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Primary structure of the *Streptomyces* R61 extracellular DD-peptidase

1. Cloning into *Streptomyces lividans* and nucleotide sequence of the gene

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An 11450-base DNA fragment containing the gene for the extracellular active-site serine DD-peptidase of *Streptomyces* R61 was cloned in *Streptomyces lividans* using the high-copy-number plasmid pIJ702 as vector. Amplified expression of the excreted enzyme was observed. Producing clones were identified with the help of a specific antiserum directed against the pure DD-peptidase. The coding sequence of the gene was then located by hybridization with a specific nucleotide probe and sub-fragments were obtained from which the nucleotide sequence of the structural gene and the putative promoter and terminator regions were determined. The sequence suggests that the gene codes for a 406-amino-acid protein precursor. When compared with the excreted, mature DD-peptidase, this precursor possesses a cleavable 31-amino-acid N-terminal extension which has the characteristics of a signal peptide, and a cleavable 26-amino-acid C-terminal extension. On the basis of the data of Joris et al. (following paper in this journal), the open reading frame coding for the synthesis of the DD-peptidase was established. Comparison of the primary structure of the *Streptomyces* R61 DD-peptidase with those of several active-site serine β-lactamases and penicillin-binding proteins of *Escherichia coli* shows homology in those sequences that comprise the active-site serine residue. When the comparison is broadened to the complete amino acid sequences, significant homology is observed only for the pair *Streptomyces* R61 DD-peptidase/*Escherichia coli ampC* β-lactamase (class C). Since the *Streptomyces* R61 DD-peptidase and β-lactamases of class A have very similar three-dimensional structures [Kelly et al. (1986) *Science (Wash. DC) 231*, 1429–1431; Samraoui et al. (1986) *Nature (Lond.) 320*, 378–380], it is concluded that these tertiary features are probably also shared by the β-lactamases of class C, i.e. that the *Streptomyces* R61 DD-peptidase and the β-lactamases of classes A and C are related in an evolutionary sense.

For the purpose of defining the interaction between β-lactam antibiotics and their bacterial enzyme targets at the molecular level, the active-site serine DD-peptidase of *Streptomyces* R61 is being actively investigated [1, 2]. X-ray crystallography has led to the identification of the polypeptide chain folding at a resolution of 0.28 nm [3–5]. In parallel to this, the nucleotide sequence of the corresponding gene has been established. This paper describes experiments which led to (a) cloning of the gene for the *Streptomyces* R61 DD-peptidase; (b) determination of the nucleotide sequence; (c) identification of possible features related to the processing and excretion of the primary translation product; (d) comparison of the amino acid sequence of the protein with those of several active-site serine β-lactamases and penicillin-binding proteins.

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Abbreviations. Ac, acetyl; bp, base pair; kb, 1000 base pairs; PBP, penicillin-binding protein.

Enzymes. Bacterial alkaline phosphatase (EC 3.1.3.1); β-lactamases (EC 3.5.2.6); DD-peptidases (EC 3.4.16.–); lysozyme (EC 3.2.1.17); restriction endonucleases (EC 3.1.21.4); T_4 DNA ligase (EC 6.5.1.1).

MATERIALS AND METHODS

Bacterial strains and plasmids

Streptomyces R61 was from the Microbiology Department of the University of Liège. *S. lividans* TK24 (str-6; a strain cured of its natural plasmids) [6] and the non-conjugative high-copy-number plasmids pIJ702 [7] and pIJ385 [8] were from the John Innes Institute, Norwich, UK. *Escherichia coli* HB101 [9] and plasmid pBR322 [10] were also used.

Growth conditions and media

Growth of *Streptomyces* cultures was carried out at 28 °C with vigorous orbital shaking. The following media were used: YEME medium [11]; Merck peptone medium and E_9 broth [12]; Difco brain heart infusion; 2 xTY broth (Amersham handbook); and glycerol-casein medium [13]. R2YE agar [11] was also used. *E. coli* HB101 was grown at 37 °C in Luria-Bertani or M9CA medium [14] with vigorous shaking.

Enzymes, antiserum, proteins and antibiotics

The DD-peptidase of *Streptomyces* R61 was prepared as described in [15]. Rabbit anti-(R61 DD-peptidase) antiserum was prepared by Gamma S.A. (Tavier, Belgium). The enzymes used in the recombinant DNA techniques were from Bethesda

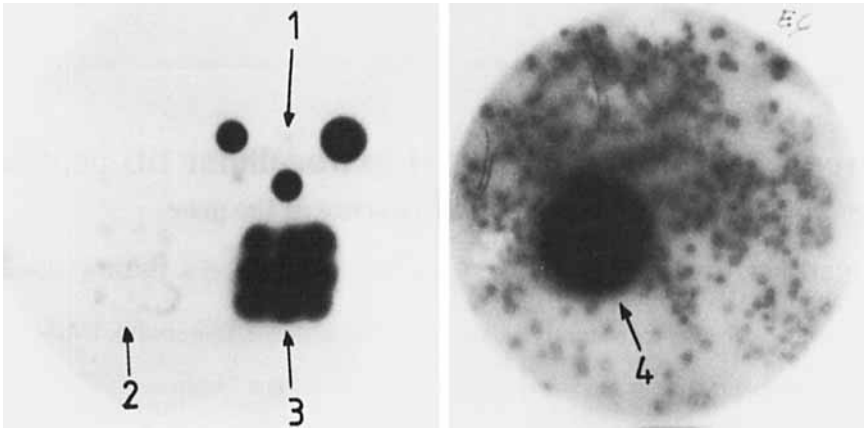

Fig. 1. Streptomyces *R61 DD-peptidase-producing colonies as detected with the immunological test.* (1) Three samples of the purified DD-peptidase; (2) nine colonies of the *Streptomyces lividans* TK24 recipient strain; (3) nine colonies of the *Streptomyces* R61 donor strain; (4) one colony of the *Streptomyces lividans* CC1 recombinant

Research Laboratory (Gaithersburg, MD, USA); New England Biolabs (Beverly, MA, USA); Sigma Chemical Co. (St Louis, MO, USA); Boehringer, Mannheim, FRG; Amersham International, Amersham, UK. Radioactive 3-(4-hydroxy-5-[^{125}]iodophenyl)propionamide-protein A (product IM144; specific radioactivity 35 mCi/mg) was from Amersham. Thiostrepton was a gift from Dr. R. B. Sykes (Squibb and Sons, New Brunswick, NJ, USA). The other antibiotics and bovine serum albumin were from Sigma.

