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Transcriptional analysis of the DD-peptidase/penicillin-binding protein-encoding *dac* gene of *Streptomyces* R61: Use of the promoter and signal sequences in a secretion vector

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Summary. The promoter region of the gene encoding the extracellular DD-peptidase/penicillin-binding protein of *Streptomyces* R61 has been identified by in vivo promoter probing and S1 mapping. A secretion vector, pDML116, was constructed by inserting into the multicopy *Streptomyces* plasmid pIJ702, a 247 bp DNA sequence that contained the transcriptional, translational and secretory signals and the 12 amino acid N-terminal region-encoding sequence of the mature *Streptomyces* DD-peptidase/penicillin-binding protein. Insertion, downstream of this 247 bp segment, of the *Streptomyces* R61 DD-peptidase-encoding gene or the *Escherichia coli* R-TEM β-lactamase-encoding gene yielded plasmids pDML120 and pDML128, respectively, which allowed expression and secretion of the relevant enzymes by *Streptomyces lividans*. The maximal secretion levels obtained were 42 mg protein/ml for the autologous *Streptomyces* DD-peptidase and 0.9 mg protein/ml for the heterologous *E. coli* β-lactamase.

Key words: In vivo promoter probing – S1 mapping – Protein secretion – R-TEM β-lactamase – *Streptomyces* R61 DD-peptidase/PBP

Introduction

The use of gram-positive bacteria harbouring appropriate secretion vectors as a means to produce proteins of procaryotic and eucaryotic origin is well documented (Chang 1987). *Streptomyces lividans* is a very useful organism for this purpose (Gray et al. 1984; Munoz et al. 1985; Kieser et al. 1986; Pulido et al. 1986; Lichenstein et al. 1988; Noack et al. 1988; Chang and Chang 1988; Piron-Fraipont et al. 1989). Indeed, its RNA polymerase recognizes promoters of other bacterial species (Bibb and Cohen 1982), its DNA restriction capability is limited and its proteolytic activities are low. Moreover, *Streptomyces* are generally regarded as safe organisms which are able to grow to high densities and produce large amounts of extracellular proteins on an industrial scale. However, when genes encoding extracellular proteins of foreign origin are cloned into *Streptomyces* with their own signal sequences, complete secretion of synthesized proteins is rare (Dubus 1986; Ali and Dale 1986). In contrast, expression of genes encoding chimaeric proteins consisting of a *Streptomyces* signal peptide located upstream of the desired mature protein generally results in complete secretion of the gene product (Lichtenstein et al. 1988; Chang and Chang 1988; Nagashima et al. 1989).

Streptomyces and *Actinomadura* spp. produce extracellular β-lactamases and DD-peptidases/penicillin-binding proteins (PBPs) and several genes encoding these proteins, which all interact with penicillin, have been cloned (Dehottay et al. 1986; Duez et al. 1987; Lenzini et al. 1987; Piron-Fraipont et al. 1989) and sequenced (Duez et al. 1987; Dehottay et al. 1987; Lenzini et al. 1988; Houba et al. 1989). The nucleotide sequence containing the transcriptional, translational and secretory signals of the *dac* gene that encodes the DD-peptidase/PBP of *Streptomyces* R61 has been used to construct a *Streptomyces* expression-secretion vector. The efficacy of the vector has been tested using the autologous *Streptomyces* R61 enzyme itself and the heterologous R-TEM β-lactamase of *Escherichia coli* as models.

Materials and methods

Bacterial strains and growth conditions. Streptomyces R61 was from this laboratory. *S. lividans* TK24 (Hopwood et al. 1983) was from the John Innes Institute,

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Norwich, UK. Both were grown at 28° C with vigorous orbital shaking in YEME (Hopwood et al. 1985), mYEME (Erpicum et al. 1990) or glycerol-casein (Leyh-Bouille et al. 1971) liquid media. R2YE (Hopwood et al. 1985) and MMT (Katz et al. 1983) agar plates were also used. Promoter-probing experiments were carried out on kanamycin-gradient MMT agar plates (Ward et al. 1986).

