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Characterization of the Promoter, Signal Sequence, and Amino Terminus of a Secreted β-Galactosidase from "*Streptomyces lividans*"

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The gene for a secreted 130-kilodalton β-galactosidase from "*Streptomyces lividans*" has been cloned, its promoter, signal sequence, and amino terminal region have been localized, and their nucleotide sequence has been determined. The signal sequence extends over 56 amino acids and shows the characteristic features of signal sequences, including a hydrophilic amino terminus followed by a hydrophobic core near the signal cleavage site. The secretion of β-galactosidase depends on the presence of the signal sequence. β-Galactosidase is the major protein in culture supernatants and extracts of strains expressing the cloned β-galactosidase gene and represents a valuable tool in the study of protein secretion in *Streptomyces* spp.

Studies of protein secretion in bacteria show that most secreted proteins are synthesized in a precursor form with a signal sequence at the amino-terminal end (24). The signal sequence directs the emerging polypeptide chain to the membrane and presumably aids the peptide chain in crossing the membrane. At some point in this process, the signal sequence is cleaved off the precursor molecule by a signal peptidase and the mature protein is released from the cell membrane. In gram-negative organisms secreted proteins are generally trapped in the periplasmic space between the two membranes surrounding the bacterium, whereas gram-positive organisms such as *Bacillus* spp. or *Streptomyces* spp. secrete proteins directly into the surrounding medium. Although the size of the exported proteins can vary widely, the export of large proteins of over 100 kilodaltons (kDa) has not been extensively studied.

Streptomyces spp. are filamentous, gram-positive soil organisms mainly known for the production of commercially important antibiotics. We are interested in exploring the mechanism of protein secretion in these organisms because we have found that *Streptomyces* spp. are able to efficiently secrete both low- and high-molecular-weight proteins. Eventually, these secretion pathways might be used to develop *Streptomyces* spp. as host-vector systems for the production of heterologous proteins. To examine protein secretion in *Streptomyces* spp. and compare it with that in gram-negative and other gram-positive organisms (e.g., *Bacillus* spp.), we have cloned, identified, and partially sequenced the gene coding for an exported 130-kDa β-galactosidase from "*Streptomyces lividans*."

MATERIALS AND METHODS

Bacterial strains and plasmids. "*S. lividans*" 1326 is the wild-type strain producing β-galactosidase. Strain 1326-9R is a β-galactosidase-deficient mutant isolated from strain 1326 after two rounds of mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (13). Plasmid pSKL1 (Fig. 1). contains the cloned β-galactosidase gene from "*S. lividans*" (7). To localize the β-galactosidase promoter, a plasmid containing a promoterless β-galactosidase gene was constructed in *Escherichia coli*, using a plac3Stu derivative of pBR322 (6) as the vector. Plasmid plac3Stu (see Fig. 6) contains the *Pvu*II-to-*Eco*RI region of pBR322, including the origin of replication and the ampicillin resistance marker. A polylinker fragment, *Sph*I-*Xho*I-*Bcl*I-*Sac*I, is located at the *Pvu*II site, and a 188-base-pair (bp) *Sau*3A fragment containing the terminator t_{oop} from bacteriophage lambda was inserted at the *Eco*RI site. This terminator reduces the transcriptional readthrough in *E. coli* and *Streptomyces* spp. (T. Eckhardt, unpublished results). A *Stu*I-*Pst*I-*Stu*I-*Bam*HI linker region is located past the terminator at the *Eco*RI site, and the *E. coli lacZ* gene was inserted in clockwise direction between the *Stu*I and *Sph*I sites. pUC9 (29) and plac3Stu were used to generate promoter fragments with *Bam*HI ends (see Fig. 7). Plasmid pbgal4 was constructed by replacing the *Pst*I-*Sph*I *lacZ* region of plac3Stu with the 6.4-kilobase (kb) *Pst*I-*Sph*I β-galactosidase fragment from pSKL4 (see Fig. 6).

Culture media and antibiotic selection. Techniques for culturing *Streptomyces* spp. are described in detail in reference 13. For β-galactosidase assays, mRNA isolation, and protein gels, *Streptomyces* strains were grown in YEME medium (13) containing 20% sucrose and 1% galactose at 28°C with the spores from a single R2YE plate as inoculum. For β-galactosidase assays, the cultures were grown for 48 h, as β-galactosidase levels reach a plateau after that time. The cultures contained around 200 mg (wet weight) of cells per ml. R2YE plates were used for regeneration of transformed protoplasts. After a 24-h incubation at 28°C, selection of transformants was carried out directly by overlaying the plates with 3 ml of aqueous solution containing 0.1 mg of thiostrepton and 0.8 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) per ml. The plates were allowed to air dry and were incubated for 48 to 96 h at 28°C. β-Galactosidase-positive clones formed a blue halo after 48 h due to the hydrolysis of X-gal by the extracellular β-galactosidase. Selection of *E. coli* transformants was done on LB plates (17) containing 50 μg of ampicillin per ml.