Screening of DD-peptidase-excreting clones (immunological test)

Nitrocellulose filters (Millipore HATF) were (a) laid for 30 min on *Streptomyces* colonies grown on R2YE agar medium; (b) dried for 20 min at room temperature; (c) incubated for 1 h at room temperature and then for 2 h at 37 °C with a 2.5% bovine serum albumin solution made in NaCl/P_i buffer pH 7.4 [NaCl/P_i buffer was a mixture of A (8 vol.), B (1 vol.) and C (1 vol.) where A = 1% NaCl, 0.025% KCl, 0.143% Na_2HPO_4, 0.025% KH_2PO_4; B = 0.1% $CaCl_2 \cdot 2\,H_2O$; and C = 0.1% $MgCl_2 \cdot 6\,H_2O$]; (d) incubated at 37 °C with a $^1/_{1000}$ dilution of the anti-(R61 DD-peptidase) antiserum made in NaCl/P_i buffer; e) washed five times with NaCl/P_i buffer at 20 °C; (f) incubated with the ^{125}I-labelled protein A for 2 h at room temperature (10 μCi of the radioactive protein in 50 ml of NaCl/P_i buffer was sufficient to treat 10 filters at the same time); (g) incubated at room temperature, twice (for 30 min and 16 h, successively) with the NaCl/P_i buffer and then twice (for 30 min each time) with the NaCl/P_i buffer supplemented with 0.1% Triton X-100; (h) dried for 1 h at room temperature; and (i) exposed for 3–4 days at −70 °C to an X-ray film (Fuji Film Co. Ltd, Medical) in a Kodak X-omatic cassette with intensifying screen. Steps c–g were carried out with slow orbital shaking.

Estimation of DD-peptidase activity in culture fluids

The tripeptide Ac_2-L-Lys-D-Ala-D-Ala was used as substrate and the amount of released D-Ala was measured as described in [15].

Recombinant DNA techniques

Essentially, the procedures described in [11] were used for the preparation of *Streptomyces* R61 chromosomal DNA, the large-scale and mini (alkaline lysis) preparations of plasmid DNA, the preparation of protoplasts of *S. lividans* TK24 and the transformation experiments. However, lysis of *Streptomyces* R61 with lysozyme was performed at 0 °C for 150 min in the presence of 80 mM EDTA. Protoplasts (10^7 in 100-μl samples), DNA (100 ng in 10-μl samples) and poly(ethyleneglycol) (66 μl of a 28.5% solution) were mixed together. Before plating, the transformation mixtures were diluted with P buffer [11].

Essentially, the procedures described in [14] were used for digestion with the various endonucleases, treatment with bacterial alkaline phosphatase, ligation, agarose gel electrophoresis of digested and ligated DNAs and elution of separated DNA samples. However, restriction was carried out for 1 h at the optimal temperature of the enzyme using 1 μg DNA for two enzyme units. Digestion of restricted DNAs with the alkaline phosphatase was carried out in 50 mM Tris/HCl buffer pH 8 using 1 enzyme unit/pmol phosphate and the mixtures were incubated at 37 °C for 1 h (in the case of 5′ protruding ends) or at 56 °C for 2 h (in the case of 3′ protruding ends). Ligation mixtures contained 2 μg restricted genomic DNA, 1 μg restricted and alkaline-phosphatase-treated vector DNA and 1 unit of T_4 DNA ligase; they were incubated at 4 °C for 16 h and then at 16 °C for at least 3 h. All the digested DNAs were submitted to extraction with phenol/chloroform mixture and then with chloroform alone, precipitated with ethanol and stored at −28 °C in TE buffer [11].

DNA probe and hybridization experiments

Peptide T_2 isolated from a trypsin digest of carboxymethylated DD-peptidase [16] contained the sequence Asp-Asp-Asn-Gly-Thr-Ile, on the basis of which a 17-nucleotide-long probe 3′ CT^A_G-CT^A_G-TT^A_G-CCN-TGN-TA 5′ (N meaning A, G, C or T) was synthesized [17]. The polynucleotide was labelled with [γ-^{32}P]ATP as described in [18]. The hybridization experiments were carried out at 37 °C for 18 h using the Southern procedure [19, 20].

Nucleotide sequencing

The dideoxynucleotide chain-termination method [21] was used. Several lengths of sequence were proved to be difficult to read because of base compression, probably caused by the high G + C content of *Streptomyces* DNA, and were resolved

