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β-Lactamase Expression in *Streptomyces cacaoi*

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Plasmids were prepared by inserting genomic DNA fragments from *Streptomyces cacaoi* within the *mel* gene of plasmid pIJ702. The inserted DNA fragments contain the β-lactamase-encoding *bla* gene and upstream nucleotide sequences of various lengths. The transcription start point of *bla* was identified by nuclease S1 mapping. Upstream nucleotide sequences of sufficient lengths had an enhancing effect on β-lactamase production by the *Streptomyces* host. The dot blot hybridization assay revealed that this effect was exerted at the transcriptional level. Experimental evidence strongly suggests that the underlying mechanism involves, at least in part, one or several *trans*-acting elements. In one of the constructs, in which the upstream nucleotide sequence was reduced to 0.3 kb, the *bla* promoter was present but the *bla* gene was expressed by readthrough from a promoter, possibly the *mel* promoter, of the pIJ702 vector.

β-Lactamases are enzymes that effectively hydrolyze the β-lactam antibiotics into biologically inactive metabolites. Depending on the bacterial species, different regulatory mechanisms are involved in the expression of the β-lactamase structural gene (*bla*). Thus, for example, in *Bacillus licheniformis*, inducible β-lactamase synthesis is controlled by at least two genes, *blaI* and *blaR*. *blaI* encodes a repressor (9) whose expression is autoregulated. *blaR* is transcribed together with *blaI* as a polycistronic mRNA from the *blaI* promoter (18), and the product of *blaR*, a membrane-bound penicillin-binding protein, functions as a penicillin sensory transducer (15a, 42). A repressor also seems to be involved in β-lactamase expression in *Enterobacter cloacae* (11) and *Citrobacter freundii* (21), while in *Escherichia coli*, *bla* appears to be regulated by a growth rate-dependent attenuator (15).

Streptomyces spp. produce a wide range of antibiotics. In order to protect themselves, they also provide a wide range of enzymes that modify the antibiotics and/or their targets (24, 26, 35). Often, but not always, the genes that code for these defensive enzymes and those that code for antibiotic synthesis are grouped into clusters, providing the possibility of coordinated regulation of the antibiotic-synthesizing and -modifying machineries (4). This picture, however, does not apply to β-lactamase production in *Streptomyces* spp. *bla* is not connected to the genes involved in β-lactam synthesis. Moreover, β-lactamase production appears not to be a major defense mechanism, and penicillin resistance might be due to the presence of targets of relatively low affinity for the drug (29).

In previous studies, the gene encoding the β-lactamase of *Streptomyces cacaoi* (30) was cloned and sequenced (19, 20). Experiments were then undertaken to identify the transcriptional start point and to explore the regulatory effects that upstream DNA sequences may exert on gene expression.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *Streptomyces lividans* 1326 (22) and *Streptomyces cacaoi* KCC-S0352 were from D. A. Hopwood (John Innes Institute, Norwich, U.K.) and A. Seino (Kaken Chemical Co., Tokyo, Japan), respectively. pIJ385 (13) and pIJ486 (40) were from T. Kieser and M. J. Bibb (John Innes Institute). pSL1 (27, 39), pDML51, pDML52, pMCP38, and pMCP39 (20), and pMCP28 (25) were described previously. *Bam*HI linker, *Escherichia coli* HB101 (3), and pUC18 (41) were from Takara Shuzo Co. (Tokyo, Japan).

Recombinant DNA techniques. *Streptomyces* plasmid DNA was prepared by alkaline lysis (31) followed by spermine treatment (16). *E. coli* DNA was isolated by the method of Holmes and Quigley (10). Treatment with restriction endonucleases and other enzymes was carried out as recommended by the commercial suppliers. DNA fragments were purified by electroelution (36). Transformation was performed by the method of Thompson et al. (38) for *Streptomyces* spp. and by that of Hanahan (7) for *E. coli*. Nucleotide sequencing was performed with the Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio).

Enzymes and antibiotics. Exonuclease III, mung bean nuclease, restriction endonucleases, T4 polynucleotide kinase, and 8-bp *Bam*HI linker (5′-CGGATCCG-3′) were from Takara Shuzo Co. (Tokyo, Japan). The Klenow fragment of DNA polymerase I, T4 DNA ligase, and calf intestine alkaline phosphatase were from Boehringer (Mannheim, Federal Republic of Germany). S1 nuclease was from Sigma Chemical Co. (St. Louis, Mo.). Thiostrepton was a gift from S. J. Lucania, Squibb and Sons Inc. (New Brunswick, N.J.). Other antibiotics were from Sigma.