DNA manipulations. The general procedures for DNA isolation in *Streptomyces* spp. are described in reference 13. The procedures for DNA manipulation, including plasmid

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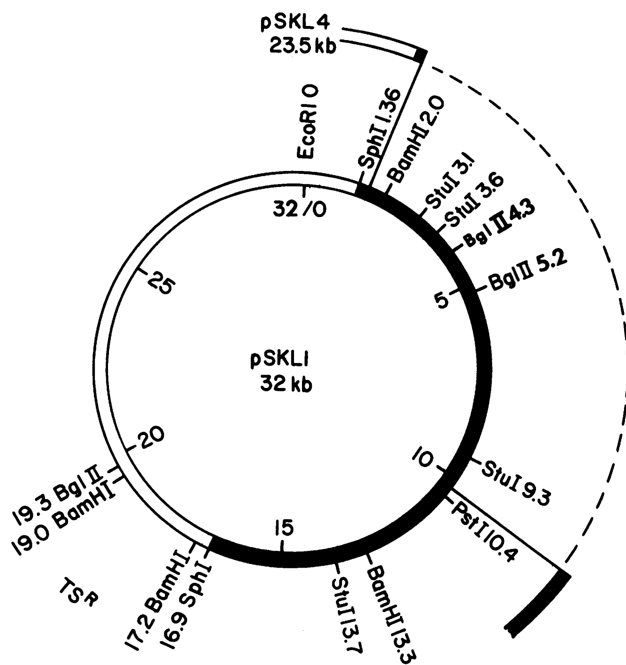

FIG. 1. Restriction map of pSKL1 (7) and pSKL4, indicating the approximate position of the relevant restriction sites. pSKL1 contains the β-galactosidase gene on a 15.5-kb *Sph*I chromosomal insert (solid bar). The open bar indicates the *Sph*I region from the vector pJI6. TS indicates the thiostrepton resistance gene. pSKL4 (outer circle) was obtained as a spontaneous β-galactosidase-positive deletion of pSKL1. The broken line indicates the chromosomal region deleted from pSKL1. Numbers are coordinates in kilobases.

isolation from *E. coli*, restriction enzyme cleavage, and DNA ligation, are described in reference 17. DNA sequence analysis was performed by the Maxam-Gilbert (18) and dideoxy-chain termination methods (22). In vitro mutagenesis with hydroxylamine is described in reference 8.

Nuclease S1 mapping. The 5′ ends of in vivo transcripts of the β-galactosidase gene were mapped by nuclease S1 mapping (1). RNA was isolated from 38-h cultures by using the hot phenol procedure described previously (13). The *Pvu*I-to-*Xma*III fragment spanning the transcription start site (see Fig. 4) was labeled with [γ-^{32}P]ATP. The DNA strands were separated by using a denaturing gel (17), and the DNA strand labeled at the *Xma*III site was used as probe for mRNA hybridization.

β-Galactosidase assay. β-Galactosidase was assayed by the method of Miller (19). β-Galactosidase activity is expressed as nanomoles of *o*-nitrophenyl-β-D-galactoside hydrolyzed per minute per milliliter of cell culture at 37°C. Cell-associated β-galactosidase was determined by using toluenized cells (19). For this purpose 1 ml of culture was spun down, the culture supernatant was removed, and the cells were suspended in β-galactosidase assay buffer to a final volume of 1 ml. Toluene (20 μl) was added, and after vigorous shaking the mixture was incubated for 15 min at 37°C. Samples were used for enzyme assay. Extracellular β-galactosidase levels were determined by using portions of culture supernatants after removal of the cells by centrifugation in an Eppendorf microfuge for 5 min.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (16). For separation of β-galactosidase, an 8% separating gel and a 5% stacking gel were used. The ratio of acrylamide to *N,N′*-methylenebisacrylamide (BIS) was 39:1.

Protein sequencing. The N-terminal sequence of the mature protein was determined using a Beckman 890M sequenator. Medium from "*S. lividans*" secreting β-galactosidase was precipitated with 70% (final concentration) $(NH_4)_2SO_4$; the protein was redissolved, dialyzed against deionized H_2O, and lyophilized. The protein was reduced, *S*-dansylaminoethylated (23), recovered by precipitation with ethanol, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The fluorescent 130-kDa band was electroeluted essentially as described previously (14). The electroeluted protein (~2 nmol) was again recovered by ethanol precipitation and redissolved in 0.1% sodium dodecyl sulfate, and one-half was used for sequence analysis.

RESULTS

β-Galactosidase gene. We previously cloned the gene for an exported 130-kDa β-galactosidase from "*S. lividans*" 1326. Plasmid pSKL1 carried the β-galactosidase gene on a 15.5-kb *Sph*I DNA fragment (7). Plasmid pSKL4 (Fig. 1) was isolated as a spontaneous deletion of pSKL1, having lost 8.5 kb of the chromosomal insert but retaining the ability to express the extracellular β-galactosidase. Strains containing pSKL4 or pSKL1, in fact, produced similar amounts of β-galactosidase, indicating that the remaining 6.7-kb chromosomal insert contained not only the coding region for the enzyme but also its promoter. The structural region of the β-galactosidase gene was further localized by deletion analysis (Fig. 2). A *Bcl*I-to-*Sph*I deletion, as well as deletion of two *Mlu*I fragments, resulted in a β-galactosidase-negative phenotype. The *Nae*I-to-*Xmn*I deletion affected the synthesis and localization of β-galactosidase as outlined below. This deletion analysis indicates that the β-galactosidase gene spans at least 3.7 kb of the chromosomal insert.

mRNA start site. To localize and characterize the promoter region of the β-galactosidase gene, the entire 6.7-kb *Pst*I-to-*Sph*I fragment carrying the chromosomal insert was scanned for an mRNA start site by using standard S1 mapping techniques (1). Only the 1.5-kb *Pst*I-*Xmn*I fragment labeled 5′ at the *Xmn*I site showed protection of a 300-nucleotide-long fragment by mRNA, indicative of an mRNA start site to the left of the *Xmn*I site (Fig. 2).

