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Nucleotide sequence of the gene encoding the *Streptomyces albus* G β-lactamase precursor

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A 1400-base DNA fragment, which contains the gene encoding the extracellular active-site serine β-lactamase of *Streptomyces albus* G previously cloned into *Streptomyces lividans* [Dehottay et al. (1986) *Gene 42*, 31 – 36], was sequenced. The gene codes for a 314-amino-acid precursor, the N-terminal region of which has the characteristics of a signal peptide. The β-lactamase as excreted by the host strain *S. lividans* PD6 has a ragged N-terminus, indicating either the presence of a leader peptidase of poor specificity or the action of an aminopeptidase. The primary structure (as deduced from the nucleotide sequence) was confirmed by amino acid sequencing of a 16-residue stretch at the amino terminus of the protein, a 12-residue stretch containing the active-site serine [De Meester et al. (1987) *Biochem. J. 244*, 427 – 432] and a 23-residue stretch obtained by trypsin digestion of the protein. The β-lactamase belongs to class A, has three half-cystine residues (one of which occurs on the amino side of the active-site serine) and is inactivated by thiol reagents. Putative ribosome binding site and terminator region were identified.

In previous studies, a 1.4-kb *SstI – PstI* chromosomal DNA fragment containing the gene that encodes the extracellular β-lactamase of *Streptomyces albus* G was cloned in *Streptomyces lividans* TK24 on the high-copy number plasmid pIJ702 [1]. The resulting strain, *S. lividans* PD6 carrying the modified plasmid pDML6, was a mucher better producer of β-lactamase than *S. albus* G grown under optimal production conditions. In parallel to these studies, derivatization of the *S. lividans* PD6 β-lactamase by β-iodopenicillanate under conditions causing complete enzyme inactivation, followed by trypsin digestion, led to the isolation of a dihydrothiazine-labelled dodecapeptide (Ala-Asp-Glu-Leu-Phe-Pro-Met-Cys-Ser-Val-Phe-Lys) that possessed the sequence Phe-Xaa_3-Ser-Xaa_2-Lys characteristic of all the active-site serine β-lactamases [2]. Serine was the binding site of β-iodopenicillanate since the preceding cysteine in the β-iodopenicillanate-derivatized (and guanidine chloride unfolded) protein remained accessible to the thiol reagent 5,5′-dithiobis-(2-nitrobenzoate). The 1.4-kb β-lactamase-encoding *SstI – PstI* DNA fragment of *S. lividans* PD6 has now been sequenced allowing establishment of the primary structure of the precursor form of the enzyme and isolation of the promoter region.

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Abbreviations. ClHgBzOH, *p*-chloromercuribenzoate; DD-peptidase, D-alanyl-D-alanine carboxypeptidase; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); PBP, penicillin-binding protein.

Enzymes. D-Alanyl-D-alanine carboxypeptidase (EC 3.4.16.–); aminoglycoside phosphotransferase (EC 2.7.1.95); bacterial alkaline phosphatase (EC 3.1.3.1); DNA polymerase I (EC 2.7.7.7); endo-β-*N*-acetylglucosaminidase (EC 3.2.1.96); β-lactamase (EC 3.5.2.6); restriction endonucleases (EC 3.1.21.4); trypsin (EC 3.4.21.4); lysozyme (EC 3.2.1.17).

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

S. lividans TK24 and its derivative M336 bearing the promoter probe plasmid pIJ424 were from the John Innes Institute (Norwich, UK) [3]. *S. lividans* PD6, plasmid pDML6 (see the introduction) and the growth conditions were described in [1].

Promoter probing in vivo

pIJ424 uses the promoterless aminoglycoside phosphotransferase *aph* gene of transposon Tn*5* as the indicator for promoter activity. Insertion of a promoter-containing DNA fragment in front of the *aph* gene confers resistance to kanamycin on *S. lividans* TK24 used as a host. pIJ424 also contains the thiostrepton resistance (*tsr*) marker as well as the major terminator (*ter*) of the *Escherichia coli* phage fd so that readthrough from upstream vector promoters into the *aph* gene is largely prevented [3].