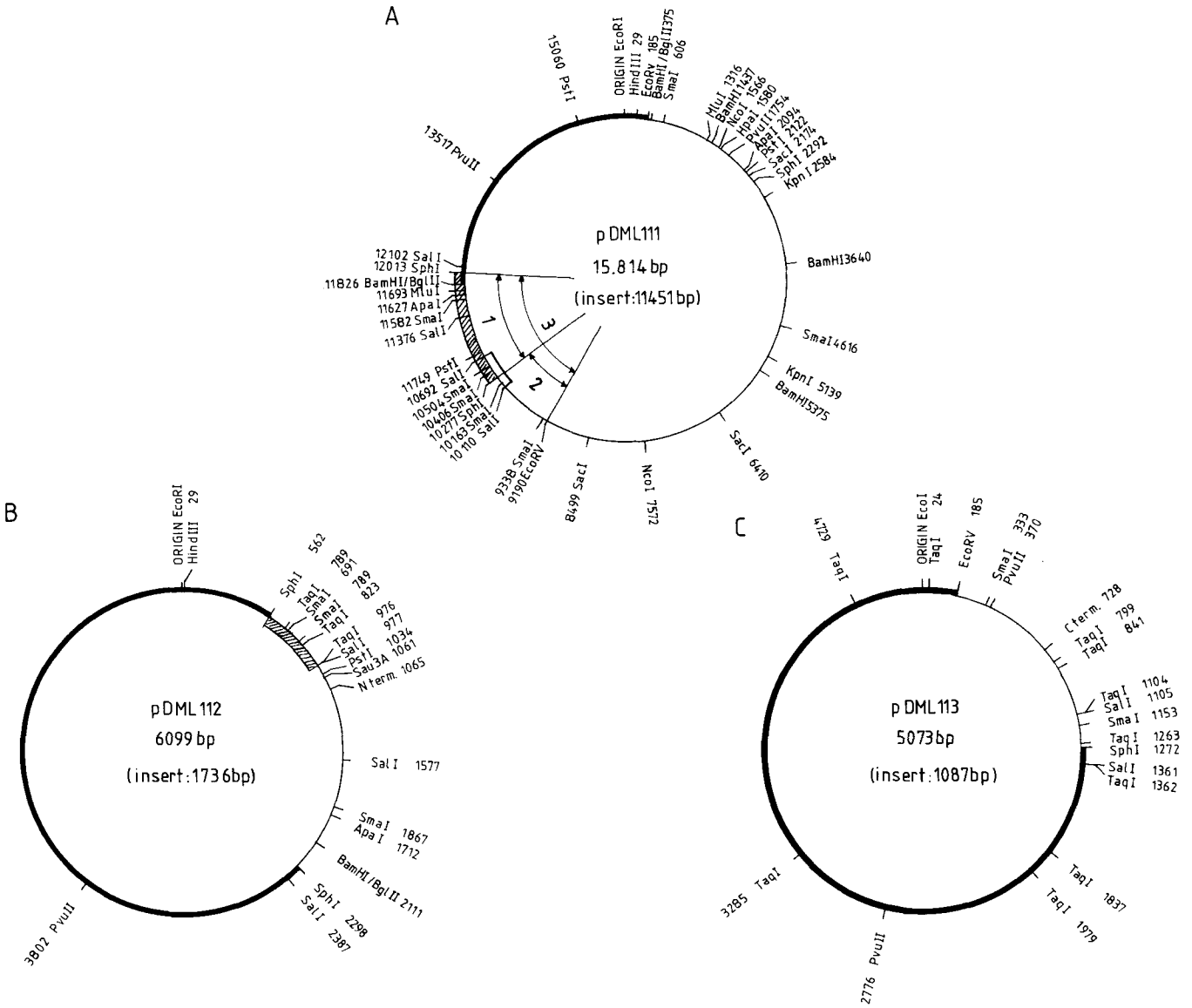

Fig. 2. *Restriction maps of pDML111 (A), pDML112 (B) and pDML113 (C).* (A) The 11.45-kb *Bgl*II insert (light line) in pBR322 (heavy line). The *Sph*I fragment (hatched box) and *Sal*I fragment (open box) hybridize with the nucleotide probe (Fig. 3). (1) Segment cloned in pDML112 (Fig. 2B); (2) segment cloned in pDML113 (Fig. 2C); (3) segment cloned in pDML114 (see text). (B) The 1.75-kb *Sph*I insert (light line) in pBR322 (heavy line). The *Sph*I-*Sal*I insert (hatched box) hybridizes with the nucleotide probe. (C) The 1.1-kb *Sph*I-*Eco*RV insert (light line) in pBR322 (heavy line)

using the formamide procedure described in [8]. For each of the three possible reading frames, the codon usage was analysed with Staden's program [22] using the neomycin phosphotransferase gene of *Streptomyces fradiae* as reference for codon usage [23]. Possible errors indicated by frame shifts were corrected by careful re-examination of the X-ray films (and using the aforementioned formamide procedure).

Comparison of the amino acid sequence of the DD-peptidase with those of β-lactamases and penicillin-binding proteins

Segments of varying span length (11, 15, 25 and 31 residues, respectively) of a pair of proteins were analysed with the McLachlan's procedure [24] which compares protein sequences on the basis of the relative amino acid substitution frequencies found among families of homologous proteins. Matrices expressing the probability (P) that similarity between pairs of segments occurred by chance were scored: blank ($1\times10^{-2} < P \ll 1$); 1 ($5\times10^{-3} \leqq P \leqq 1\times10^{-2}$); 2 ($1\times10^{-3} \leqq P \leqq 5\times10^{-3}$); 3 ($1\times10^{-4} \leqq P \leqq 1\times10^{-3}$) and 4 ($0 \leqq P \leqq 1\times10^{-4}$). The statistical significance of the global comparison between two proteins was estimated by calculating the χ^2_{app} value as described by Fitch [25]. McLachlan's test and Fitch's test were carried out with the help of programs developed by C. Wuilmart (Laboratoire de Physiologie animale, Université Libre de Bruxelles, Belgium).

RESULTS

Cloning of the DD-peptidase gene

When applied to colonies (grown for 3–5 days at 20°C on R2YE medium), the immunological test was positive in the case of *Streptomyces* R61 and negative in the case of *S. lividans* TK24 (Fig. 1).

pIJ702 carries thiostrepton resistance (*tsr*) and melanin production (*mel*) genes. pIJ385 carries thiostrepton resistance

Table 1. Streptomyces *R61 DNA (partial) libraries*

Library	Endonuclease used to cut *Streptomyces* R61 chromosomal DNA	Plasmid vector/endonuclease	Number of transformants/μg of vector DNA	Recombinants %	Number of recombinants screened
1	*Bam*HI	pIJ702/*Bgl*II	3000	95 (*mel*$^-$)	3200
2	*Bgl*II	pIJ702/*Bgl*II	15000	90 (*mel*$^-$)	14300
3	*Sac*I	pIJ702/*Sac*I	50000	2 (*mel*$^-$)	450
4	*Cla*I	pIJ385/*Cla*I	2500	75 (*tsr*$^-$)	3150
5	*Pst*I	pIJ385/*Pst*I	2500	50 (*aph*$^-$)	3400
					24500