Plasmids. The high copy-number plasmid pIJ702 (Katz et al. 1983) and the promoter probe pIJ424 (Ward et al. 1986) were from the John Innes Institute, Norwich, UK. pIJ424 contains the aminoglycoside phosphotransferase-encoding gene of Tn*5*, conferring kanamycin resistance.

pDML111 and pDML112, derivatives of pBR322, and pDML110, a derivative of pJI702, were from this laboratory (Duez et al. 1987). They all contained the *Streptomyces* R61 DD-peptidase/PBP-encoding *dac* gene. pJBS633, a derivative of pBR322, containing the signal peptide-free R-TEM β-lactamase Ap gene (Broome-Smith and Spratt 1986), was from the University of Sussex, Brighton, UK.

Estimation of the expressed DD*-peptidase and β-lactamase.* DD-carboxypeptidase activity in culture fluids was assayed by measuring the amount of D-Ala released from the diacetyl tripeptide (Ac_2)-L-Lys-D-Ala-D-Ala (Frère et al. 1976). DD-carboxypeptidase-producing *Streptomyces* colonies were detected using the immunological test described in Duez et al. (1987) except that the [^{125}I]-labelled protein A was replaced with alkaline phosphatase coupled to goat antibodies directed against rabbit IgG (Bio-Rad immunoblot assay kit). β-Lactamase activity in culture fluids was estimated with nitrocefin as substrate (O'Callaghan et al. 1972) and β-lactamase-producing *Streptomyces* colonies were detected as described in Dehottay et al. (1986).

Cell fractionation. Cells from 10 ml culture samples were washed twice with 10 ml of 100 mM potasmium phosphate, pH 7.0, suspended in 2.5 ml of the same buffer containing 1×10^{-4} M phenylmethylsulfonyl fluoride and 1×10^{-4} M EDTA and the cell suspension was sonicated for 150 s (MSE ultra-disintegrator MR2; power 12). Centrifugation for 30 min at 18000 rpm (SS34 Sorvall rotor) yielded a supernatant and a pellet, i.e. cytoplasmic and membrane fractions. The pellet was washed once and suspended in 0.2 ml phosphate buffer.

Enzymes and antibiotics. The enzymes used for recombinant DNA experiments were from Boehringer, Mannheim, FRG, Bethesda Research Laboratory (Gaithesburg, MD, USA), Amersham International (Amersham, UK), Sigma Chemical Co. (St Louis, MO, USA) and New England Biolabs (Beverly, MA, USA). Nitrocefin was purchased from BBL Microbiology Systems (Cockeysville, MD, USA), and kanamycin was from Sigma Chemcial Co. (St Louis, MO, USA). Thiostrepton was a gift from S.J. Lucania (Squibb and Sons, New Brunswick, NJ, USA).

DNA recombinant techniques. The procedures used were essentially those described in Maniatis et al. (1982) and Hopwood et al. (1985). S1 mapping was carried out according to Bibb et al. (1986) at a hybridization temperature of 45° C. RNA was isolated as described in Hopwood et al. (1985) from cultures of *S. lividans* TK24 harbouring pDML110, grown in YEME containing 34% sucrose, 5 mM $MgCl_2$ and 0.5% glycine, for 96 h, after which time the production of DD-peptidase was maximal. Nucleotide sequencing was carried out using the M13 dideoxynucleotide (Sanger et al. 1977, 1980) and the Maxam and Gilbert (1980) procedures.