Nuclease S1 mapping. *Streptomyces* strains were grown in E medium (28) with or without 50 μg of thiostrepton per ml. The RNAs were isolated as described before (31) by a modification of the method of Penn et al. (32). The probe (see Results), ^{32}P labeled at the 5′ end by T4 polynucleotide kinase (23), was separated into single-stranded fragments by electrophoresis, and one of the fragments was hybridized with 50 μg of total RNA (or 50 μg of carrier tRNA as a control) in 20 μl of hybridization buffer (1). After 3 h at 61°C,

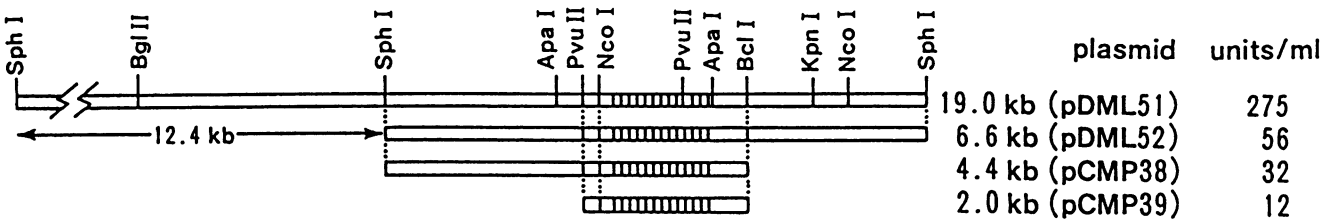

FIG. 1. Restriction maps of *S. cacaoi* chromosomal DNA fragments carrying *bla* inserted into pIJ702 and β-lactamase production (units per milliliter of culture) by *S. lividans* 1326 harboring the indicated plasmids. The *bla* gene is indicated by the hatched bars between the dotted lines.

hybridization was stopped by addition of 300 μl of ice-cold S1 digestion buffer (280 mM NaCl, 30 mM sodium acetate, 4.5 mM zinc acetate) containing 200 U of S1 nuclease and 20 μg of single-stranded carrier DNA from salmon sperm. After a 45-min incubation at 37°C, the protected hybrids were extracted with phenol, precipitated with 2-propanol, and redissolved in formamide-containing loading buffer (90% formamide, 0.1% xylenecyanol, 0.1% bromophenol blue). Samples were denatured for 2 min at 90°C, quickly chilled and analyzed by electrophoresis in the presence of 7 M urea.

Dot blot and Southern hybridization. Dot blot experiments were carried out by the method of Ward et al. (40). The probe used (see Results) was labeled by the nick translation method (34). The method of Southern (37) was used as modified by Ishihara et al. (14).

β-Lactamase assay. β-Lactamase activity was measured (28) by using benzylpenicillin as a substrate. One unit of activity was defined as the amount of enzyme that catalyzed hydrolysis of 1 μmol of benzylpenicillin per min at 30°C.

Nucleotide sequence accession number. The nucleotide sequence data have been deposited in the DDBJ, EMBL, and GenBank data bases under accession number D90201.

RESULTS

In previous studies, DNA fragments carrying the *S. cacaoi bla* gene were inserted into the *mel* gene *Bgl*II site of pIJ702, giving rise to pDML51, pDML52, pMCP38, and pMCP39 (Fig. 1). *S. lividans* harboring these recombinant plasmids produced the β-lactamase in various amounts, depending on the size of the DNA insert (Fig. 1). The nucleotide sequence of *bla*, as determined by the procedure of Sanger et al. (34a), was unsatisfactory in several respects. The proposed initiator GTG codon was not preceded by a likely Shine-Dalgarno sequence or followed by a typical signal peptide-encoding sequence. The use of the Sequenase kit yielded the revised version shown in Fig. 2. The open reading frame that encodes the 325-amino-acid precursor of the β-lactamase began 200 bp downstream of an *Nco*I site and 4 bp downstream of an *Ava*II site. The putative ribosome-binding site is also indicated in Fig. 2.

Promoter mapping: plasmids pMCP67 and pMCP122. Promoter mapping was carried out on the four aforementioned plasmids. Note that the length of *S. cacaoi* DNA which is present upstream of *bla* is 15.1 kb long in pDML51, 2.7 kb long in pDML52 and pMCP38, and 0.3 kb long in pMCP39. For that purpose, pMCP67 and pMCP122 were constructed as follows. pMCP67 was constructed by inserting the 2.2-kb *Bgl*II-*Sac*I DNA segment of pMCP39 into pUC18 previously digested with *Sac*I and *Bam*HI (Fig. 3A). pMCP122 was constructed by inserting the 1.1-kb *fd* terminator-containing *Kpn*I-*Bgl*II DNA segment of pIJ486 into the *Kpn*I and *Bgl*II sites of pMCP39 (Fig. 3B). The restriction maps of pMCP67 and pMCP122 are shown in Fig. 3C. pMCP67 was used to prepare the four nucleotide probes (Pr.) 1, 2, 3, and 4 (Fig. 3D).