To identify the start site of this mRNA at the nucleotide level, the DNA sequence of a 664-bp *Sma*I-*Pvu*II fragment spanning the putative promoter region was determined by the sequencing strategy outlined in Fig. 3. The nucleotide sequence is shown in Fig. 4. The precise mRNA start site was obtained by S1 nuclease mapping (see Materials and Methods), and the result of such an experiment is shown in Fig. 5. The region upstream of this start site shows sequences characteristic for a promoter (Fig. 4). The region is relatively A+T rich compared with the average G+C content of 70 to 76% for *Streptomyces* DNA (12). The −10 region of the proposed β-galactosidase promoter has similarity to −10 regions from other *Streptomyces* promoters (15). In fact, the *tsrP1* promoter (3, 15) has the identical −10 region TAGGGT, a sequence close to the consensus sequence TAGGAT found for some of the *Streptomyces* promoters (R. Hütter and T. Eckhardt, *in* M. Goodfellow, ed., *Actinomycetes in Biotechnology*, in press). The −35 region has homology to the corresponding sequences in *E. coli* and *Streptomyces* spp. (15).

β-Galactosidase promoter probe vector. To study the function of this proposed β-galactosidase promoter region, we

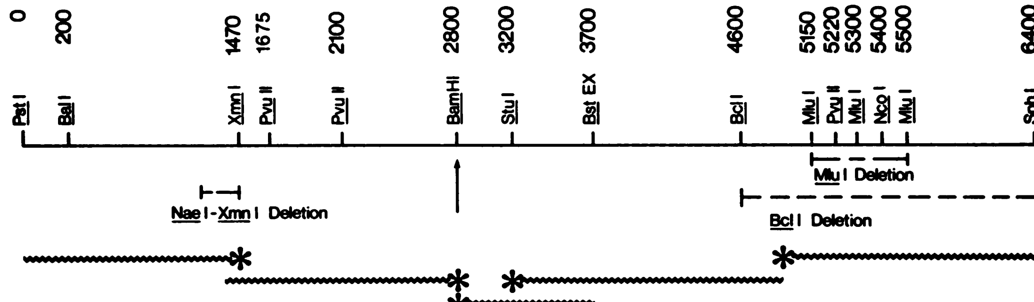

FIG. 2. Extented restriction map of the chromosomal *Pst*I-*Sph*I region of pSKL4, indicating the deletions (– – –) used to localize the β-galactosidase gene and the mutation introduced at the *Bam*HI site (arrow) during construction of pPS7. Numbers are base pair coordinates. Wavy line, DNA fragments used for S1 mapping with the labeled 5′ end (asterisks).

examined the effect of various-length sequences upstream of the mRNA start site on the expression of the β-galactosidase gene. For this purpose, a promoterless β-galactosidase gene was constructed which could serve as the probe for promoter activity. We chose to introduce a unique *Bam*HI site at the start site of the β-galactosidase and to remove the *Bam*HI site within the β-galactosidase structural gene (Fig. 2). This would allow promoter fragments to be inserted as *Bam*HI or *Sau*3A fragments into the newly introduced *Bam*HI site. pbgal4 (Fig. 6) was mutated in vitro by using hydroxylamine, and mutant plasmids having lost the single *Bam*HI site were identified by restriction analysis. The mutant plasmids lacking the *Bam*HI site were converted into shuttle vectors by insertion of the *Sph*I fragment of pIJ6 (Fig. 1) into the *Sph*I site present on pbgal4. After transformation into "*S. lividans*," these mutants were analyzed for β-galactosidase production. Three mutant plasmids, including pbgal4-1, produced β-galactosidase at levels identical to pSKL4, indicating that the mutation of the *Bam*HI site had no effect on β-galactosidase expression or stability. Restriction endonuclease digestion of pbgal4-1 revealed that a *Hin*fI site was located at the former *Bam*HI site. A base transition induced by hydroxylamine apparently converted the *Bam*HI recognition sequence GGATCC to the *Hin*fI recognition sequence GAATCC or GGATTC. Either mutation is most likely to be a silent mutation in the third position of the reading frame, since the reading frame of a *Bam*HI recognition sequence preferred by *Streptomyces* spp. is NGG-ATC-CNN with a G or C residue in the third position (5, 21). The construction of plasmid pPS7 containing the promoterless β-galactosidase gene is outlined in Fig. 6.