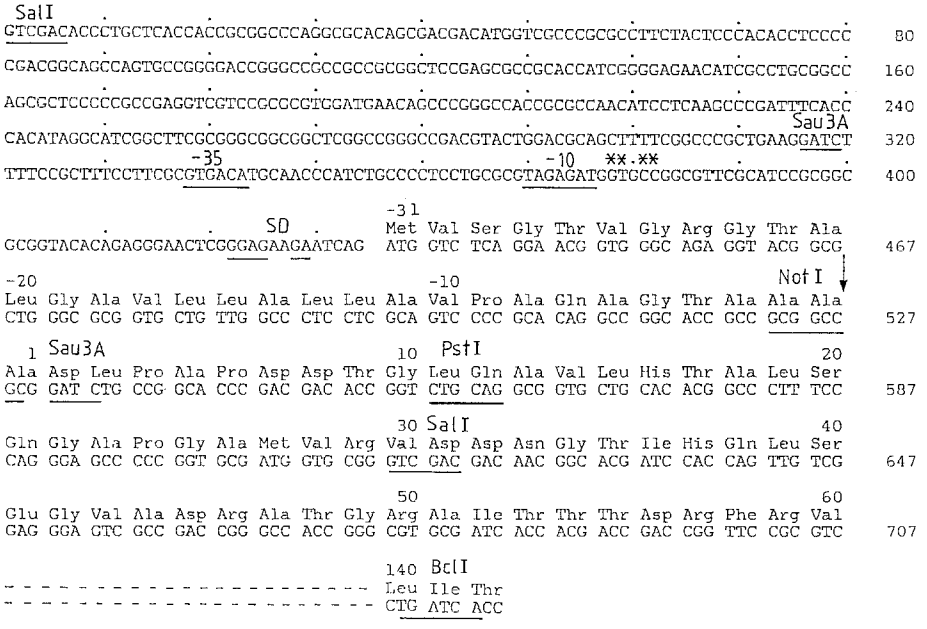

Fig. 1. Sequence of the 434 nucleotides located upstream of the *Streptomyces* R61 DD-peptidase/PBP-encoding *dac* gene and position of the main restriction sites. The −10, −35 and Shine-Dalgarno sequences (SD), the transcriptional starts (*asterisks*) and the site of cleavage of the signal peptide (*vertical arrow*) are shown

Results

The dac *gene nucleotide sequence*

Figure 1 shows the revised sequence of the 434 nucleotides located upstream of the ATG initiation codon of the *dac* gene. Compared with the sequence published previously (Duez et al. 1987), G338, G340 and the triad G396 C397 G398 have been added; a dyad GG (between T330 and C331) and a G (between C360 and T361) were eliminated; G391 and C392 were inverted.