Enzymes, antibiotics and recombinant DNA techniques

The enzymes were from Amersham International, Amersham, UK; Boehringer, Mannheim, FRG; Bethesda Research Laboratory, Gaithersburg, MD, USA; New England Biolabs, Beverly, MA, USA. Thiostrepton was a gift from Dr R. B. Sykes (The Squibb Institute for Medical Research,

Princeton, NJ, USA) and kanamycin was from Sigma Chemical Co., St Louis, MO, USA. The preparation of plasmid DNA [4] and digestion with restriction endonucleases, treatment with Klenow fragment of DNA polymerase I, treatment with bacterial alkaline phosphatase, ligation experiments, agarose gel electrophoresis and DNA elution [5, 6] were carried out as described. Transformation of protoplasted host cells was as described [3]. Lysozyme used for protoplasting was from Belovo, Bastogne, Belgium.

Nucleotide sequencing

The dideoxynucleotide chain-termination method [7, 8] was used. Zones of base compression were resolved using the formamide procedure [3]. For each possible reading frame, the codon usage was analyzed with Staden's program [9, 10] and the neomycin phosphotransferase gene of *Streptomyces fradiae* as reference [11]. Possible errors indicated by frame shifts were corrected by careful re-examination of the X-ray films (and using the aforementioned formamide procedure).

β-Lactamase of Streptomyces lividans *PD6: chromatofocusing and measurement of β-lactamase activity*

The *S. lividans* PD6 β-lactamase was purified as described [12]. The preparation thus obtained was further resolved into several enzymatically active species by chromatofocusing of 30 nmol β-lactamase on a column (0.2 × 20 cm) of Mono P (Pharmacia FPLC system) [13]. The column was equilibrated in 25 mM *N*-methylpiperazine/HCl buffer pH 5.7, supplemented with 8% ethyleneglycol and 8% glycerol. Elution ($0.7\ \text{min} \times \text{min}^{-1}$) was carried out with 27 ml Pharmacia polybuffer 74 pH 4.0 diluted 10 times with water and supplemented with ethyleneglycol and glycerol as above. The β-lactamase activity was measured by the method of O'Callaghan et al. [14] at 30°C in 500 µl of 50 mM sodium phosphate buffer pH 7.0 containing 100 µM nitrocefin.

Trypsin digest: isolation of peptide AG3

The β-iodopenicillanate-derivatized *S. lividans* PD6 β-lactamase (see the introduction) was digested with trypsin and the products were separated by FPLC on a Pro-RPC column as described [2]. Elution yielded in addition to the active-site serine-containing dodecapeptide, several unlabelled peptides. One of them, peptide AG3, was of high purity; it was further characterized.

Amino acid composition and sequencing

Amino acid sequencing was done with a 470-A Applied Biosystem gas-phase sequenator as described [15].

Derivatization of thiol groups

p-Chloromercuribenzoate (ClHgBzOH) and 5,5′-dithiobis(2-nitrobenzoate) (Nbs_2) were from Sigma (St Louis, MO, USA). The presence of S-S bonds was investigated by Nbs_2 treatment of the sodium-borohydride-reduced protein [16].

RESULTS

The 1.4-kb *SstI*–*PstI* insert of pDML6 contained the structural gene encoding the *S. albus* G β-lactamase and had