β-Galactosidase promoter. Using the promoterless β-galactosidase gene from pPS7, we tested various DNA regions from the region upstream of the β-galactosidase mRNA start site for their ability to promote β-galactosidase

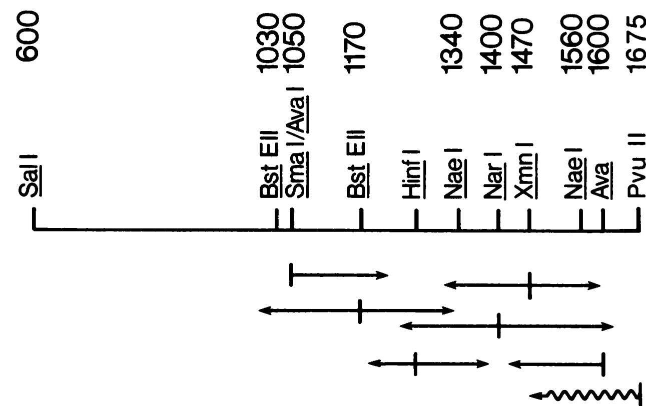

FIG. 3. Detailed map and sequencing strategy used to sequence the promoter and amino-terminal regions of the β-galactosidase gene. The coordinates correspond to those in Fig. 2. The straight arrows indicate the start, direction, and length of the DNA sequence obtained by using the Maxam-Gilbert method (18). The waved arrow indicates the DNA sequence obtained by the method of Sanger et al. (22). Only the relevant *Hin*fI and *Nar*I sites are indicated.

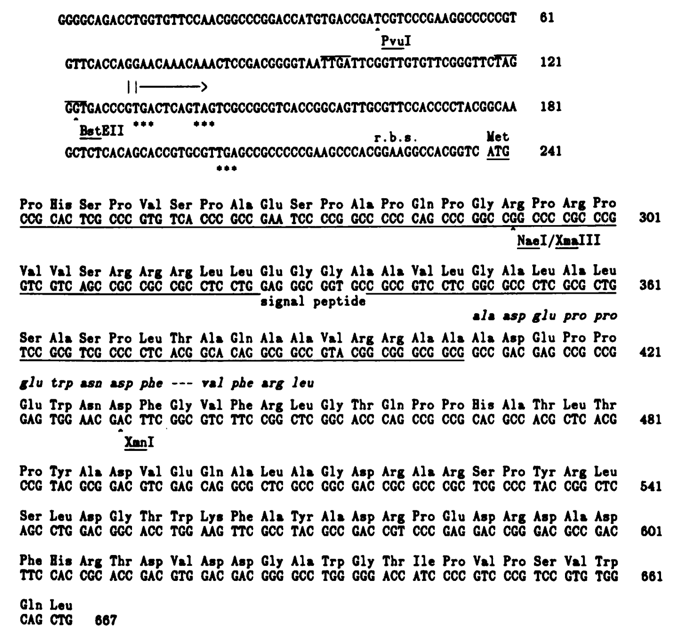

FIG. 4. Nucleotide sequence of the *Sma*I-*Pvu*II fragment spanning the promoter, untranslated, and amino-terminal regions of the β-galactosidase gene. Proposed −35 and −10 regions are overlined. The arrow indicates the start site and direction of mRNA transcription. Translated stops in the untranslated region are indicated by asterisks. r.b.s indicates the ribosome-binding site preceding the initiation codon with bases complementary to the "*S. lividans*" 16S RNA (4). The proposed signal sequence is underlined. Amino acid residues of the extracellular β-galactosidase as determined by protein sequencing are given in italics. Relevant restriction sites are indicated. The cleavage sites of *Nae*I and *Xma*III restriction endonucleases are identical. The *Nae*I restriction endonuclease generates blunt-ended DNA; the *Xma*III restriction endonuclease generates DNA fragments with a 4-base single-stranded extension at the 5′ end.

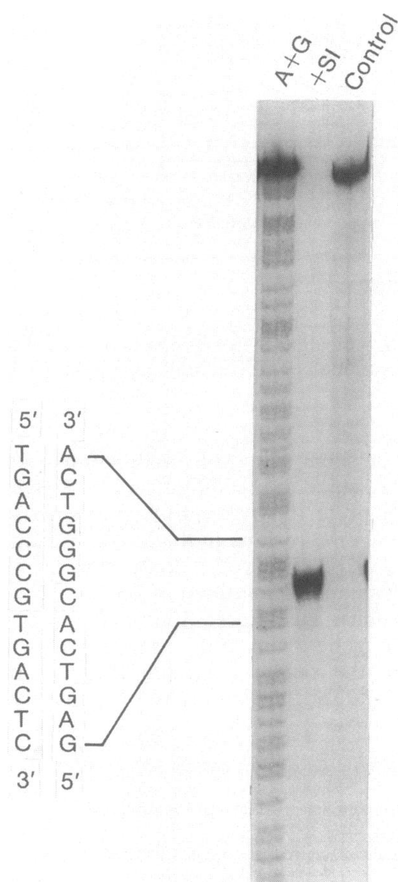

FIG. 5. S1 nuclease protection mapping of total RNA isolated from 38-h cultures of "*S. lividans*" 1326-9R carrying pSKL4, grown and isolated as described in Materials and Methods. Lanes: A+G; Maxam-Gilbert A+G sequencing reactions of the probe as reference for the start site; +S1, mRNA, probe, and 100 U of S1 nuclease; Control, mRNA and probe hybridized without S1 nuclease. The nucleotide sequence across the mRNA start site is indicated to the left.

synthesis. To insert these fragments into the single *Bam*HI site of pPS7, the fragments of interest were modified such that they were flanked by *Bam*HI sites as outlined in Fig. 7. The resulting constructs were subsequently converted to shuttle vectors by insertion of the *Sph*I fragment from pIJ6 and were introduced into "*S. lividans*" 1326-9R for analysis of β-galactosidase expression. The intra- and extracellular β-galactosidase levels of the various constructs are given in Table 1. Strains carrying plasmid Bst13PS7-IJ6 produced β-galactosidase at wild-type levels, indicating that the 140-bp *Bst*EII fragment originating from immediately upstream of the start site of the β-galactosidase mRNA is sufficient to express β-galactosidase when inserted in the correct orientation upstream of the β-galactosidase gene.