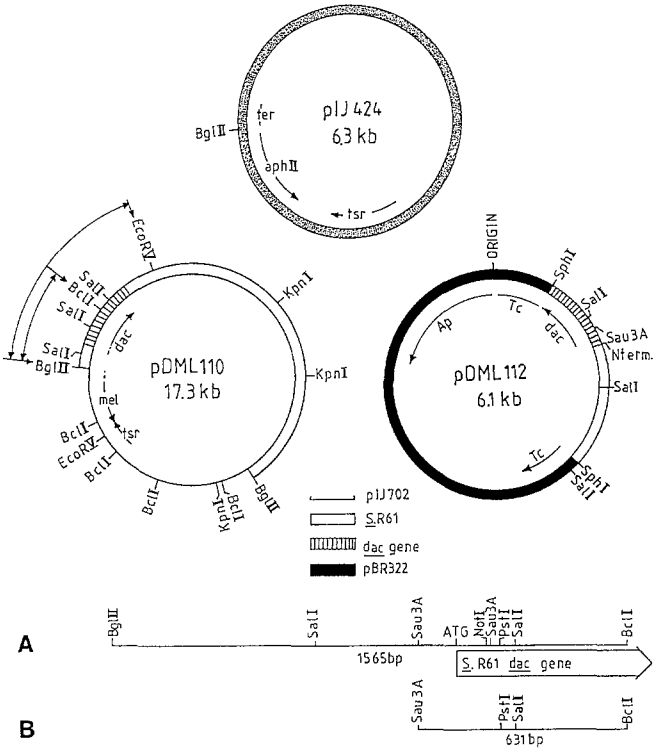

Fig. 2. Promoter-probing constructs. *tsr*, thiostrepton resistance gene; Tc, tetracycline resistance gene; *mel*, tyrosinase gene; Ap, ampicillin resistance gene. The *dac* gene in pDML112 lacks the carboxy terminal-encoding region. **A** The 1565 bp DNA segment. The 1565-bp *Bgl*II-*Bcl*I fragment was prepared from pDML110 and inserted into pIJ424, which had been cleaved with *Bgl*II and dephosphorylated. *Streptomyces lividans* TK24 cells transformed with the ligation mixture and selected on kanamycin (100 μg/ml)-MMT agar were found to contain a plasmid of the expected size (7865 bp). The orientation of the cloned fragment was deduced from the sizes of the fragments produced by *Pst*I. **B** The 631 bp DNA segment. The 631 bp *Sau*3A-*Bcl*I fragment was inserted in pIJ424 by means of a triple ligation procedure. The ligation mixture included: (i) The 300 bp *Sau*3A-*Sal*I fragment obtained from pDML112 by *Sal*I digestion, isolation and partial *Sau*3A digestion of the 615 bp *Sal*I fragment. (ii) The 331 bp *Sal*I-*Bcl*I fragment obtained by *Sal*I and *Bcl*I digestion of the 2630 bp *Bgl*II-*Eco*RV fragment of pDML110. (iii) pIJ424 cleaved with *Bgl*II and dephosphorylated. After transformation of *S. lividans* TK24, kanamycin-resistant (100 μg/ml) colonies were isolated. Given that the 331 bp stretch had no *Pst*I site while the 300 bp stretch and pIJ424 had one and three *Pst*I sites, respectively, one recombinant plasmid possessing the expected 6900 bp size and four *Pst*I sites was selected. The orientation of the 631 bp insert was deduced from the sizes of the fragments produced by *Pst*I

The transcriptional and translational signals of the Streptomyces dac *gene*

The transcriptional and translational signals of the *dac* gene were identified by in vivo promoter probing and S1 mapping experiments. As shown below, the transcriptional and translational signals were inclued in a 122 nucleotide stretch, from nucleotide 316 to nucleotide 437 (Fig. 1). These experiments made use of three plasmids: pDML110, pDML112 and the promoter-probe pIJ424 (Fig. 2).

Promoter probing. The recombinant plasmid obtained by inserting the 1565 bp *Bgl*II-*Bcl*I sequence of pDML110 into the promoter-probe pIJ424 (Fig. 2) allowed *S. lividans* TK24 to grow in the presence of kanamycin up to a concentration of 500 μg/ml (as observed by streaking spores on kanamycin-gradient agar plates). As also shown in Fig. 2, another plasmid was obtained by inserting into pIJ424 the 631 bp DNA fragment that contained the same amino terminal-encoding region of the *dac* gene as that present in the 1565 bp DNA fragment described above, but had a much shorter nucleotide sequence upstream of the ATG initiation codon (nucleotides 316–946 in Fig. 1). This plasmid allowed *S. lividans* to grow in the presence of kanamycin up to a concentration of 750 μg/ml.

S1 mapping. pDML112 containing the 631 bp DNA segment mentioned above was cut at the *Not*I site. After dephosphorylation, the linearized plasmid was ^{32}P-labelled at the 5′-OH end and digested with *Sal*I. Among the four DNA fragments thus produced, the 523 bp *Sal*I-*Not*I fragment (nucleotides 1–523 in Fig. 1) bearing the ^{32}P-label on the 5′-OH end of the *Not*I site, was hybridized with the total RNA fraction prepared from a culture of *S. lividans* harbouring pDML110 (and thus containing the complete *dac* gene). Non-hybridized strands were eliminated by treatment with S1 nuclease and the remaining double-stranded fragments were analysed by polyacrylamide gel electrophoresis and aligned with a Maxam-Gilbert sequence of the probe. Four major hybrids were localized (see Fig. 3), allowing identification of the transcription starts at nucleotides 378, 379, 381 and 382 (Fig. 1), i.e. at positions −53, −54, −56 and −57 upstream of the ATG initiation codon.