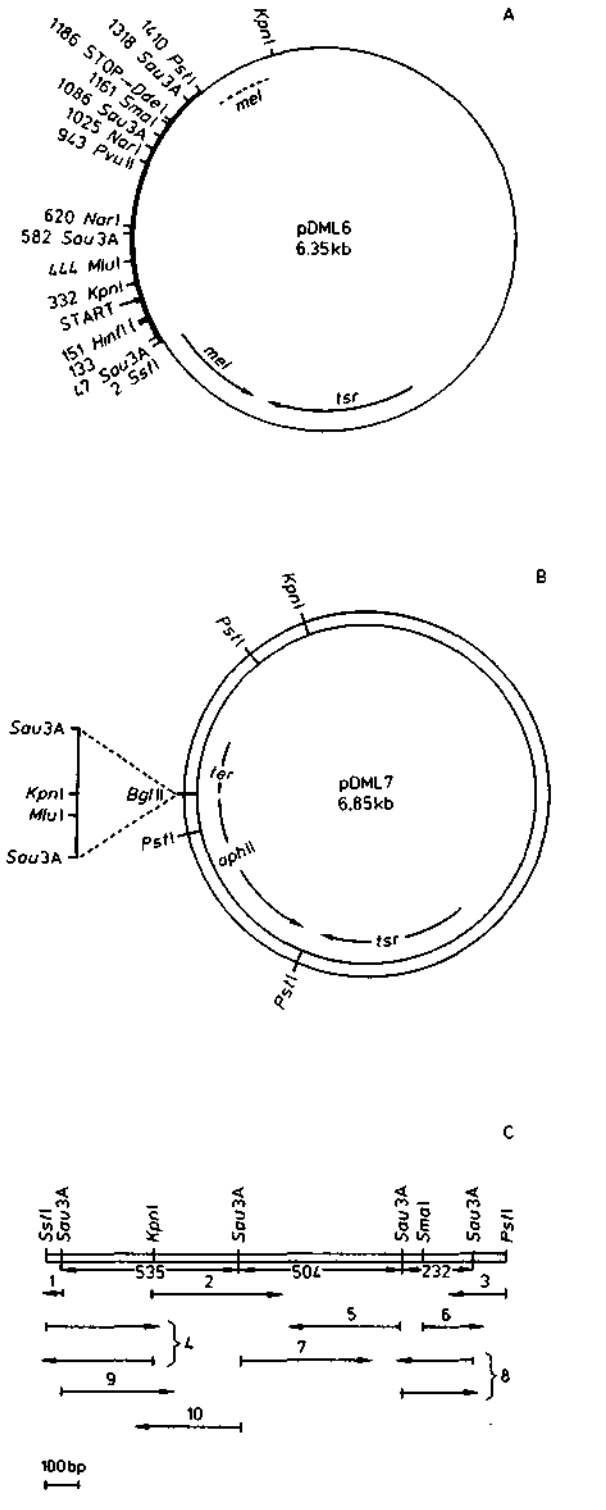

Fig. 1. *Restriction map of the 1.4-kb* SstI–PstI *fragment inserted in plasmid pDML6 (A) and of the plasmid pDML7 with the inserted 530 bp by* Sau3A *fragment (B), and strategy of sequencing (C).* (A) Light line: plasmid pIJ702. Heavy line: *SstI–PstI* insert; the four restriction sites for *Sau*3A and the single restriction site for *MluI* and *KpnI* are underlined. Arrows: translation start and stop points of the *S. albus* G β-lactamase precursor gene. (B) Only the restriction sites relevant to the present work are shown. *aph* and *tsr:* see Materials and Methods; *ter:* major terminator of fd phage. (C) The phage vectors M13mp10, M13mp11, M13mp18, M13tg130 and M13tg131 were used to clone the various sub-fragments