Hybridization of the 192-bp *Nco*I-*Ava*II probe (Pr.2 in Fig. 3D) with the RNAs prepared from *S. cacaoi*, *S. lividans* 1326 (pDML51), *S. lividans* (pDML52) and *S. lividans* (pMCP38) resulted in partial protection of the probe, leading to the conclusion that transcription of the gene starts at position 112(T), 113(C), or 114(G) (Fig. 2), 113(C) behaving as a major transcriptional start point (Fig. 4). Hence the −35 and −10 boxes were located as shown in Fig. 2.

At variance with the above results, hybridization assays with the same *Nco*I-*Ava*II probe and the RNAs prepared from *S. lividans* 1326(pMCP39) resulted in full protection of the probe, leading to the conclusion that the promoter was located upstream of the *Nco*I site. In turn, hybridization

```
1                                                                                                    100
CATGGCCCCGACTGTAAGCGGAGCCGCTCCCAGCAGGCATGTATTCCGTTTCCGCATGAACCGGCGCACTCGGGTCTTCGACCGTATGTCCGCACCGGGA
NcoI                                                                                    -35
                ***                                                                                  200
CGAGAGTGTTCTCGCAGCTCACGGCCGGGCCGCCCGCGGTGCCCGGCCGTTCCGTCTTCCCGTGCGCCCCGGCGTACGGCACGGCACAGGAGGTCCGGAC
  -10                                                                                      RBS
                                                                                             AvaII
  1                                                                                  30
ATGCGTATCCGTCCCACCCGTCGTCTTCTCCTCGGCGCGGTCGCGCCGCTCGCCCTCGTTCCGCTGGTGGCCTGCGGTCAGGCGTCGGGC
MetArgIleArgProThrArgArgLeuLeuLeuGlyAlaValAlaProLeuAlaLeuValProLeuValAlaCysGlyGlnAlaSerGly

 31                                                                                  60
TCCGAGAGCGGCCAGCAGCCCGGCCTCGGCGGTTGCGGGACGAGCGCACACGGCTCGGCGGACGCCCACGAGAAGGAGTTCCGGGCGCTG
SerGluSerGlyGlnGlnProGlyLeuGlyGlyCysGlyThrSerAlaHisGlySerAlaAspAlaHisGluLysGluPheArgAlaLeu