Construction of a Streptomyces *expression-secretion vector*

(i) *Plasmid pDML116.* The DNA stretch extending from nucleotide 316 to nucleotide 562 (Fig. 1) contained the transcriptional, translational and secretory signals of the *dac* gene and, in addition, that portion of the *dac* gene encoding the 12 amino acid N-terminal region of the mature protein. Hence, the site of cleavage by the leader peptidase is included. On this basis, insertion of the corresponding *Sau*3A-*Pst*I, 247 bp DNA fragment (nucleotides 316–562 in Fig. 1) into the high-copy number plasmid pIJ702 should give rise to a plasmid that can serve as an expression-secretion vector in *Streptomyces*.

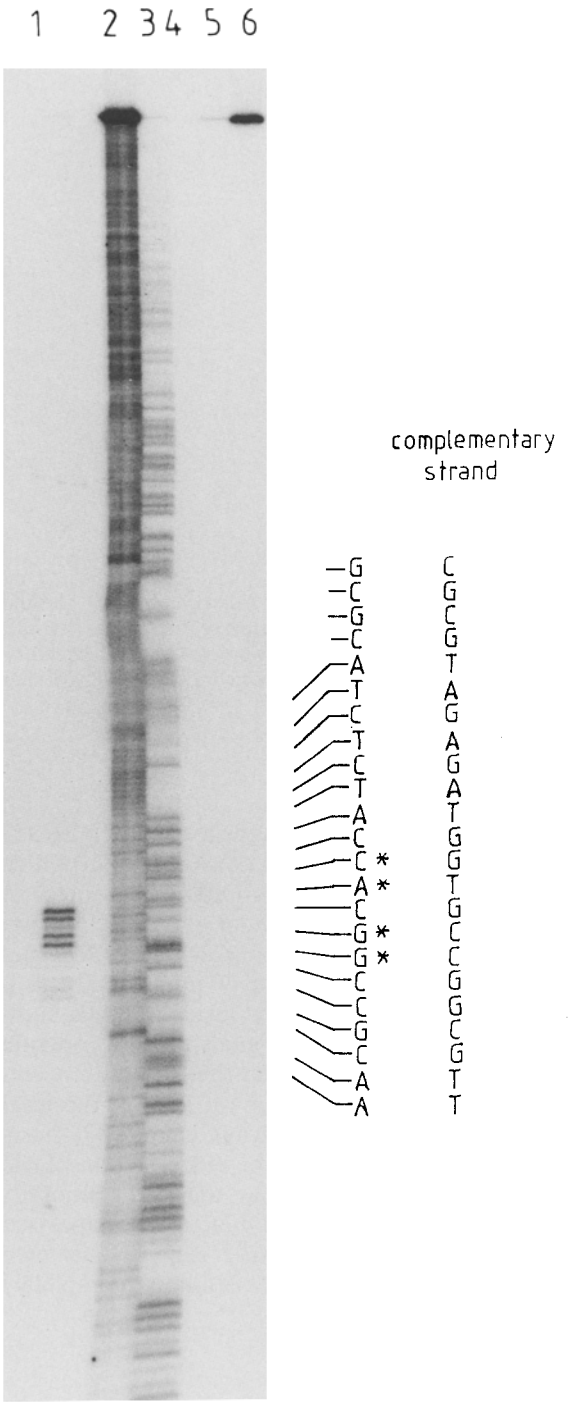

Fig. 3. S1 nuclease mapping of the promoter of the *dac* gene. *Asterisks* indicate the four major start-points. Hybridizations contained RNA from: lane 1, *S. lividans* TK24+pDML110 (bearing *dac* gene); lane 4, *S. lividans* TK24+pIJ702; lane 5, self-hybridized DNA probe; lane 6, untreated DNA probe. Maxam and Gilbert sequence ladders for DNA probe are in lanes 2 (G+A) and 3 (T+C)

pDML112 (Fig. 2) was doubly digested with *Sal*I and *Pst*I (nucleotides 2–562 in Fig. 1) and the isolated 562 bp DNA fragment was partially digested with *Sau*3A. The 247 bp DNA fragment was isolated by 5% acrylamide