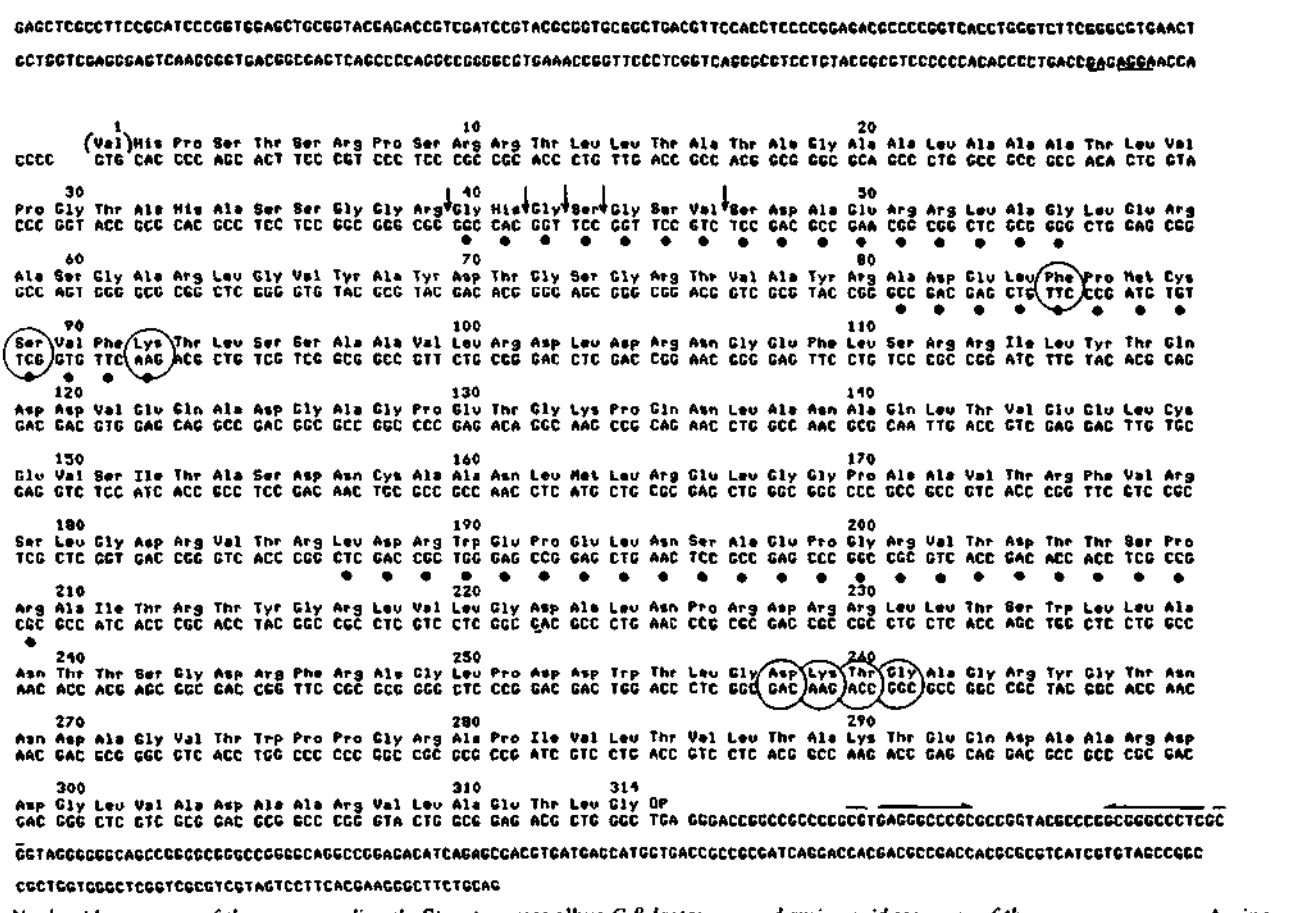

Fig. 2. *Nucleotide sequence of the gene encoding the* Streptomyces albus *G β-lactamase and amino acid sequence of the enzyme precursor.* Amino acids of particular importance (see text) are circled. Residues marked with a black dot were determined by amino acid sequencing. The putative ribosome binding site is underlined. The inverted repeat of the putative transcription termination signal is shown by horizontal arrows. The possible processing sites of the protein precursor are shown by vertical arrows

promoter activity. The restriction map of this insert is shown in Fig. 1A. It had four sites for *Sau*3A and one each for *Mlu*I and *Kpn*I.

Localization of the promoter region within the 1.4-kb SstI–PstI *DNA fragment*

The 1.4-kb *Sst*I–*Pst*I DNA insert (isolated by agarose gel electrophoresis) was cut with *Sau*3A, the digest was ligated to the promoter-probe pIJ424 (previously cleaved with *Bgl*II and treated with bacterial alkaline phosphatase) and the resulting ligation mixture was used to transform *S. lividans* TK24. Thiostrepton-resistant transformants showing resistance to kanamycin were selected on MMT agar [17] plates containing 150 μg kanamycin × ml^{-1} and then on kanamycin-gradient plates. Seven clones (out of 10 that could grow in the presence of 250 μg kanamycin × ml^{-1} or more) had a plasmid larger than pIJ424. In particular, *S. lividans* strain PD7, carrying the recombinant plasmid pDML7 (Fig. 1B), grew in the presence of 500 μg kanamycin × ml^{-1}.

Digestion of pDML7 with *Mlu*I caused linearization of the DNA and digestion with *Kpn*I (which has two restriction sites, one in the *Sau*3A insert and the other in the pIJ424 vector; see Fig. 1B), yielded two fragments of 5.35 and 1.48 kb, respectively. These results demonstrated that the promoter activity was located within the larger (535-bp) sub-fragment produced by *Sau*3A digestion of the 1.4-kb *Sst*I–*Pst*I insert. In addition, double digestion of pDML7 with *Mlu*I and *Pst*I gave rise to four fragments of 4320, 1250, 920 and 360 bp, respectively. Isolation of the smallest fragment demonstrated that the orientation of insertion of the 535-bp *Sau*3A sub-fragment in pDML7 was that shown in Fig. 1B.