Table 2. *Excretion of the* DD*-peptidase by* Streptomyces lividans *CC1,* Streptomyces lividans *TK24 and* Streptomyces *R61 in various liquid media after 72 h of growth at 28°C*
References describing the media are given in Materials and Methods

Streptomyces strains	Medium	DD-Peptidase excreted
		mg · l^{-1}
CC1	YEME	5.79
	Luria-Bertani	6.69
	Merck peptone	9.60
	2xTY	9.22
	glycerol-casein	16.19
	brain heart	0.11
	E9	8.57
TK24	Merck peptone	0
R61	glycerol-casein	0.5

and neomycin resistance (*aph*) genes. The *mel* segment contains restriction sites for *Bgl*II, *Sac*I and *Sph*I, the *aph* segment site for *Pst*I and the *tsr* segment sites for *Cla*I, *Eco*RV and *Pvu*II. Consequently, *Streptomyces* R61 gene libraries were prepared in pIJ702 and pIJ385 as indicated in Table 1.

Using *S. lividans* TK24 as recipient, the transformation mixtures were grown on R2YE agar medium for 15 h at 28 °C, then overlaid with 2.5 ml of soft agar containing thiostrepton (250 μg/ml; libraries 1, 2, 3 and 5) or neomycin (50 μg/ml; library 4). After a further 2.5–5 days, recombinants were identified on the basis of their white appearance (*mel*$^-$; libraries 1, 2 and 3), sensitivity to thiostrepton (library 4)

Table 4. *Amino acid composition of the* Streptomyces *R61* DD*-peptidase*
The number of residues per molecule as found by amino acid analysis [16], of the excreted protein and as deduced from the nucleotide sequence of the gene are given. The main discrepancies between the values are underlined

Amino acid	Residues in DD-peptidase molecule	
	from protein	from gene
Ala	34	34
Arg	13	14
Asx	38	39
Cys	3	2
Glx	28	27
Gly	32	30
His	8	9
Ile	9	9
Leu	33	33
Lys	8	7
Met	6	8
Phe	12	12
Pro	11	12
Ser	29	27
Thr	38	40
Trp	4	2
Tyr	13	14
Val	30	30

Table 3. *Codon usage for the synthesis of the* Streptomyces *R61* DD*-peptidase*

TTT/Phe	0	TCT/Ser	0	TAT/Tyr	2	TGT/Cys	0
TTC/Phe	12	TCC/Ser	11	TAC/Tyr	12	TGC/Cys	2
TTA/Leu	0	TCA/Ser	2	TAA/OC	0	TGA/OP	1
TTG/Leu	3	TCG/Ser	7	TAG/AM	0	TGG/Trp	2
CTT/Leu	1	CCT/Pro	0	CAT/His	0	CGT/Arg	4
CTC/Leu	13	CCC/Pro	6	CAC/His	10	CGC/Arg	9
CTA/Leu	0	CCA/Pro	1	CAA/Gln	2	CGA/Arg	0
CTG/Leu	23	CCG/Pro	8	CAG/Gln	19	CGG/Arg	4
ATT/Ile	0	ACT/Thr	0	AAT/Asn	0	AGT/Ser	1
ATC/Ile	11	ACC/Thr	23	AAC/Asn	16	AGC/Ser	7
ATA/Ile	0	ACA/Thr	0	AAA/Lys	0	AGA/Arg	1
ATG/Met	9	ACG/Thr	20	AAG/Lys	7	AGG/Arg	0
GTT/Val	0	GCT/Ala	2	GAT/Asp	1	GGT/Gly	6
GTC/Val	19	GCC/Ala	21	GAC/Asp	26	GGC/Gly	21
GTA/Val	0	GCA/Ala	4	GAA/Glu	2	GGA/Gly	4
GTG/Val	15	GCG/Ala	21	GAG/Glu	8	GGG/Gly	8