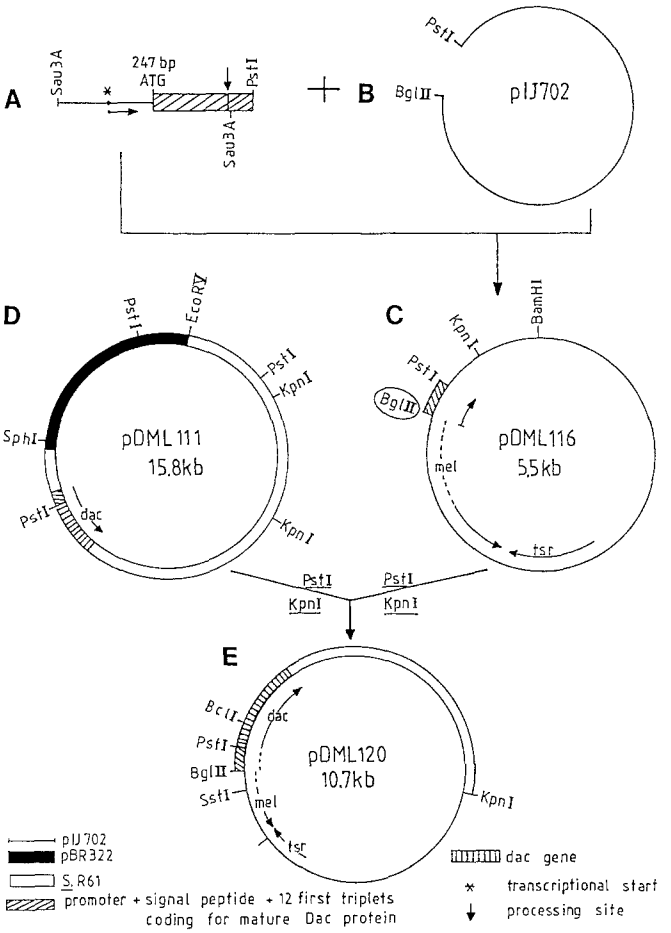

Fig. 4A–E. Construction of pDML116 (upper part) and pDML120 (lower part)

gel electrophoresis and ligated to pIJ702, from which the 510 bp *Bgl*II-*Pst*I, DNA fragment had previously been eliminated (Fig. 4, upper part). The resulting plasmid was designated pDML116 (Fig. 4, upper part). *S. lividans* TK24 transformants harbouring pDML116 were thiostrepton-resistant. Note that the *Bgl*II-*Sau*3A fusion reconstituted the *Bgl*II site. Upon digestion with *Bgl*II and *Pst*I, pDML116 regenerated the 247 bp DNA fragment.

(ii) *Plasmid pDML120.* In order to test the ability of pDML116 to encode secretory information, the entire *dac* gene was reconstituted in this plasmid, yielding pDML120 (Fig. 4, lower part). When grown in the glycerol-casein medium containing 25 µg thiostrepton per ml, *S. lividans* TK24/pDML120 produced twice as much extracellular DD-peptidase/PBP as *S. lividans* TK24/pDML110 (42 mg versus 20.6 mg/l), showing the increased efficiency of pDML120 compared to the original pDML110. Note that the growth conditions (120 h at 28° C) were optimal and identical for both strains and that *S. lividans* TK24/pIJ702 did not produce any detectable extracellular DD-peptidase or β-lactamase (Duez et al. 1987; Lenzini et al. 1987).