Nucleotide sequencing of the 1.4-kb SstI–PstI *DNA fragment*

Fig. 1C illustrates the strategy used; 80% of the sequence were determined on both strands. Fig. 2 gives the complete nucleotide sequence of the 1.4-kb *Sst*I–*Pst*I DNA fragment and translates the open reading frame into amino acid sequence. From the translation start point (GTG) to the stop codon (an opal TGA triplet), the gene contained 942 nucleotides and had the information for the synthesis of a 314-amino-acid polypeptide.

Primary structure of the S. lividans *PD6 β-lactamase*

The amino acid composition of the extracellular β-lactamase of *S. albus* G as reported earlier [12] is in good agreement with that of the *S. lividans* PD6 β-lactamase as deduced from the nucleotide sequence of the gene, assuming that Gly-40 is at the amino terminus of the mature, excreted

enzyme (*vide infra*). The peptide stretch extending from Ala-81 to Lys-92 is the active-site serine-containing dodecapeptide described in [2] (see the introduction). In addition, the peptide stretch extending from Leu-187 to Arg-209 is the 23-amino-acid peptide AG3 isolated from the tryptic digest (see Materials and Methods). As shown in Fig. 3 (and as explained in the legend), two or three thiol groups occurred per enzyme molecule. These data were in agreement with the amino acid composition and the primary structure (Fig. 2) of the protein. Prior reduction of the protein by borohydride had no effect, suggesting that no S-S bonds were present. On incubation of the β-lactamase (50 mM) with ClHgBzOH at 1, 2, 4 and 20 μM (or more) for 30 min at 37°C (in 50 mM sodium phosphate buffer pH 7.0 containing 10% ethylene-glycol and 5% glycerol), the enzyme was inhibited by 25%, 40%, 73% and 100%, respectively. Inactivation proceeded with a second-order rate constant of 165 $M^{-1} s^{-1}$.

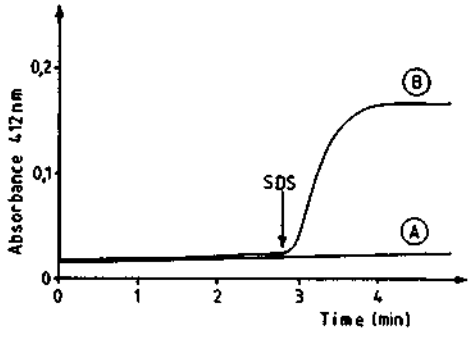

Fig. 3. *Estimation of free sulphydryl groups in the* Streptomyces lividans *PD6 β-lactamase by Nbs_2 treatment.* β-Lactamase (4.75 μM) was incubated at 37°C with 1 mM Nbs_2 in 50 mM sodium phosphate buffer pH 7.0 10% ethyleneglycol, 5% glycerol. Time of addition of SDS (0.15% final) is indicated by the arrow. The increase in absorbance at 412 nm (0.16) induced by this denaturing agent indicated the presence of 2 or 3 free sulphydryl groups [0.16/13600 (ε_{412} of Nbs_2) = 11.76 μM [SH]; hence 11.76/4.75 = 2.48]. (A) No SDS added; (B) SDS (0.15%, final) added at the time indicated by the arrow

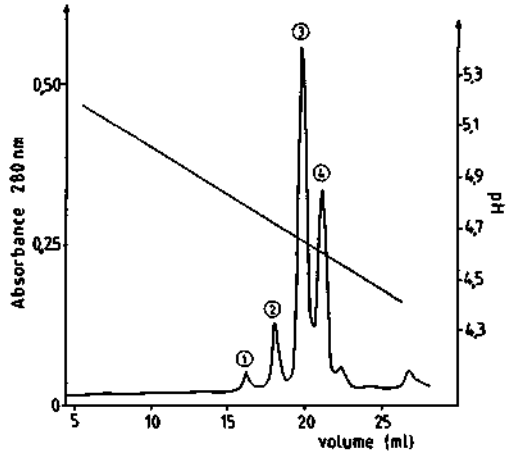

Fig. 4. *Chromatofocusing of the purified β-lactamase synthesized by* Streptomyces lividans *PD6.* For details, see Materials and Methods