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FIG. 2. Partial nucleotide sequence of *S. cacaoi bla* gene (revised from reference 19). The asterisks indicate the transcription start site. *Nco*I and *Ava*II sites, the Shine-Dalgarno sequence (RBS), and the −10 and −35 promoter hexamers are underlined. Nucleotides are numbered in the two upper lines. The open reading frame starts with the third line, at which point the numbering refers to the deduced amino acid sequence. An inverted repeat is shown by horizontal arrows.

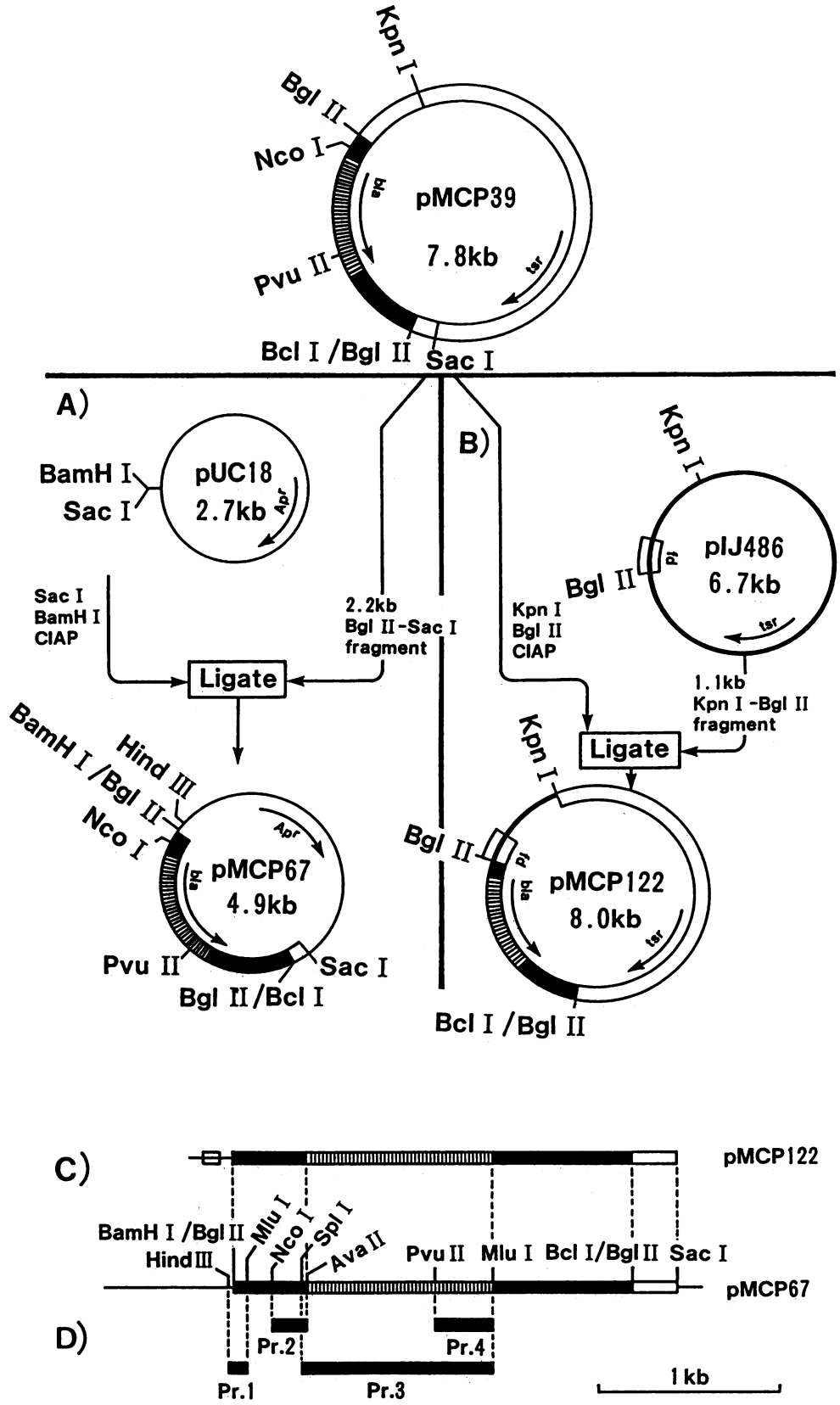

FIG. 3. (A and B) Construction of pMCP67 (A) and pMCP122 (B). Ap^r, *E. coli* ampicillin resistance gene; *bla*, *S. cacaoi* β-lactamase gene; *tsr*, *Streptomyces* thiostrepton resistance gene. Symbols: open bars, pIJ702; solid bars, *S. cacaoi*; hatched bars, *bla* gene; thin solid line, pUC18; thick solid line, pIJ486; open box, *fd* terminator. (C and D) Partial restriction maps of pMCP122 and pMCP67 (C) and locations of the four probes (Pr.1, Pr.2, Pr.3, and Pr.4) used for hybridization assays (D).

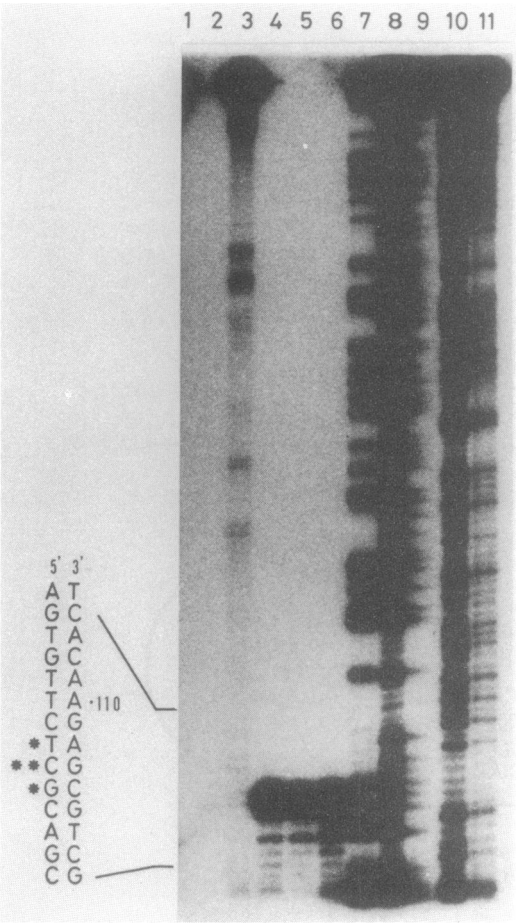

FIG. 4. High-resolution S1 mapping of various clones. The 192-bp *Nco*I-*Ava*II probe (Pr.2, Fig. 3D) was hybridized with tRNA (lane 1); RNA from *S. cacaoi* (lane 2); and RNAs from *S. lividans* 1326 carrying pMCP39 (lane 3), pMCP38 (lane 4), pDML52 (lane 5), and pDML51 (lane 6). Sequence ladders are G (lane 7), G plus A (lane 8), A plus C (lane 9), T plus C (lane 10), and C (lane 11). Asterisks indicate a major and two minor transcription start sites. Nucleotide −110 refers to the numbering in Fig. 2.

with the 110-bp *Hin*dIII-*Mlu*I probe (Pr.1 in Fig. 3D) resulted in the protection of the *Bam*HI/*Bgl*II-*Mlu*I portion of the probe, indicating that in *S. lividans* 1326(pMCP39), *bla* was expressed by readthrough from a promoter of the pIJ702 vector (13) used to construct pMCP39 (20). *S. lividans* 1326 (pMCP122) (bearing the *fd* terminator; see above) produced much less β-lactamase than *S. lividans* 1326(pMCP39) (1 versus 12 U of enzyme per ml of culture filtrate).

Effect of nucleotide sequence upstream of *bla* transcriptional start point. pMCP141 through pMCP146, used in the experiments described below, are deletion plasmids in which nucleotide sequences of decreasing lengths were made upstream of the *bla* transcriptional start point (Fig. 5A). These plasmids were constructed as follows. (i) The 5.8-kb *Bcl*I *S. cacaoi* DNA segment was excised