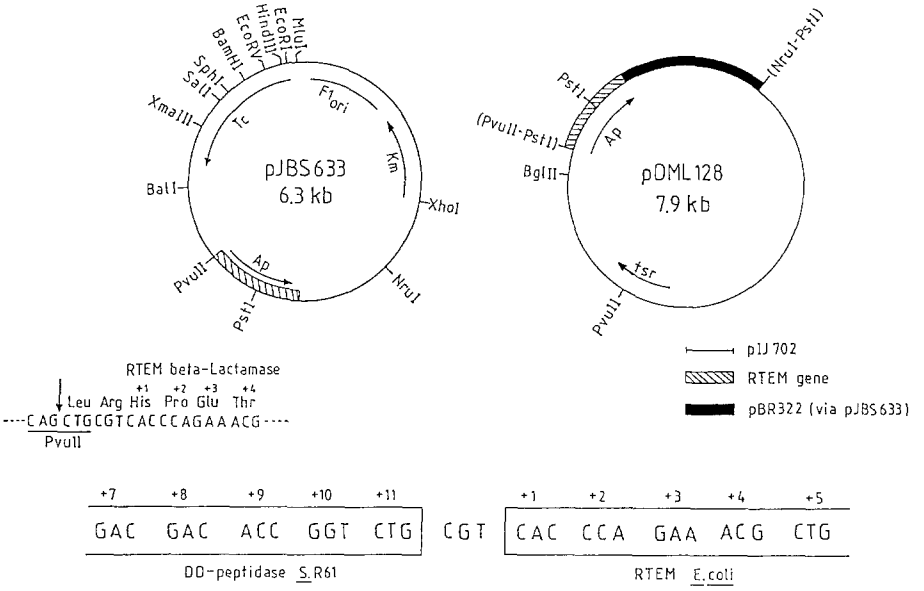

Fig. 5. Construction of pDML128. pDML116 was linearized with *Pst*I. *Pst*I ends were blunted by ligation with the synthetic oligonucleotide 5'-CTGCA followed by addition of dGTP with Klenow enzyme to add the missing base. Blunt-end ligation was carried out between this modified plasmid and the *bla*-containing 2430 bp *Pvu*II-*Nru*I fragment of pJBS633. One *S. lividans* TK24 β-lactamase-producing clone was found. The recombinant plasmid (pDML128) was isolated and the junction sequenced (lower part of figure), revealing that two codons were missing. The reading frame was thus preserved, but the expected *Pst*I and *Pvu*II sites were lacking

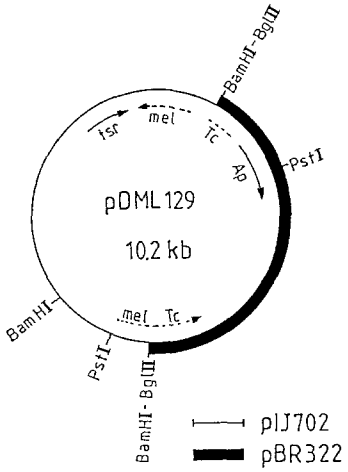

Fig. 6. Construction of pDML129

(iii) *Plasmid pDML128: a* Streptomyces *expression-secretion vector of the heterologous* E. coli *R-TEM β-lactamase.* The R-TEM β-lactamase gene was excised from pJBS633, where it occurs without its own signal peptide-encoding nucleotide sequence (Broome-Smith and Spratt 1986), and inserted into pDML116. The resulting plasmid pDML128 (Fig. 5) thus contained the region of the *dac* gene encoding the 11 N-terminal amino acids, fused to the mature R-TEM β-lactamase-encoding gene via the triplet CGT (Fig. 5). *S. lividans* TK24/pDML128 grown in mYEME medium for 96 h produced maximally 0.9 mg of extracellular β-lactamase/l. Fractionation of the cells showed that at least 96% of the expressed protein was secreted.