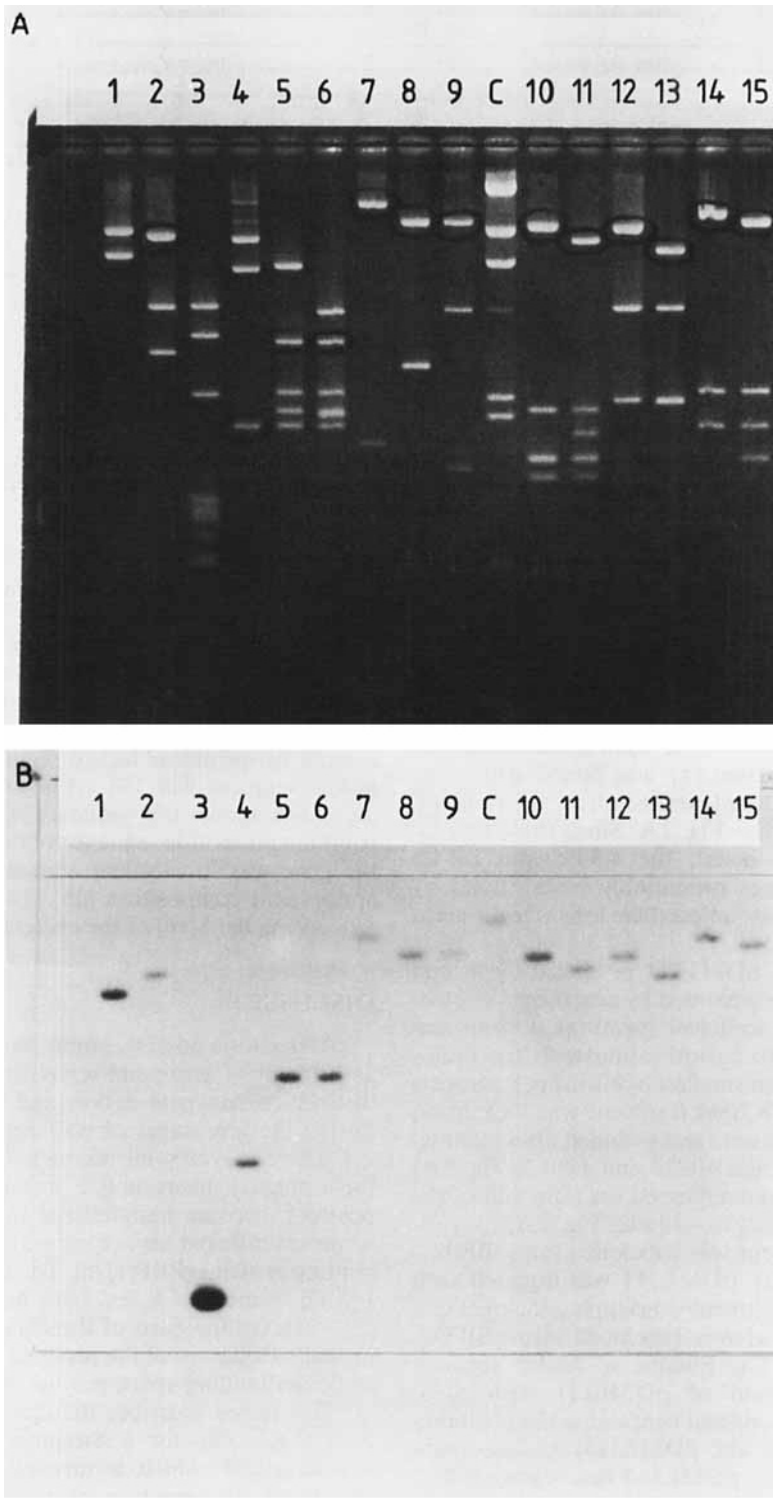

Fig. 3. *Agarose gel electrophoresis of pDML111 digests (A) and hybridization with the nucleotide probe (B).* (A) Ehtidium bromide stain of gel. (B) Autoradiography of the Southern blot. The restriction endonucleases were (1) *Eco*RV; (2) *Pst*I; (3) *Sal*I; (4) *Sph*I; (5) *Apa*I; (6) *Apa*I + *Hin*dIII; (7) *Hpa*I + *Hin*dIII; (8) *Kpn*I + *Hin*dIII; (9) *Mlu*I + *Hin*dIII; (10) *Nco*I; (11) *Nco*I + *Hin*dIII; (12) *Sst*I; (13) *Sst*I + *Hin*dIII; (14) *Bam*HI; (15) *Bam*HI + *Hin*dIII. C for control: *Hin*dIII-restricted λ DNA

or sensitivity to neomycin (library 5) (by replica plating to antibiotic-containing R2YE) (Table 1). Of the 24500 recombinants screened for DD-peptidase excretion, eight had a positive immunological reaction (Fig. 1). All of them originated from library 2. As judged from the size of the zones, they produced larger quantities of the DD-peptidase than the original *Streptomyces* R61. The best producer, *S. lividans* CC1, was grown in several liquid media. The levels of DD-peptidase activity in the culture fluids showed that gene cloning had indeed resulted in amplification of the expressed protein but variations occurred depending on the medium (Table 2).

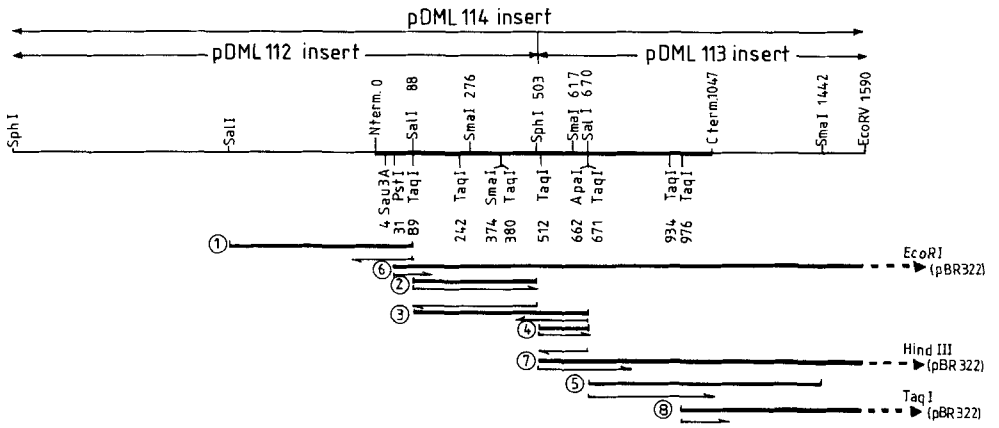

Fig. 4. *Strategy of nucleotide sequencing.* M13mp11 was used to clone subfragments 1 (*Sal*I, 602 bp) and 3 (*Sal*I, 582 bp). M13tg130 was used to clone subfragments 2 (*Sal*I-*Sph*I, 415 bp), 4 (*Sph*I-*Sal*I, 167 bp), 5 (*Sal*I-*Sma*I, 772 bp), 6 (*Pst*I-*Eco*RI, 1644 bp), 7 (*Sph*I-*Hin*dIII, 1243 bp) and 8 (*Taq*I, 614 bp). M13tg131 was also used to clone subfragments 2 and 4

Localization of the DD*-peptidase gene*

Of the eight DD-peptidase-producing clones isolated, five had acquired an 11.45-kb insert, two a 4.3-kb insert and one a 2.4-kb insert (as shown by agarose gel electrophoresis of mini-preparations). The 11.45-kb insert present in pDML110 of *S. lividans* CC1 was subcloned in the *Bam*HI site of pBR322 and the resulting plasmid pDML111 was prepared in large quantities from *E. coli* HB101 and purified on a CsCl gradient. Its restriction map is shown in Fig. 2A. Since there was no *Bgl*II site in the 11.45-kb insert, the 4.3-kb and 2.4-kb fragments in the other clones presumably were caused by post-cloning internal deletions, unless there are three different genes in R61.

Restriction fragments of pDML111 produced by several different endonucleases were separated by agarose gel electrophoresis, transferred to nitrocellulose membranes (Southern procedure) and submitted to hybridization with the radioactive DNA probe. Of the two smallest hybridizing fragments detected (Fig. 3), the 1.75-kb *Sph*I fragment was located on the left side of the original insert (and included about 200 bp of pBR322 between nucleotides 10277 and 12013; Fig. 2A) and the 0.6-kb *Sal*I fragment overlapped the right end of the *Sph*I fragment (nucleotides 10110–10692; Fig. 2A).

