhimurium* (Dale *et al.*, 1985). Overall homology scores for pairwise alignment is shown. The scores for every pairwise alignment of the selected sequences are based on the number of matching amino acids and the number of amino acid deletions from the sequences required to keep the common regions in register, as calculated in GENALIGN, using the region method and conservative amino acid replacement alphabet. Boxes indicate groups of highly similar sequences. Repeated randomization of the selected amino acid sequences and subsequent calculation of homology scores, resulted in homology score values between 0 and -100. Numbers 1 to 12 represent class A β -lactamases.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. <i>S. badius</i>		211	137	123	143	147	168	173	159	117	100	94	-56	-64	-29
2. <i>S. cacaioi</i> (this paper)			137	128	138	131	151	162	142	106	108	102	-57	-63	-39
3. <i>S. fradiae</i>				230	209	128	136	132	132	90	126	119	-48	-28	-31
4. <i>S. lavendulae</i>					209	126	131	118	121	89	119	119	-48	-72	-12
5. <i>S. albus</i>						121	120	124	131	80	95	95	-26	-63	-39
6. <i>S. cacaioi</i> (Lenzini <i>et al.</i> , 1988)							102	122	109	106	101	88	-46	-74	-8
7. <i>B. licheniformis</i>								181	207	135	105	103	-39	-70	-2
8. <i>B. cereus</i> type I									175	126	110	106	0	-71	-3
9. <i>B. cereus</i> type III										117	90	89	-23	-61	-18
10. <i>Staph. aureus</i>											106	104	0	-96	-2
11. <i>E. coli</i> TEM-1												201	-24	-91	-2
12. <i>K. pneumoniae</i>													-22	-92	1
13. <i>B. cereus</i> class B														-120	-1
14. <i>E. coli</i> class C															-102
15. <i>Sal. typhi</i> class D															

reported here, as well as those reported previously (Fig. 5). Thus it seems likely that the β -lactamases described by us are representatives of active-site serine β -lactamases, as has been shown for the *S. albus* G enzyme (De Meester *et al.*, 1987) and the *E. coli* TEM-1 β -lactamase (Fisher *et al.*, 1981).

Streptomyces β -lactamases are extracellular enzymes (Ogawara, 1981). When cells of *S. lividans* containing the cloned β -lactamases of *S. badius*, *S. cacaioi*, *S. fradiae* and *S. lavendulae* were grown, the majority of the enzyme activity was secreted (Jaurin *et al.*, 1988a; data not shown). This indicated that these enzymes should be synthesized in a precursor form containing an NH₂-terminal signal peptide, as are most extracellular proteins of *Streptomyces* (Hütter & Eckhardt, 1988). Using the method of von Heijne (1986), the cleavage site between the signal peptide and the mature protein of the four β -lactamases was predicted (Fig. 6). Based on this analysis, both *S. badius* and *S. cacaioi* β -lactamases contained signal peptides 26 amino acids long. Predicted signal peptides of the *S. fradiae* and *S. lavendulae* enzymes were 34 amino acids long. All four signal peptides conformed to the criteria characteristic of such sequences: a hydrophilic amino terminus containing charged amino residues such as arginine; a central hydrophobic transmembrane region; and the -1 to -3 cleavage site rule (von Heijne, 1986). The lengths of

signal peptides vary, e.g. 18 to 25 for *E. coli* and 30 to 40 for *Bacillus*; for *Streptomyces* signal peptides the range is much wider, from 24 to 56 amino acid residues (Hütter & Eckhardt, 1988). The four predicted β -lactamase signal peptides (Fig. 6) also conform to the notion that *Streptomyces* signal peptides have a predominance of arginine residues as charged residues in the hydrophilic part of the signal peptide, whereas lysine residues are preferred by *E. coli* or *Bacillus* (Hütter & Eckhardt, 1988).

The roles of β -lactamases in *Streptomyces* are not completely known (Ogawara, 1981). Whereas β -lactamases cause drug resistance in pathogenic bacteria, a change in target specificity is the main resistance mechanism in *Streptomyces* (Ogawara, 1981). It has been suggested that β -lactamases in *Streptomyces* are ancestors of those in pathogenic bacteria (Ogawara, 1981). The class A *Bacillus* β -lactamase group showed higher similarities to β -lactamases of *Streptomyces* than to β -lactamases of the enterobacteria (Table 1). The β -lactamase of *Staphylococcus aureus* exhibited highest homology to the *Bacillus* group, and somewhat lower homology to the β -lactamases of *Streptomyces* and the enterobacteria (Table 1). The class A β -lactamases were aligned and divided in twenty amino acid windows and compared (Fig. 7). A high overall homology was found. Three regions that were highly conserved among the β -