Intra- and extracellular protein patterns of two constructs, the promoterless pPS7-IJ6 and P3PS7-IJ6, a pPS7 derivative carrying the β-galactosidase promoter, are shown in Fig. 8. β-Galactosidase is the major protein in the supernatant of P3PS7-IJ6 expressing the cloned β-galactosidase gene. The majority of the enzyme migrates with an apparent mobility of a 130-kDa protein. A minor band appearing at 100 kDa is a breakdown product of the 130-kDa protein (T. Eckhardt, unpublished data). The 130-kDa enzyme can also be identified in cell extracts of these strains and represents the cell-associated fraction of this enzyme. The exact nature of this form has not yet been elucidated. The high level of β-galactosidase expression by the cloned β-galactosidase gene might saturate a step in the secretion process and result in an intracellular build-up of β-galactosidase. Alternatively, the considerable size of the active enzyme might restrict its free passage through the cell wall, resulting in a periplasmic accumulation of β-galactosidase. The latter is the more favored explanation since there is no apparent difference in the apparent mobility of cell-associated and secreted forms of the enzyme, indicating that extracellular and cell-associated forms of the enzyme are both processed.

Translation of β-galactosidase. The DNA sequence downstream of the promoter region was analyzed for potential translational initiation sites and open reading frames in an effort to identify the amino-terminal coding sequence for the β-galactosidase gene and information involved for its secretion. As a consequence of the high G+C content of *Streptomyces* spp., their codon usage shows strong preferences with 90 to 95% of the triplets ending in G or C, a feature which helps to identify potential protein coding regions (5, 21). An examination of the transcribed portion of the β-galactosidase DNA sequence reveals translational stop codons in all three reading frames within the first 75 bp of the mRNA transcript. The presence of these stop codons in the leader region of the mRNA is a useful feature when the β-galactosidase gene is utilized as promoter probe, as any readthrough translation originating from the inserted promoter fragments is terminated and will not interfere with β-galactosidase expression. The first initiation codon occurs 110 bp downstream of the start site and precedes a long open reading frame. This suggests that the β-galactosidase gene contains a rather extended 5′ untranslated region. The codon usage in the long open reading frame conforms to the preference shown by *Streptomyces* spp., with 137 of the 143 sequenced codons having G or C in the third position and an overall G+C content of 82%. The initiation codon is preceded by a potential ribosome-binding site, showing complementarity to the 3′ end of 16S RNA (Fig. 3) (4). The predicted protein sequence past the initiation codon shows the characteristic features of a signal sequence, namely a hydrophilic region followed by a hydrophobic core region of 22 amino acid residues. The hydrophilic segment of this

TABLE 1. Levels of β-galactosidase produced by the promoter probe vector pPS7 carrying DNA fragments from the β-galactosidase promoter region

Construct[a]	β-Galactosidase activity[b]	
	Extracellular	Cell associated
None	10	8
pSKL1	2,100	1,900
pSKL4	2,300	1,600
DNaeXmn	22	228
pPS7-IJ6	7	10
P3PS7-IJ6	1,950	1,380
Bst13PS7-IJ6	1,887	1,450
Bst12P27-IJ6	16	7
SalBstEPS7-IJ6	3	6

[a] The various pPS7 constructs shown in Fig. 7 were converted into shuttle vectors by insertion of the *Sph*I fragment from pIJ6 as indicated in their designation. The host strain was "*S. lividans*" 1326-9R. Growth conditions and enzyme assays are described in Materials and Methods.

[b] Nanomoles of *o*-nitrophenyl-β-D-galactoside hydrolyzed per minute per milliliter of cell culture at 37°C.