Multiple amino termini in the S. lividans *PD6 β-lactamase*

The *S. lividans* PD6 β-lactamase was homogeneous in all respects except that chromatofocusing resolved the preparation into at least four catalytically active fractions (Fig. 4). Fractions 2, 3 and 4 had approximately the same specific activity (300 – 500 μmol hydrolyzed nitrocefin × min^{-1} × mg $protein^{-1}$). The pH of elution and the relative amounts were 4.79 and 2% for fraction 1, 4.71 and 9% for fraction 2, 4.65 and 55% for fraction 3 and 4.6 and 33% for fraction 4. Amino acid sequencing data (Table 1) showed that fraction 3 was still heterogeneous and that the mature β-lactamase, as isolated from the culture filtrates of *S. lividans* PD6, consisted of at least five different molecular species. Using the amino acid numbering of the protein precursor (Fig. 2), these species had Gly-40, Gly-42, Ser-43, Gly-44 and Ser-47, respectively, at the amino terminus. The higher pH of elution of fraction 2 was consistent with the presence of an extra positive charge provided by His-41. Cleavage upstream of Arg-39 might have given rise to fraction 1. Note that the catalytically active β-lactamase species of fractions 3 and 4 were histidine-free.

DISCUSSION

The β-lactamase-encoding gene is expressed constitutively in *S. albus* G [18] and its derivative *S. lividans* PD6 suggesting that gene expression depends only on the presence of a promoter sequence. *In vivo* promoter probing and positioning of the 535-bp *Sau*3A sub-fragment in the 1.4-kb *Sst*I – *Pst*I DNA fragment show that the information for promoter activity is comprised within the 200-nucleotide stretch that precedes the translation start point. Given that the transcription start point remains unknown and that various types of promoters occur in *Streptomyces* species [19], signals for promoter activity cannot be identified with certainty. Another feature of the cloned *Sst*I – *Pst*I DNA fragment is the presence immediately upstream of the translation start point of a 5′ GAGAGGA sequence which has the characteristics of a ribosome binding site [20]. Moreover, the 38-nucleotide sequence that starts at position 17 downstream of the opal stop codon TGA has an inverted repeat of 13 bases (with only one GT pair). This sequence may function as a terminator. Like all the

Table 1. *N-terminal amino acid sequences of various* Streptomyces lividans *PD6 β-lactamase species*
Fraction numbering refers to Fig. 4

Fraction	Sequence
2	Gly-His-Gly-Ser-Gly-Ser-Val-Ser-Asp
3	Gly-Ser-Gly-Ser-Val-Ser-Asp-Ala-Glu-Arg-Arg Ser-Gly-Ser-Val-Ser-Asp-Ala-Glu-Arg-Arg Gly-Ser-Val-Ser-Asp-Ala-Glu-Arg-Arg
4	Ser-Asp-Ala-Glu-Arg-Arg-Leu-Ala-Gly

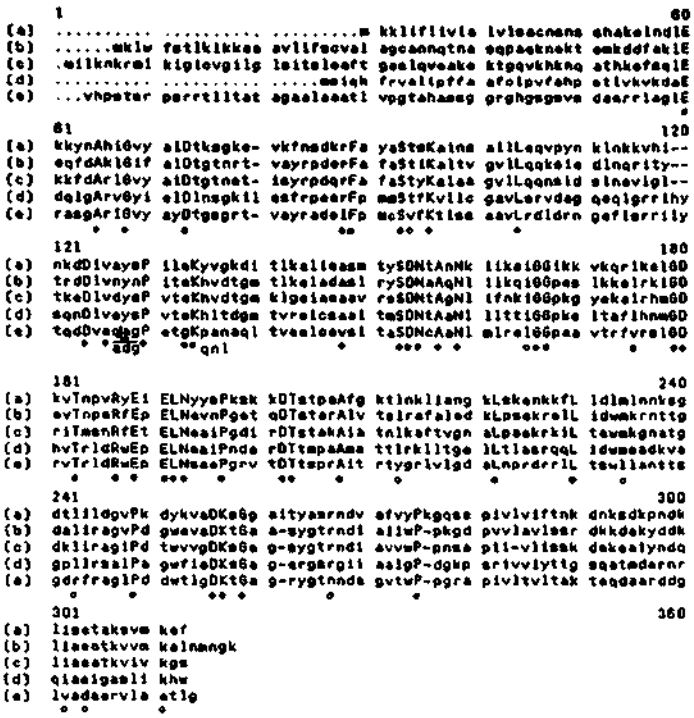

Fig. 5. *Possible alignment of the amino acid sequences of the active-site serine β-lactamase of* Streptomyces albus *G (e) and the class A β-lactamases of* Staphylococcus aureus *(a),* Bacillus licheniformis *(b),* Bacillus cereus *(c) and pBR322 (d).* The gaps introduced and the numbering are from [22]. Residues conserved in the five β-lactamases are in capital letters and marked with black dots. Open dots indicate amino acids that are conserved in the four β-lactamases used as reference but not in the *S. albus* G β-lactamase

terminators characterized so far in *Streptomyces* sp. [6, 21], it is reminiscent of the *rho*-independent terminators of *E. coli.*