from pMCP38 and transferred into pUC18, and the resulting plasmid, pMCP126, was digested with *Xba*I and *Pst*I. (ii) The linearized plasmid was treated with exonuclease III (which nibbles exclusively the *Xba*I ends), and after increasing times of incubation, the ends were blunted with mung bean nuclease and Klenow treatments and religated with T4 DNA ligase. (iii) The ligation mixtures thus obtained were used to transform *E. coli* HB101, and ampicillin-resistant transformants were identified by DNA minipreparation analyses. (iv) Recombinant plasmids containing inserts of decreasing sizes were cut with *Sac*I and *Hin*dIII, and the fragments produced were then inserted into pIJ486 downstream of the *fd* terminator, giving rise to pMCP141 through pMCP146.

pMCP172 through pMCP174, also used in the experiments described below, are pMCP38 derivatives in which a *Bam*HI linker is inserted into *Pvu*II-1, *Pvu*II-2, and *Pvu*II-3, respectively (Fig. 5B). They were constructed as shown in Fig. 6A.

The β-lactamase productions of *S. lividans* 1326 carrying each of these plasmids were estimated (Fig. 5A and B). A sharp decrease in the level of enzyme produced was observed when the size of the original DNA insert was reduced from 2.5 to 2 kb (pMCP142 to pMCP143), and another but smaller decrease was observed when the size of the DNA insert was further reduced from 1.4 to 0.4 kb (pMCP145 to pMCP146). pMCP172 and pMCP173 induced the same low level of enzyme production as pMCP146. As expected, pMCP174, in which *bla* is interrupted by the *Bam*HI linker, was silent.

Dot blot hybridization assays were carried out on clones of *S. lividans* 1326 carrying these plasmids with the *Spl*I-*Mlu*I DNA probe (Pr.3 in Fig. 3D). The amounts of mRNA hybridized were grossly proportional to the level of β-lactamase produced [except with *S. lividans* 1326 (pMCP144), in which the amounts of RNA appeared to be abnormally high], suggesting that β-lactamase production was regulated at the transcriptional level (Fig. 7).