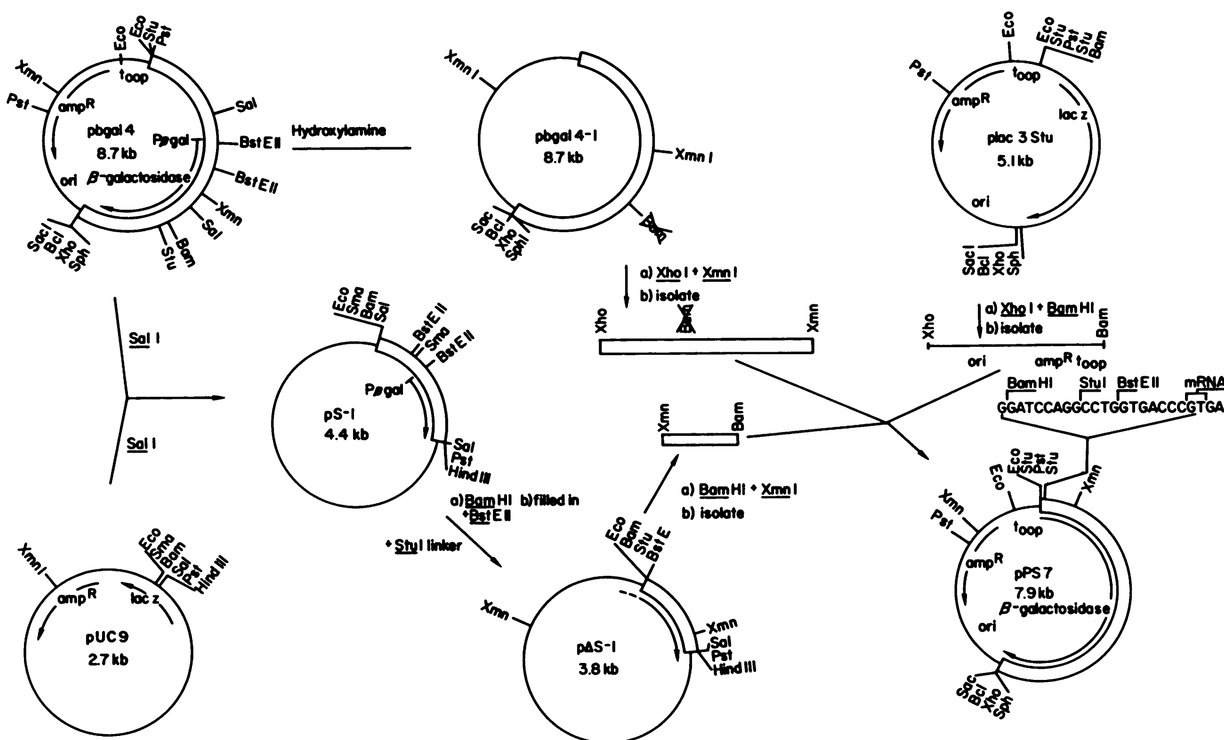

FIG. 6. Construction of the β-galactosidase promoter probe vector pPS7. *Streptomyces* sequences are indicated by open bars. The *Sal*I fragment from pbgal4 containing promoter and amino-terminal sequences of the β-galactosidase gene was cloned into pUC9 in the direction indicated. Regions upstream of the mRNA start site were modified as indicated. The *Stu*I linker has the sequence CAGGCCTG. The *Stu*I site is methylated in *E. coli dcm*$^+$ and is not recognized by the *Stu*I restriction endonuclease. The mutated *Bam*HI site in pbgal4-1 is shown crossed out. pPS7 was obtained by ligation of three fragments as indicated. The nucleotide sequence between the *Bam*HI site and the beginning of the β-galactosidase gene in pPS7 was verified and is indicated. The mRNA start site of the original β-galactosidase gene is indicated by an arrow above this sequence. Promoter fragments were inserted at the single *Bam*HI site of pPS7, and the pIJ6 *Sph*I fragment was inserted at the single *Sph*I site of pPS7. Only the relevant *Bst*EII and *Sal*I sites are given for pbgal4. Only relevant restriction sites are given for the intermediate constructs. t_{oop} is the terminator from phage lambda (see Materials and Methods).

signal sequence, however, is unusually large. It extends over 30 amino acid residues, in contrast to the 5 to 12 residues found in *Bacillus* sp. (20) or *E. coli* (24) as well as those found for the *Streptomyces* endo H gene (21). Most strikingly, the charged region is devoid of lysine residues and rich in arginine residues. A cluster of three arginine residues is found immediately preceding the hydrophobic segment. Such an arginine cluster is also found at an identical position in the signal sequence of the endo H gene (21) and in an exported protein from the *Streptomyces antibioticus* tyrosinase operon (2). The translated sequence immediately beyond the signal sequence does not show any similarity to β-galactosidase sequences from other organisms (9, 11, 25). This is not surprising as the sequenced region represents only a small fraction of the coding region of this large protein.

To verify the accuracy of the protein sequence predicted from the DNA and to identify the cleavage site of the signal peptidase, the extracellular form of the β-galactosidase was purified and its amino-terminal amino acid sequence was determined. This sequence could be aligned with the sequence predicted for the β-galactosidase as indicated in Fig. 4. The amino terminus of the mature protein is located four residues beyond the hydrophobic portion of the signal sequence. The signal peptidase cleavage apparently occurs between two alanine residues (Fig. 4), a cleavage site also present in the *Bacillus licheniformis* α-amylase (26). A minor fraction of the β-galactosidase precursor (~20%) is processed between the Ala and Asp residues.

We tested the importance of the postulated signal sequence for the secretion of the β-galactosidase by constructing an internal deletion near the N terminus which removed the signal sequence. The region between the *Nae*I and *Xmn*I sites (Fig. 4) was deleted from the β-galactosidase gene on pbgal4, removing amino acid residues −39 to +9. In this deletion, the amino terminus of the β-galactosidase is fused in frame to the rest of the β-galactosidase sequence, and most of the signal sequence, including the hydrophobic core region and processing site, is deleted. Upstream sequences, including the promoter and untranslated leader, are, however, left intact. The translation of the resulting gene fusion is initiated at the normal initiation codon of the β-galactosidase gene and proceeds in frame into the structural part of the enzyme (Fig. 4). When this construct, DNaeXmn, was analyzed for β-galactosidase activity (Table 1), we found that 90% of the β-galactosidase activity was now localized intracellularly, confirming the importance of the deleted sequence for efficient secretion of β-galactosidase. Surprisingly, the level of β-galactosidase in this construct was considerably lower than the levels obtained with the intact β-galactosidase gene. A preliminary examination of the mRNA levels by S1 analysis indicated a fivefold decrease of the β-galactosidase mRNA level, which accounts for a part of the observed reduction in β-galactosidase production (T.