(iv) *Plasmid pDML129.* The hybrid pIJ702-pBR322 plasmid pDML129 (Fig. 6) was constructed in which the β-lactamase Ap gene was left under the control of its own transcriptional, translational and secretory signals. *S. lividans* TK24/pDML129, grown in YEME medium, produced maximally about 30 μg β-lactamase/l, about one-third of which remained inside the cells, indicating that the *E. coli* secretory signals failed to operate correctly in *Streptomyces* spp. That there are differences between *E. coli* and the *Streptomyces* spp. at the level of the secretory machinery is known (Chang 1987), however, no easy generalizations can be made. Ali and Dale (1986), for example, reported that the small amounts of *Salmonella typhimurium* Oxa-2 β-lactamase expressed by *S. lividans* harbouring the *oxa*-2 gene and its own signal peptide-encoding sequence, is almost completely exported in the culture medium.

Discussion

The transcriptional signals of the *Streptomyces* R61 DD-peptidase/PBP *dac* gene are located on a DNA stretch that extends 119 bp upstream of the ATG initiation codon. Extension of this segment up to 934 bp results in a lower promoter efficiency as shown by promoter probe experiments and by the fact that *S. lividans* TK24 harbouring pDML120 is a better producer of the DD-peptidase/PBP than *S. lividans* TK24 harbouring pDML110. The reason for this effect remains obscure. An upstream open reading frame might, somehow, interfere with the activity of the *dac* promoter.

Four nucleotides, G378, T379, G381 and C382, have been identified as major transcription start points, about 55 bp upstream of the ATG initiation codon. Multiple start points have been observed in other *Streptomyces* genes (Long et al. 1987; Eckhardt et al. 1987; Buttner et al. 1987; Vögtli and Hütter 1987). The −10 box (TAGAGAT at positions 370–376) and the −35 box (GTGACA at positions 338–343) have been tentatively identified as the promoter region. The −10 box is very similar to the consensus sequence tAGgaT proposed by Hopwood et al. (1986). The −35 box also resembles the consensus sequence TTGaca. Yet, the 27 bp spanning distance between the −10 and −35 boxes exceeds the maximum length reported by Hopwood et al. (1986). Another possible candidate for the −35 box may be the ATGCAA sequence, which is 22 bp away from −10 box. Different sequences for the −35 box have been reported in the *aph* and *tsr* *Streptomyces* genes (Janssen et al. 1985). Nothing in our sequence shows homology with these.

pDML120 and pDML128 are similar constructs in which the genes that encode the mature *Streptomyces* R61 DD-peptidase/PBP and the mature *E. coli* β-lactamase, respectively, are placed under the control of the transcriptional, translational and secretory signals of the *Streptomyces* R61 *dac* gene. In pDML128, however, the secretory signal is extended by a 33 bp stretch that codes for the 11 amino acid N-terminal region of the *Streptomyces* protein and this segment is itself linked to the β-lactamase gene by an additional (CGT) codon. The yield of the autologous DD-peptidase/PBP from *S. lividans* harbouring pDML120 (≈42 mg/l) is 40-fold higher than the yield of heterologous β-lactamase from the same strain harbouring pDML128 (0.9 mg/l). In both cases, however, the expressed proteins are very effectively exported in the culture medium, suggesting that the relatively low production of the β-lactamase is not attributable to a defect in the secretory mechanism but rather to a lower efficiency of the transcriptional and/or translational mechanisms. The stability of the secreted proteins has not been carefully investigated in the present study; but it has been shown elsewhere (Erpicum et al. 1990) that the extracellular proteases of *S. lividans* TK24 are poorly active toward the enzymes under consideration. It has been shown (Palva et al. 1982; Ohmura et al. 1984) that the production of the *E. coli* β-lactamase by *Bacillus subtilis* – using constructs that place β-lactamase secretion under the control of the α-amylase signal peptide – varies substantially depending on the origin of the signal peptide (from *B. subtilis* or *Bacillus amyloliquefaciens*) and the size of the linker that joins the signal sequence and the β-lactamase gene. Further study at this level may lead to an improved *Streptomyces* expression-secretion vector for the *E. coli* β-lactamase.

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