Evidence for *trans* regulation of gene expression. The plasmids used in the experiments described below lack *bla* totally or partially but possess various portions of the nucleotide sequence that in pMCP38 occurs upstream of *bla* (Fig. 5C). These plasmids were constructed as follows (Fig. 6B). (i) The 950-bp *Sac*II DNA segment containing the neomycin resistance marker Neo^r of pIJ385 was inserted into one of the *Sac*II sites of pSL1, given rise to pMCP50. Note that pSL1 is a replicon that is compatible with the pIJ101 replicon (17) present in the constructs described above. (ii) The 1,550-bp *Mlu*I DNA segment of pMCP50 was transferred into pMCP28, giving rise to pMCP170. (iii) The *bla*-containing *Bgl*II-*Bam*HI DNA fragments of pMCP172, pMCP173, and pMCP174 were inserted into pMCP170 partially digested with *Bam*HI, giving rise to pMCP175, pMCP 176, and pMCP177, respectively.

S. lividans 1326 was cotransformed with the compatible pairs pMCP122 and pMCP175, pMCP122 and pMCP176, and pMCP122 and pMCP177. Plasmid recombination did not occur, as shown by hybridization assays with the 300-bp *Pvu*II-*Mlu*I probe (Pr.4 in Fig. 3D), and the cotransformed *S. lividans* cells were better producers of β-lactamase than *S. lividans* 1326(pMCP122) (Fig. 5C). Given that *bla* is not expressed in pMCP122 from the upstream nucleotide sequence due to the presence of the *fd* terminator (see above), these observations strongly suggested a *trans* effect of the upstream nucleotide sequences on the transcription of the pMCP122-borne *bla* gene. Note, however, that cotransformation of *S. lividans* 1326 failed to restore the same level of β-lactamase production as observed with *S. lividans* 1326 (pMCP38).

DISCUSSION

The nucleotide sequence of *bla*, as established previously (20) by the procedure of Sanger et al. (34a), was unsatisfactory in several respects (see Results). Reexamination of the

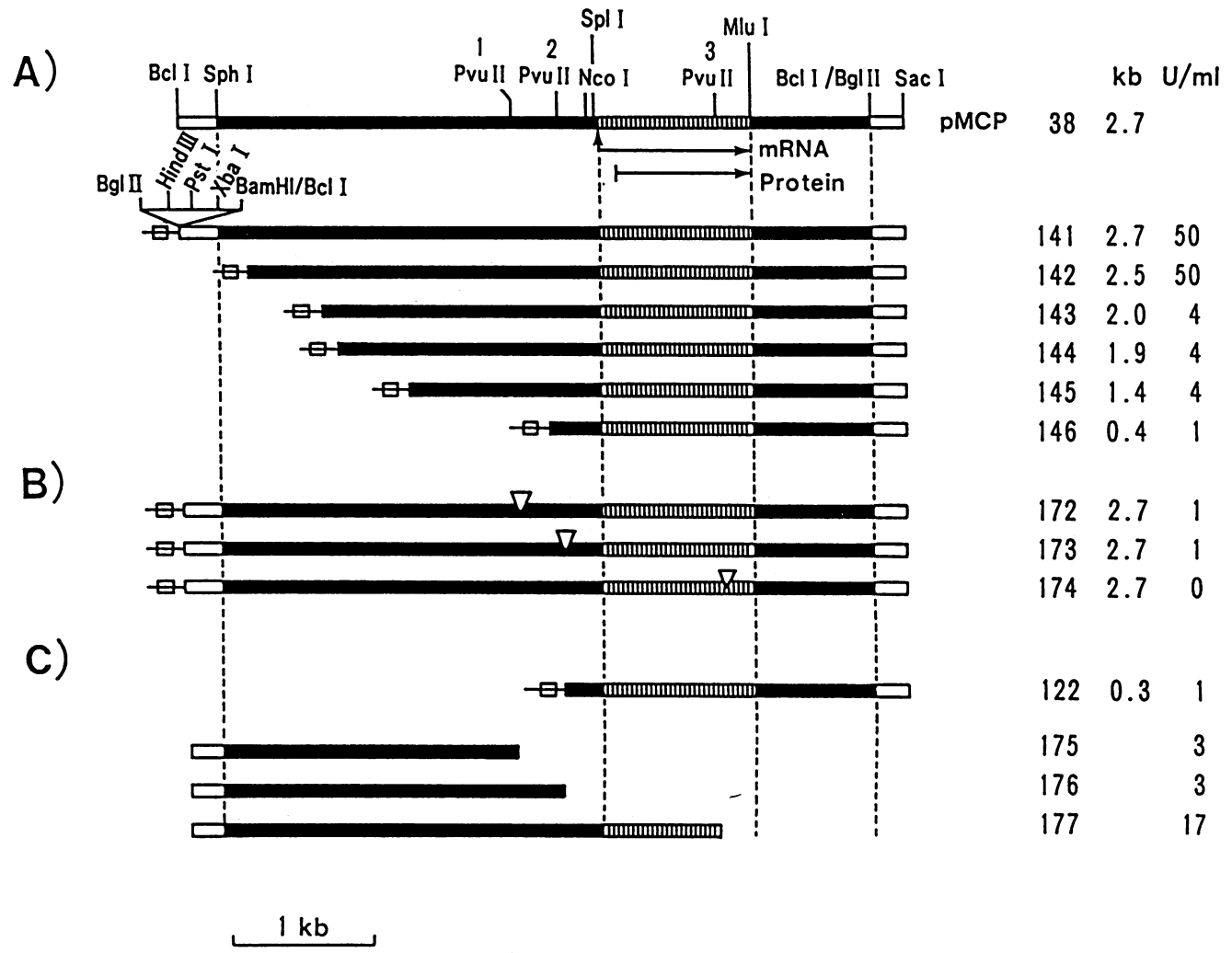

FIG. 5. Influence on *bla* expression of upstream region *S. cacaoi* DNA fragments of various lengths. For more details on the plasmids shown, see text and Fig. 1, 3, and 6. The distances between the transcription start site and the upstream junction between *S. cacaoi* and vector DNAs are given in kilobases. β-lactamase production values (units per milliliter) are those obtained with *S. lividans* 1326 harboring the indicated plasmids (A and B) or cotransformed with pMCP122 and pMCP175, pMCP176, or pMCP177 (C). ▽, *Bam*HI linker; other symbols are as in Fig. 3.