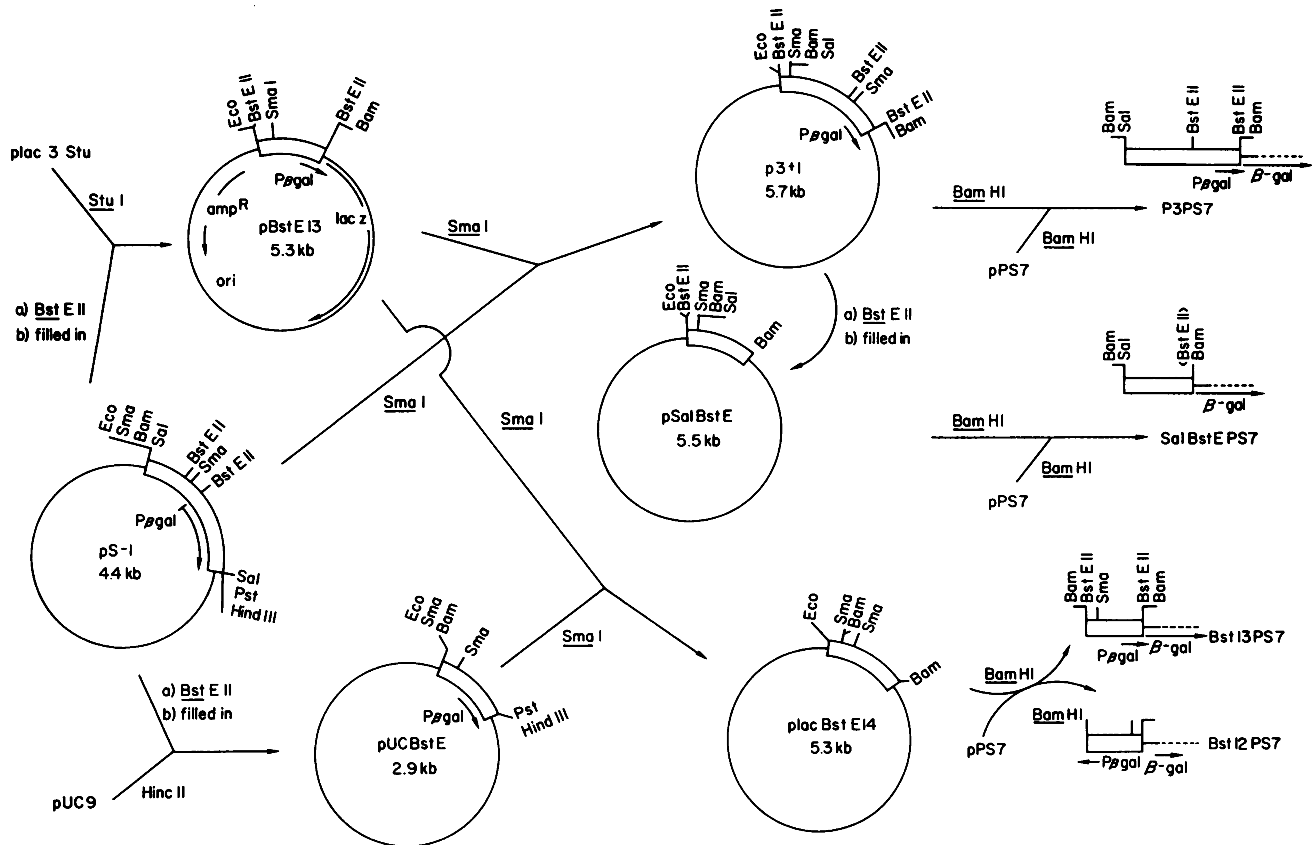

FIG. 7. Construction of plasmids carrying DNA fragments from the β-galactosidase promoter region flanked by *Bam*HI sites. *Streptomyces* sequences are indicated by open bars. A detailed map of pUC9, plac3Stu, and pPS7 is shown in Fig. 6. The *Bst*EII site from pS-1 was cloned into the *Stu*I site of plac3Stu generating a *Bam*HI site at the *Bst*EII site downstream from the promoter region. The same fragment was cloned into the *Hin*cII site of pUC9 in the orientation indicated, resulting in a *Bam*HI site at the *Bst*EII site upstream of the β-galactosidase promoter. A *Bam*HI site at the upstream *Sal*I site was already present on pS-1. Combination of the appropriate fragments as indicated resulted in the *Bst*EII fragment (placBstE14) and in the *Sal*I-to-*Bst*EII fragment (p3+1) flanked by *Bam*HI sites. The *Bst*EII fragment was deleted from p3+1 to generate pSalBstE lacking the region immediately upstream of the β-galactosidase start site. The *Bam*HI fragments from p3+1 and pSalBstE were inserted into pPS7 in the direction of the β-galactosidase gene, resulting in P3PS7 and SalBstEPS7, respectively. $P_{\beta gal}$ indicates the β-galactosidase promoter. The various promoter fragments were inserted into pPS7 as indicated at the right.