The 1.75-kb *Sph*I fragment was subcloned into pBR322, yielding pDML112 (Fig. 2B). pDML111 was digested with *Sph*I-*Eco*RV and the DNA segment overlapping the right end of the 1.75-kb *Sph*I fragment was subcloned into pBR322, yielding pDML113 (Fig. 2C). Finally, a 2.8-kb segment obtained by partial digestion of pDML111 with *Sph*I, followed by *Eco*RV restriction (and containing the combination of inserts of pDML112 and pDML113) was also subcloned into pBR322, yielding pDML114 (see segments 1, 2 and 3 in Fig. 2A). The stretch responsible for the hybridization of pDML112 with the radioactive DNA probe was between site *Sph*I 562 and site *Sal*I 977 (Fig. 2B).

Nucleotide sequencing of the DD*-peptidase-encoding gene*

Fig. 4 illustrates the strategy used for the sequencing, Table 3 shows the codon usage in the synthesis of the DD-peptidase and Fig. 5 gives the complete nucleotide sequence of the gene and its translation into amino acid sequence. This amino acid sequence was compared with the data presented in [16] and the zones of the gene coding for the N-terminal, active-site and C-terminal regions of the excreted DD-peptidase were localized in subfragments 1, 2 and 5, respectively.

The gene, from its translation startpoint (ATG for Met) to its stop codon (an opal TGA triplet), contained 1218 nucleotides and had the information for the synthesis of a 406-amino-acid polypeptide. In fact, the processed, extracellular DD-peptidase lacked segment Met-1–Ala-31. It also lacked segment Thr-380–Asp-406 from the C-terminal region. The amino acid sequence Ala-32–Thr-380 as deduced from the nucleotide sequence of the corresponding portion of the gene was in excellent agreement with both the overall amino acid composition [26] (Table 4) and the amino acid sequencing data [16] of the excreted DD-peptidase.

DISCUSSION

All bacteria possess, bound to their plasma membrane, an assortment of active-site serine DD-peptidases which catalyse distinct carboxypeptidation and transpeptidation reactions during the last stages of wall peptidoglycan synthesis [1, 2, 27]. These enzymes, mistaking a β-lactam antibiotic molecule for a normal substrate (i.e. a D-alanyl-D-alanine-terminated peptide), become immobilized in the form of a long-lived, serine-ester-linked acyl enzyme and thus behave as penicillin-binding proteins (PBPs) [28]. The genes that code for the PBPs 1A, 1B, 3 and 5 of *E. coli* have been analysed and sequenced [29–31]. On the basis of these data and others [32–34], the primary sequences of the proteins have been deduced and the penicillin-binding serine residue has been identified.

This paper describes the cloning and sequencing of the gene that codes for a *Streptomyces* active-site serine DD-peptidase/PBP which is unusual in being excreted during growth of the organism as a water-soluble enzyme [1]. In parallel with this work, the sequence of about 50% of the mature, excreted polypeptide chain has been determined after enzymatic and chemical cleavage [16].

In agreement with the current view on plasma membrane translocation of extracellular proteins [35], excretion of the *Streptomyces* R61 DD-peptidase/PBP depends on the presence of a cleavable 31-amino-acid signal peptide which is synthesized as an N-terminal segment present on a larger precursor polypeptide. This segment has one positively charged amino acid, Arg at position 8; it possesses a stretch of hydrophobic residues in the middle, and processing by a presumed leader peptidase takes place by cleavage of an Ala-Ala peptide bond.

Fig. 5. *Nucleotide sequence of the* Streptomyces *R61* DD*-peptidase precursor-encoding gene and amino acid sequence of the expressed and excreted enzyme.* The N-terminal alanine and C-terminal threonine of the mature, extracellular enzyme are in boxes. The processing sites are shown by vertical arrows. The putative ribosome binding site is underlined. The inverted repeats of the putative transcription termination signal are shown by horizontal arrows

At present, two other *Streptomyces* signal peptides have been described, namely those involved in membrane translocation of the *β-N*-glucosaminidase H in *Streptomyces* plicatus [36] and ORF 438 in *Streptomyces antibioticus* [37]. No similarity is seen between the three signal peptides. In the two latter cases, at least three arginine residues preceded the hydrophobic segment. The leader sequences of the agarase gene of *Streptomyces coelicolor* and the amylase of *Streptomyces limosus* are also known [38].

Excretion of the *Streptomyces* R61 DD-peptidase involves (or is accompanied by) alteration of the carboxy-terminal portion of the enzyme precursor. Modification is by elimination of a 26-amino-acid stretch through cleavage of a Thr-Gly peptide bond. This segment has a low hydrophobicity index and a high proportion of charged residues (4 Asp, 2 Glu, 3 Arg and 1 His). It thus differs from the membrane-anchoring peptide that occurs at the carboxy-terminal portion of the DD-peptidase/PBP5 of *Bacillus subtilis* and *Bacillus stearothermophilus* [39]. But, the *E. coli* PBP5 also possesses a C-terminal segment which plays a role in the anchoring of the protein to the plasma membrane, though it has no extended runs of hydrophobicity [40]. By analogy with this latter PBP, it may be hypothesized that, should it not be cleaved, the C-terminal segment of the *Streptomyces* DD-peptidase precursor would function as a stop-transfer sequence through which the enzyme would become membrane-bound. There is also no indication for the presence of a hydrophobic membrane-anchoring segment on the carboxy-terminal portion of the *E. coli* PBPs/DD-peptidases 1A, 1B and 3 [29–31]. PBPs 3, 5 and 6 have been shown to be synthesized as pre-proteins with amino-terminal signal peptides. The amino-terminal regions of PBPs 1A and 1B also have characteristics of a signal peptide but whether or not processing of the amino terminus occurs is not known [29].