The codon usage in the synthesis of the *S. albus* G β-lactamase shows the same biased pattern as that observed with other *Streptomyces* genes [6, 20]. Codon TGT (for Cys-88) is one of the four codons not previously reported [20] Codon GTG, instead of the usual ATG, occurs at the translation start point. Four out of thirteen sequenced *Streptomyces* genes share this peculiarity [20].

The 314-amino-acid polypeptide that is coded by the 942-nucleotide stretch extending from codon GTG to codon TGA is the precursor of the excreted, mature β-lactamase. The N-terminal portion of this precursor contains three arginine residues at positions 7, 10 and 11, followed by a long hydrophobic stretch (residues 16 – 30) and thus has the characteristics of a signal peptide. The site of cleavage of the signal peptide remains undetermined. The multiple amino termini that are present in the β-lactamase polypeptide produced by *S. lividans* PD6 may reflect either a lack of specificity of the leader peptidase of the host cell or the action of an aminopeptidase following cleavage by the leader peptidase at a site located upstream of Gly-40.

The amino acid sequence of the *S. albus* G β-lactamase as it is deduced from the nucleotide sequence of the cloned gene deserves comments. At 170 residues on the carboxyl side of the active-site serine (i.e. at position 258 in the precursor), there occurs the tetrad Asp-Lys-Thr-Gly. Given that threonine may be replaced by serine, this tetrad is found, at this position, in the active-site serine β-lactamases of class A [22]. Also, by analogy with the β-lactamases of class A, the second-order rate constant of acylation of the *S. albus* G β-lactamase by β-iodopenicillanate is high (50000 – 150000 M^{-1} s^{-1}) [23]. In contrast, the active-site serine β-lactamases of class C are characterized by the presence of histidine, instead of aspartic acid, at the amino side of the conserved sequence Lys-Thr/Ser-Gly [24, 25] and by a low value (< 1000 M^{-1} s^{-1}) of the second-order rate constant of acylation by β-iodopenicillanate [23].

The *S. albus* G β-lactamase possesses three half-cystine residues (at positions 88, 148 and 158). In all probability, there is no disulphur bond involved in the structure. Inactivation of the enzyme by ClHgBzOH indicates that at least one of these three cysteine residues is located at a crucial position in the tertiary structure. In fact, Cys-88 is part of the active site, a feature shared by the β-lactamase of *Klebsiella pneumoniae* [26]. The pBR322 β-lactamase also possesses a cysteine residue located at the seventh position on the carboxyl side of the active-site serine residue. Although the observation may be coincidental, the *S. albus* G, *K. pneumoniae* and pBR322 β-lactamases have the peculiarity to interact with β-iodopenicillanate according to a branched pathway where enzyme inactivation and turnover of the β-lactam compound take place concomitantly (so that complete inactivation of the *S. albus* G enzyme requires a molar ratio of β-lactam compound to enzyme of at least 500 [1]).

When the primary structure of the *S. albus* G β-lactamase and those of the four known β-lactamases of class A are compared pairwise, 80 – 103 identities are observed out of the 275 residues effectively aligned (Fig. 5). Yet, the *S. albus* G β-lactamase lacks 15 of the identities found in the four other β-lactamases of class A, and altogether, the five β-lactamases show only 37 identities. These enzymes, though probably homologous in an evolutionary sense, show considerable divergence. The *S. albus* G β-lactamase and the chromosomal, *ampC*, β-lactamase of *E. coli* [25] (which is currently regarded as the prototype of the β-lactamases of class C) are unrelated

in their primary structure (not shown). Yet, when the comparison is restricted to stretches of 10 amino acids around the active-site serine (Ser*), these two enzymes have six identities (Leu-Phe-Xaa$_3$-Ser*-Val-Xaa-Lys-Thr).

The β-lactamase excreted by the original strain, *S. albus* G, possesses one single histidine [12]. However, this histidine is catalytically dispensable since the host strain, *S. lividans* PD6, excreted several β-lactamase species that are histidine-free.

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