Eckhardt, unpublished data). It remains to be determined whether the fusion mRNA or protein generated in this construct is unstable or whether elements necessary for the efficient expression of the β-galactosidase gene have been deleted.

DISCUSSION

In summary, we have shown that "*S. lividans*" is capable of efficiently secreting a high-molecular-weight β-galactosidase enzyme. The secreted *Streptomyces* enzyme provides a useful tool to study protein secretion in these organisms. It is of interest that *Streptomyces* spp. export this large molecule, particularly since attempts to export the cytoplasmic *E. coli* β-galactosidase in *E. coli* or in other systems have not been successful (10, 27). The size of the β-galactosidase signal as deduced from the DNA sequence is unusual, even for *Streptomyces* spp. We believe that the cleavage observed is indeed that caused by the signal sequence peptidase and not the result of a secondary proteolytic cleavage. For one, the cleavage occurs at a classical cleavage sequence, Ala-Ala-Asp, with 80% of the cleavage occurring between Ala and Ala and 20% between Ala and Asp. Secondly, the cleavage is preceded by a typical hydrophobic transmembrane domain commonly found in signal sequences at this position and is very similar to the processing site for the secreted gene product of the open reading frame 1 of the tyrosinase operon (2). The identical processing site is used when the β-galactosidase coding region past the *Xmn*I site is replaced by other gene regions (H. Lichenstein and T. Eckhardt, unpublished results), indicating that the cleavage site does not depend on downstream domains. Some of the *Streptomyces* signal sequences have extended hydrophilic domains (2, 21). The predominance of arginine residues in these domains as well as in the β-galactosidase signal is intriguing. Their dominance over lysine residues could possibly lead to a stronger interaction of the signal with the bacterial membrane and thus stabilize the mRNA at the membrane during secretion. A more prosaic explanation would be that *Streptomyces* spp. with their high G and C content tend to prefer amino acids encoded by G+C-rich codons such as arginine codons over the A+T-rich lysine codons, as a comparison of the erythromycin resistance genes from various sources suggests (28). The observed down expression of the *Nae*I-*Xmn*I construct indicates a possible interdependence between protein expression and secretion which needs further exploration. In fact a β-galactosidase mutant which had the cluster of three

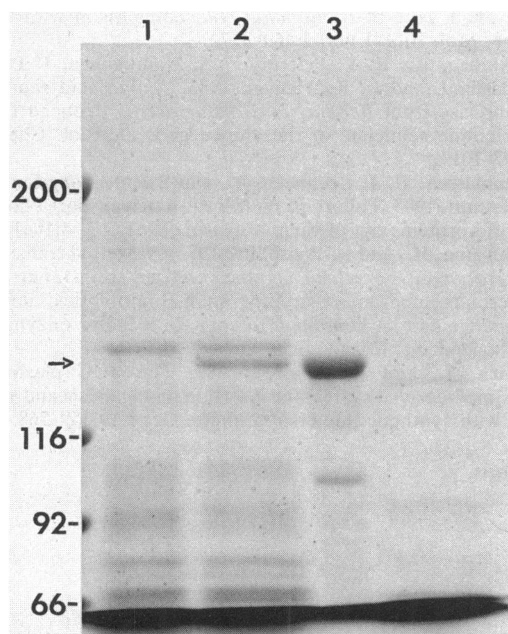

FIG. 8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of supernatants and extracts of "*S. lividans*" 1362-9R carrying the pIJ6 derivatives of pPS7-IJ6 or P3PS7-IJ6. Lanes: 1, cell extract of SalBstEPS7-IJ6, 100 μg of protein loaded; 2, cell extract of P3PS7-IJ6, 100 μg of protein loaded; 3, culture supernatant of P3PS7-IJ6, 40 μl loaded; 4, culture supernatant of pPS7-IJ6, 200 μl loaded. The positions of the "*S. lividans*" β-galactosidase (→) and molecular size standards (in kilodaltons) are indicated at the left.

arginine residues in the signal sequence mutated to glycine residues has shown a similar down expression of β-galactosidase (T. Eckhardt, unpublished results).

The "*S. lividans*" β-galactosidase is an excellent subject for the study of protein secretion in *Streptomyces* spp. β-Galactosidase can be easily monitored by using various chromogenic substrates such as X-gal or 4-methylumbelliferyl-β-D-galactosidase. The secreted enzyme forms a blue halo on solid media containing X-gal, facilitating the identification of secretory mutants. The abundance of β-galactosidase in culture supernatants of "*S. lividans*" helps the recovery of this protein. The "*S. lividans*" β-galactosidase has been expressed in a variety of *Streptomyces* species, but appears not to be expressed in *E. coli*. As our studies of promoter activity show, the promoterless β-galactosidase gene has all the necessary features for a useful promoter probe. This aspect is currently being developed further, and various promoters are being studied using this gene (H. Lichenstein, unpublished results).

The ability of *Streptomyces* spp. to secrete large proteins may be helpful in developing these organisms into host-vector systems for heterologous protein secretion, and the elucidation of the mechanisms of protein export in *Streptomyces* spp. can lead to a better understanding of export of large proteins in other systems.

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