Analysis of the gene that codes for the *Streptomyces* R61 DD-peptidase precursor reveals other interesting features. The 5′ GGAG-A-A 3′ segment, at position −13 to −6 upstream of the translation start codon (ATG for Met) complements

	Protein	−20 · −10 · · +10 · +20	Ref.
E. coli PBPs	(a)	G f d f n q s k f n r a T q a l r q V G S n i K p F l y t a a m d k g l t l a s m	(29)
	(b)	G s e p q f a G y n r a m q a R r s i G S l a K p a t y l t a L s q p k i y r L n	(29)
	(c)	s g t p k e a m R n r T i T D v F e p G S t v K p m v v m t a L Q r g v v r e n s	(30)
	(d)	d y n s g k v l a e q n a d v R r d p a S l T K m m t s y v i g Q a m k a g k f k	(31)
	(e)	d y A s g k v l a e g n a d e k l d p a S l T K i m t s y v v g	(31)
Bacillus PBPs	(f)	d a q t g k i l y e k n i d t v l g i a S m T K m	(44)
	(g)	e a s s g k i l y s k n a d k R l p i a S m T K m m t e y l l l e a i d q g k v k	(44)
S. R61 DD-peptidase	(h)	*G V A D R A T G R A I T T T D R F R V G S V T K S F S A V V L L Q L V D E G K L D*	
Class A β-lactamases	(i)	d t k s g k e - v k f n s d k R F a y a S t s K a i n s a i L L e q V p y n K L n	(45)
	(j)	d t g t n r T - v A y r p d e R F a f a S t i K a l t v g V L L Q q k s i e d L n	(45)
	(k)	d t g t n e T - i s y r p d q R F a f a S t y K a l a A g V L L Q q n s i d s L n	(45)
	(l)	d l n s g k i l e s f r p e e R F p m m S t f K v l l c g a v L s r V D a G q e q	(45)
	(m)	d t g s g r T - v A y r a d e l F p m c S V f K t l S s a a v L r d l D r n g e f	(46)
Class C β-lactamases	(n)	G y A D i A k k q p v T q q t l F e l G S V s K t F t g V l g g d a i a r G e i k	(47)
	(o)	G k A D i A n n h p v T q q t l F e l G S V s K t F n g V l g g d r i a r G e i k	(48)
	(p)	k k A D i A a n k p v T p q t l F e l G S i s K t F t g	(49)
	(q)	v T p e t l F e i G S V s K	(50)
Oxa-2 β-lactamase	(r)	a d r a m l v f d p v r s k k R y s p a S t f K i p h t l f a L d a g a v r d e f	(51)
Subtilisin	(s)	p g v s i q s t l p g g T y g a y n g t S m a t p h v A g a a a l i l s k h p t t	(52,53)

Fig. 6. *Amino acid sequences around the active-site serine residue of the extracellular* DD-*peptidase of* Streptomyces *R61 (h), various membrane-bound penicillin-binding proteins (PBPs) (a–g), β-lactamase (i–r) and subtilisin (s).* Capital letters show identities with the *Streptomyces* R61 DD-peptidase. (a, b, c, d and e) PBPs 1A, 1B, 3, 5, and 6 of *E. coli*; (f and g) PBPs (DD-carboxypeptidases) of *Bacillus stearothermophilus* and *Bacillus subtilis;* (i, j, k, l and m) class A β-lactamases of *Staphylococcus aureus, Bacillus licheniformis, Bacillus cereus, Escherichia coli* (RTEM plasmid) and *Streptomyces albus* G; (n, o, p and q) class C β-lactamases (chromosome *ampC* gene) of *E. coli, Citrobacter freundii, Enterobacter cloacae* and *Pseudomonas aeruginosa;* (r) oxa-2 β-lactamase (*E. coli*)

six bases out of eight of the 3′ end of the 16 S rRNA of *S. lividans* [41] and resembles a ribosome binding site [42]. In addition, the 31-nucleotide sequence that starts at position 9 downstream of the opal stop codon TGA, exhibits an inverted repeat of 12 bases with only one mismatch and thus may function as a terminator [42, 43]. Up to 263 nucleotides have been sequenced upstream of the translation start codon. *In vivo* promoter probing, using the promoter-probe plasmid pIJ424 [8], has shown that the promoter region is included in this sequence (data not shown). However, since the transcription start is not known, the consensus −10 and −35 sequences cannot be identified with certainty.

Comparison of the amino acid sequences of the *Streptomyces* R61 DD-peptidase with those of various active-site serine β-lactamases and DD-peptidase/PBPs shows that, remarkably, the sequence Ser*-Xaa_2-Lys, where Ser* is the active-site serine, is conserved in all these proteins. Alignment of the 20-amino-acid sequences that flank the amino and carboxyl sides of the active-site serine (Fig. 6) leads to the following observations. (The gaps introduced in the sequences of several β-lactamases of class A are those proposed by Ambler [45] for optimal matching.)

a) The sequence Phe-Xaa_3-Ser*-Xaa_2-Lys which is present in all the β-lactamases of class A and C (but not in the oxa-2 β-lactamase) occurs also in the *Streptomyces* R61 DD-peptidase. In addition to the triad Phe ... Ser ... Lys, the *Streptomyces* R61 DD-peptidase and the *E. coli* and *C. freundii* β-lactamases of class C have identities at positions −20, −18, −17, −15, −9, −1, +1, +5, +8 and +17. Similarily, the *Streptomyces* R61 DD-peptidase and the *B. licheniformis* β-lactamase of class A have identities at positions −14, −11, −5, +9, +10, +11, +12 and +19. Strikingly, the similarity between the *Streptomyces* R61 DD-peptidase and the β-lactamases of either class A or class C is greater than that between the β-lactamases of class A and C.

b) A phenylalanine at the fourth position on the amino side of the active-site serine (as it occurs in the β-lactamases of class A and C and in the *Streptomyces* R61 DD-peptidase) is found in the *E. coli* PBP3 and an arginine at the fifth position on the amino side of the active-site serine (as it occurs in four β-lactamases of class A and in the *Streptomyces* R61 DD-peptidase) is found in the *E. coli* PBPs 1B and 5 and in the *B. subtilis* DD-peptidase. In addition to the conserved Ser*-Xaa_2-Lys sequence, the pair *Streptomyces* R61 DD-peptidase/*E. coli* PBP1A have five identities at positions −20, −8, −2, −1 and +5 and the pair *Streptomyces* R61 DD-peptidase/*E. coli* PBP3 have eight identities at positions −12, −9, −7, −6, −4, −1, +11 and +12 (and an inverted diad Thr-Val or Val-Thr at positions +1 and +2).