<i>S. badius</i>	AsnAspArgAlaArg	Phe	AlaTyrHis	SerThrPheLys	AlaLeuGlnAlaAla
<i>S. cacaot</i> (this study)	ArgAspGlyGluArg	Phe	ProTyrAsn	SerThrPheLys	AlaLeuGlyCysGlu
<i>S. fradiae</i>	ArgAlaAspGluArg	Phe	ProIleAla	SerMetPheLys	ThrIleAlaValAla
<i>S. larendulae</i>	ArgAlaGluGluArg	Phe	ProMetCys	SerValPheLys	ThrLeuAlaValAla
<i>S. albus</i> G	ArgAlaAspGluLeu	Phe	ProMetCys	SerValPheLys	ThrLeuSerSerAla
<i>S. cacaot</i> (Lenzini <i>et al.</i> , 1988)	ArgAlaAspGluArg	Phe	AlaTyrGly	SerThrPheLys	AlaLeuGlnAlaGly
<i>E. coli</i> TEM-1	ArgProGluGluArg	Phe	ProMetMet	SerThrPheLys	ValLeuLeuCysGly

Fig. 5. Active-site comparison between β -lactamases of various *Streptomyces* spp. and of *E. coli* (for references see Table 1). Regions with strong similarity are boxed.

<i>S. badius</i>	MetHisProArgValArgThrAlaValAlaGlyLeuValAlaValLeuSerLeuValProLeuAlaAlaCysGly	
<i>S. cacaot</i>	MetLeuHisThrArgIleArgAlaThrLeuGlyAlaValAlaAlaLeuSerLeuValProValMetAlaCysGly	
<i>S. cacaot</i> *	MetArgIleArgProThrArgArgLeuLeuGlyAlaValAlaProLeuAlaLeuValProLeuValAlaCysGlyAlaSerGlySerGlyGluGluProGlyLeuGlyGlyCysGlyThrSerAla	
<i>S. fradiae</i>	ValAspArgThrThrAlaArgProAsnArgAlaValLeuAlaThrGlyValGlyAlaAlaLeuAlaAlaThrAlaAlaAlaGlyProAlaHisAla	
<i>S. larendulae</i>	ValGlyThrThrGlyAlaArgProSerArgArgAlaValLeuThrAlaAlaAlaGlyAlaValAlaGlyIleProLeuGlyGlySerThrAlaPheAla	
<i>S. albus</i>	ValHisProSerThrSerArgProSerArgThrLeuLeuThrAlaThrAlaGlyAlaAlaLeuAlaAlaAlaThrLeuValProGlyThrAlaHisAlaSerSerGlyGlyArg	

Fig. 6. Signal peptide compilation. The cleavage site for *S. badius*, *S. cacaot*, *S. fradiae* and *S. larendulae* signal peptides was predicted by the method of von Heijne (1986). The proposed cleavage site for each signal peptide is located immediately after the last amino acid shown. The signal peptide of *S. albus* β -lactamase has been described previously (Dehottay *et al.*, 1987). The sequence of the signal peptide of the *S. cacaot* β -lactamase described by Lenzini *et al.* (1988) (denoted *S. cacaot**) has been corrected (M. V. Lenzini and J. Dusart, personal communication).

Fig. 7. Alignment of the amino acid sequences of class A β -lactamases (for references see Table 1). Optimal alignment of the sequences was achieved using the program GENALIGN (Sobel & Martinez, 1986). The alignment was done using the Jimenez-Montano and Zamora-Cortina conservative amino acid replacement alphabet. Each sequence was divided into successive 20 amino acid windows. Every window was compared with the amino acid consensus sequence derived from the alignment. Homology was defined as identity plus conservative replacements of amino acids. The windows are represented as follows: open boxes represent 0–9 amino acid homology to the consensus sequence; light-grey boxes indicate 10–14 amino acid homology to the consensus; and stippled boxes show 15–20 amino acid homology to the consensus sequence. I, II and III indicate conserved regions, and correspond to positions 61–99, 110–208 and 225–301, respectively, of the precursor of the *S. albus* G β -lactamase (Dehottay *et al.*, 1987).

lactamases could be identified. The first conserved block spanned the active-site region, the second was located in the middle of the primary sequence and the third conserved block was found at the C-terminus (Fig. 7). The Gram-positive class A β -lactamases exhibited a similar pattern of conserved segments in these three regions. However, the two enterobacterial class A β -lactamases showed a lack of homology to the Gram-positive class A enzymes in the region preceding the active site and in the C-terminus region. This probably indicates that these regions are not essential for a functional β -lactamase, and therefore have not been under evolutionary constraint. Also, we suggest that *Streptomyces* class A β -lactamases are representatives of a Gram-positive superfamily of genes, from which class A β -lactamases of Gram-negative bacteria may have evolved.

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