sequence with the Sequenase kit resulted in the elimination of one nucleotide between the A at position 201 and the T at 202 and yielded a much clearer picture. In all likelihood, the ATG triplet at 201 to 203 and the AGGAGG stretch from 211 to 216 are the initiator codon and the Shine-Dalgarno sequence, respectively. The amino-terminal region of the β-lactamase precursor, as deduced from the nucleotide sequence, has all the features of a signal peptide with four Arg's at positions 2, 4, 7, and 8 and a highly hydrophobic stretch from Leu-9 to Ala-24. The inverted repeat that occurs between nucleotides 101 and 114 may explain the difficulties encountered with the Sanger et al. procedure (34a). As derived from S1 mapping data, the *bla* transcriptional start point, T at 112, C at 113, or G at 114, is located about 90 bp upstream of the ATG initiator codon at 201 to 203. Finally, the proposed −10 and −35 sequences are in fair agreement with the consensus sequences proposed for *Streptomyces* promoters (12).

Recombinant plasmids were previously prepared by inserting *S. cacaoi* DNA fragments that contain *bla* and upstream nucleotide sequences of various lengths into the *mel* gene of pIJ702 (20). S1 mapping experiments showed that the *bla* promoter is active in pDML51, pDML52, and pMCP38 but not in pMCP39. In this last case, transcription is made by readthrough from a promoter, most likely the *mel* promoter (2) of the pIJ702 vector. The *bla* promoter may be silent in pMCP39 because of a higher affinity of the RNA polymerase for the *mel* promoter and/or a shift of the *bla* promoter to a conformation in which the aforementioned inverted repeat might be involved. Insertion of an *fd* terminator in pMCP39 caused a decreased level of the β-lactamase produced by the *Streptomyces* host. Closure and opening of the *bla* promoter might thus be under the control of a regulating gene that would not be present in pMCP39.

Although the *bla* promoter functions in pDML51, pDML52, and pMCP38, these plasmids produced progressively less β-lactamase, in the indicated order, in the *Streptomyces* host (20). In order to shed light on this phenomenon, the size of the nucleotide sequence in pMCP38 upstream of the *bla* operator was decreased stepwise from 2.7 to 0.4 kb and an *fd* terminator was inserted in order to protect these sequences from any possible readthrough from