When, using the method of McLachlan, the comparison is broadened to the whole amino acid sequences, homology vanishes except between the β-lactamases of a given class (A or C), the pair PBP1A−PBP1B of *E. coli* and the pair *Streptomyces* R61 DD-peptidase/*E. coli* β-lactamase of class C. The χ^2_{app} values are 5.8 for the pair *Streptomyces* R61 DD-peptidase/*E. coli* β-lactamase and 1.3 for the pair *Streptomyces* R61 DD-peptidase/*Bacillus licheniformis* β-lactamase, indicating probabilities for relatedness due to evolutionary relationship of 98.2% and 74%, respectively. A χ^2_{app} of 10, indicating a probability of 99.6%, is currently considered as a very strong index of such a relationship [25]. Restriction of the analysis to the N-terminal 150 residues, thus comprising the active-site serine area, does not increase the χ^2_{app} values.

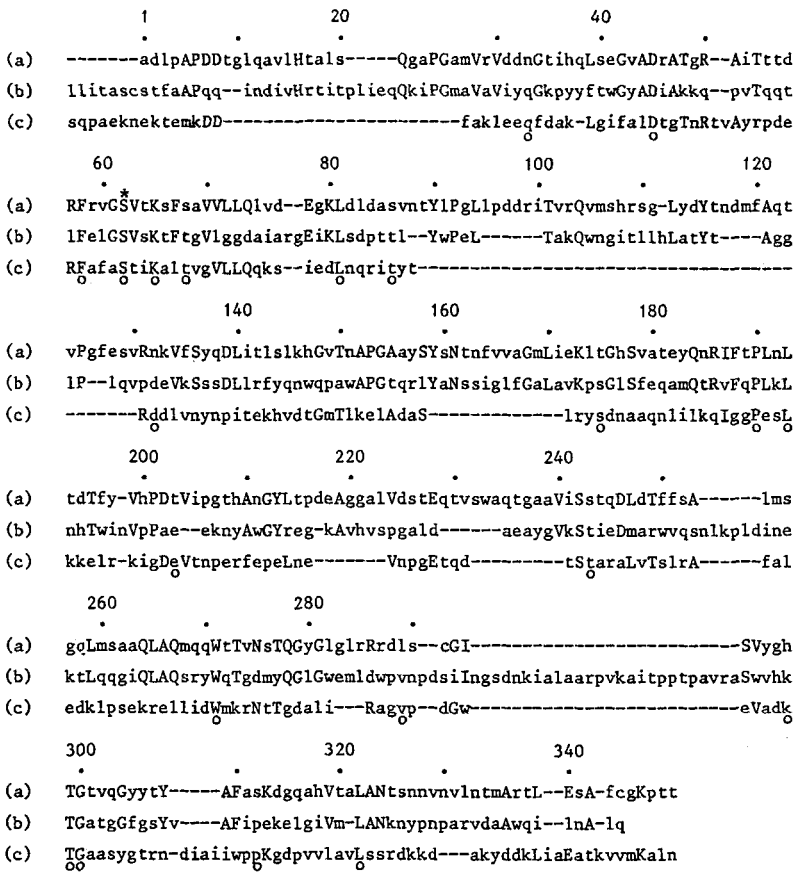

Fig. 7. *Possible amino acid sequence alignment of the active-site serine extracellular* DD*-peptidase of* Streptomyces *R61 (a), the class C β-lactamase of* Escherichia coli *(chromosome) (b) and the class A β-lactamase of* Bacillus licheniformis *(c).* Numbering starts from the N-terminus of the DD-peptidase and ignores the gaps postulated in the sequence to obtain optimal matching. Residues that are identical in the DD-peptidase and a given β-lactamase (or both β-lactamases) are in capital letters. Residues that are identical in the two β-lactamases are marked by an open circle (○). Closed circles (●) indicate every 10th residue

Segments having a matrix score of 3 or 4 served as a basis for the possible alignments and gaps shown in Fig. 7. These alignments maximize the similarities between the enzymes and, on that basis, the pair *Streptomyces* R61 DD-peptidase/*E. coli* β-lactamase have 89 identities out of 315 residues effectively aligned. The figure drops to 47 (for 252 aligned residues) for the pair *Streptomyces* R61 DD-peptidase/*B. licheniformis* β-lactamase and to 22 for the pair *E. coli* β-lactamase/*B. licheniformis* β-lactamase.

Finally, an interesting feature of the comparison is that the triad His-Thr-Gly in the R61 *Streptomyces* DD-peptidase or the triad Lys-Thr-Gly in the *B. licheniformis* and *E. coli* β-lactamases occurs at the same position 298–300, i.e. at position 136–139 on the amino side of the active-site serine. Moreover, the triad Lys-Thr-Gly is also conserved in the *E. coli* PBPs 1A, 1B, 3 and 5 away from the active-site serine (at positions 716–718, 698–670, 494–497 and 213–215, respectively; the active-site serine being at position 465, 510, 307 and 44, respectively).

In spite of a low level of relatedness in the primary structures, it has been shown [5, 54] that the spatial arrangement of the secondary structure elements (helices and strands of β-sheet) of the *Streptomyces* R61 DD-peptidase and the *B. licheniformis* (*Bacillus cereus*) β-lactamase of class A is so great that these two enzymes are obviously homologous in an evolutionary sense. Since as shown above, the *Streptomyces* R61 DD-peptidase and the β-lactamases of class C show higher similarities in their amino acid sequences, it may be concluded that the β-lactamases of class C probably share all the tertiary structural features that are common to the β-lactamases of class A and the *Streptomyces* R61 DD-peptidase.

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