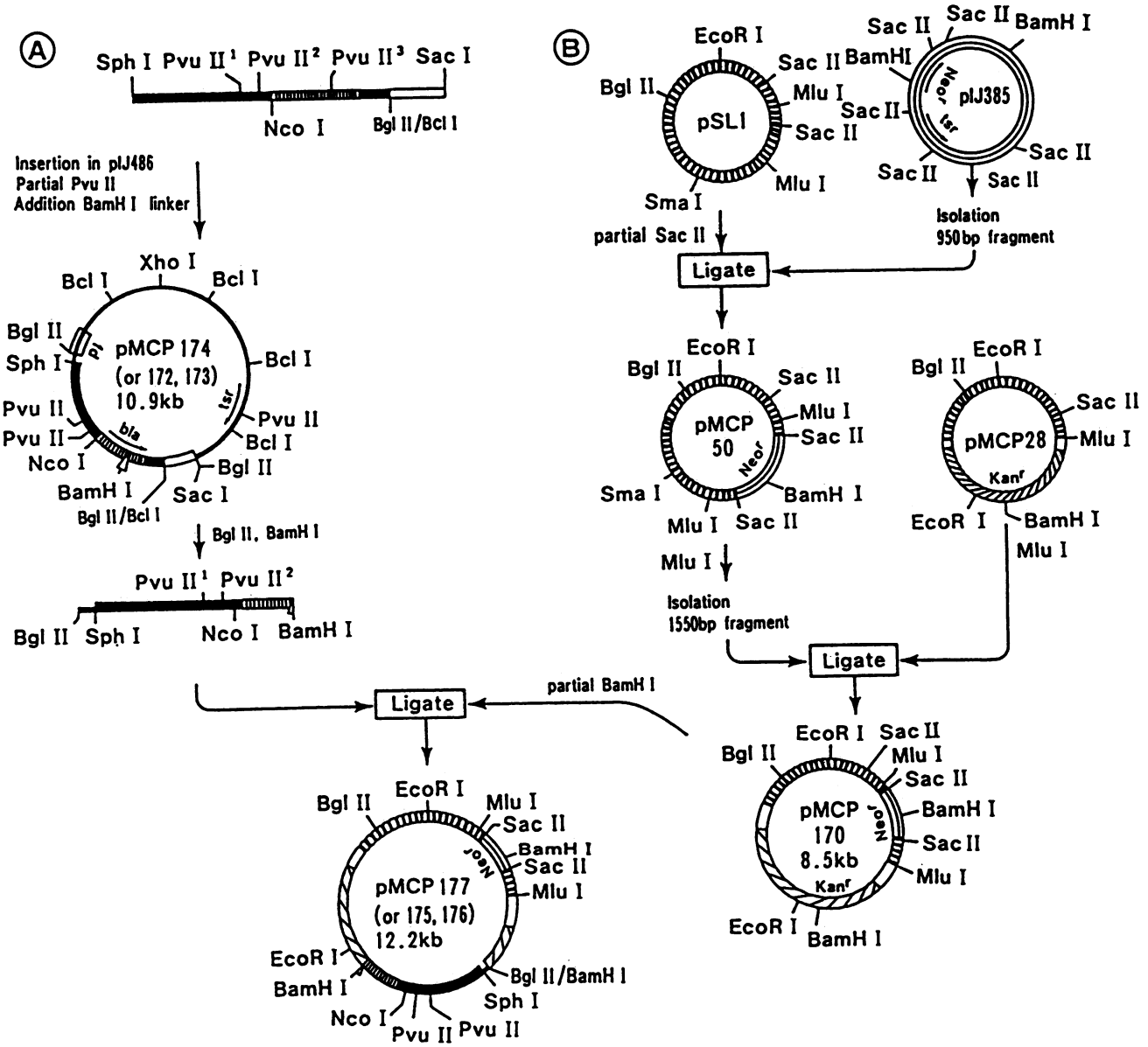

FIG. 6. Plasmid constructions. Ap^r, *E. coli* ampicillin resistance gene; tsr, *Streptomyces* thiostrepton resistance gene; Neo^r, *Streptomyces* neomycin resistance gene; Kan^r, *Streptomyces* kanamycin resistance gene. Vertically striped bars, pSL1; horizontally striped bars, pIJ385; diagonally striped bars, kanamycin resistance gene from *Streptomyces kanamyceticus*. Other symbols are as in Fig. 3.

the vector. Alternatively, a *Bam*HI linker was inserted into the upstream nucleotide sequence of pMCP38. Finally, the *S. lividans* host was cotransformed with several pairs of plasmids. One of these plasmids always carries *bla* preceded by the *fd* terminator, and the accompanying plasmid carries an upstream sequence of given size. Based on the levels of β-lactamase production that these plasmids induced in the host, it appears that the 0.7-kb nucleotide stretch located downstream of *Sph*I and the 1.1-kb nucleotide stretch located upstream of the *Pvu*II-2 site enhance β-lactamase expression and that, in all likelihood, the underlying mechanism is, at least in part, a *trans* effect of these nucleotides on the transcription of *bla*. This mode of gene regulation differs from those described so far for β-lactamase expression (see Introduction). Enhancing elements or activators, however, have been detected in bacteria (8, 33). More specifically, *Streptomyces* gene products known to be in-

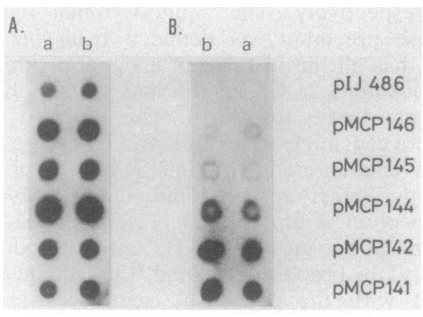

FIG. 7. Dot blot hybridization with clones containing pMCP141 through pMCP146. The probes were (A) the *tsr*-containing 1.1-kb *Bcl*I fragment of pIJ702 and (B) Pr.3 (Fig. 3D). The amount of total RNA used for blotting was 25 μg (a) and 50 μg (b).

volved in antibiotic synthesis (5) are assumed to be regulators or activators, though at present, quantitative data are not available. Finally, mention should also be made of an *S. cacaoi* strain in which β-lactamase synthesis is inducible (6, our unpublished result).

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