JOURNAL OF BACTERIOLOGY, Nov. 1995, p. 6033–6040 Vol. 177, No. 21
0021-9193/95/$04.00+0

Isolation and Characterization of Two Genes Encoding Proteases Associated with the Mycelium of *Streptomyces lividans* 66

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Received 12 June 1995/Accepted 28 August 1995

A strain of *Streptomyces lividans* 66 deleted for a major tripeptidyl aminopeptidase (Tap) was used as a host to screen an *S. lividans* genomic library for clones overexpressing activity against the chromogenic substrate Ala-Pro-Ala-β-naphthylamide. In addition to reisolation of the *tap* gene, clones representing another locus, *slpD*, were uncovered. *slpD* was analyzed by deletion subcloning to localize its functional sequence. Nucleotide sequence determination revealed an open reading frame encoding a 55-kDa protein exhibiting significant amino acid sequence homology to Tap, particularly around the putative active-site serine residue. No secreted protein was observed for strains harboring the *slpD* clone, but inspection of the predicted protein sequence revealed a putative lipoprotein signal peptide (signal peptidase II type), suggesting a mycelial location for the SlpD proteinase. In an attempt to isolate an endoprotease known to be active against some heterologous proteins, a second clone was isolated by using a longer substrate (*t*-butyloxycarbonyl [Boc]-APARSPA-β-naphthylamide) containing a chemical blocking group at the amino terminus to prevent aminopeptidase cleavage. This locus, *slpE*, appeared to also encode a 55-kDa mycelium-associated (lipoprotein) proteinase, whose predicted protein sequences showed significant amino acid homology to Tap and SlpD, particularly around the putative active-site serine residues. Chromosomal integration and deletion analysis in both the wild-type and Tap-deficient backgrounds appeared to indicate that SlpD was essential for viability and SlpE was required for growth on minimal media.

Streptomyces lividans 66 has been used by a number of workers for the expression of heterologous proteins. Recent reviews have documented the successful production of a variety of heterologous proteins which can be directly secreted into the culture medium and correctly folded to allow the formation of appropriate disulfide bonds (1, 3, 10). Relatively little is known about the proteases present in this host strain, although such information is clearly crucial to the ability to make particular heterologous proteins. A gene, *slpA*, encoding an endoprotease was previously identified (7) by expression screening of a genomic library for the ability to cleave skim milk. However, strains in which this chromosomal locus had been specifically deleted retained the ability to degrade at least a number of heterologous proteins (unpublished observations).

Aminopeptidase activity in *S. lividans* 66 cultures has been suspected because of the observation of processed forms of some secreted proteins, notably, the endogenous secreted serine protease inhibitor (17). We have recently identified a gene encoding the major aminopeptidase activity in *S. lividans* 66 (6) which was shown to be a previously unknown tripeptidyl aminopeptidase (Tap). Characterization of the Tap protein (15) demonstrated its ability to remove tripeptides from the amino termini of proteins. Hydrolysis of chromogenic peptide substrates by Tap was also demonstrated. The *tap* gene was indeed cloned by virtue of overexpression of its ability to hydrolyze Gly-Pro-Leu-β-naphthylamide (GPL-βNA). Deletion of the chromosomal *tap* gene eliminated the ability of the strain to hydrolyze GPL-βNA and drastically reduced the tripeptidyl aminopeptidase activity. However, low levels of *tap* activity were still observed for several of the heterologous proteins produced in strains carrying the *tap* deletion. Many of these proteins contained amino-terminal residues consisting of the amino acids Ala-Pro-Ala. We therefore, set out to characterize and eliminate the remaining minor aminopeptidase activities present in the *S. lividans* Δ*tap* strain MS7. This approach resulted in the isolation and deletion of the gene encoding a subtilisin-like protease (Ssp) from *S. lividans* (4). The resulting deletion strain MS11 (Δ*tap* Δ*ssp*) exhibited reduced but significant Tap activity compared with its parental strain, MS7. In an effort to identify genes encoding the residual activity against the tripeptide substrate, two loci which appear to encode mycelium-associated proteinases important for growth and viability of this organism have been identified. An analysis of these genes and their products is presented in this report. To our knowledge, this is the first description of the existence of a cell wall-associated proteinase in a *Streptomyces* sp.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Streptomyces* strains shown in Table 1 were maintained and cultured as described by Hopwood et al. (13). *S. lividans* MS5 was derived by sequential deletion of *pepP1*, and *-2* (5) and *slpA* and *-C* (7). Deletion of *tap* from MS5 produced the MS7 strain (6). Deletion of *ssp* (4) then produced the MS11 strain. *Escherichia coli* strains were maintained and manipulated as described by Maniatis et al. (16). The pINT2 integration vector carries the Amp^r gene for propagation in *E. coli* and a thiostrepton resistance gene for selection in *Streptomyces* spp. The *xylE* gene is expressed from the *aph* promoter and is used as an independent reporter of stable integrative transformation into

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference(s)
Strains		
S. lividans 66		John Innes Institute[a]
MS5	Δ*pepP1* Δ*pepP2* Δ*slpA* Δ*slpC*	5, 7
MS7	MS5 Δ*tap*	6
MS11	MS7 Δ*ssp*	Unpublished data
MS20	Δ*slpE*	This study
S. alboniger 504		P. Redshaw[b]
S. ambofaciens ATCC 23877		ATCC[c]
S. coelicolor M130		John Innes Institute
S. fradiae ATCC 14544		ATCC
S. griseus ATCC 10137		ATCC
S. rimosus ATCC 10970		ATCC
S. parvulus 2238		John Innes Institute
E. coli		
HB101		Bethesda Research Laboratories
*DH5*α		Bethesda Research Laboratories
Plasmids		
pT7T318U	Amp^r	Pharmacia
pINT2	pUC-based vector for integrative transformation into *Streptomyces* (*xylE*$^+$ Amp^r, thiostrepton resistant)	7

[a] Norwich, United Kingdom.
[b] Austin College, Austin, Tex.
[c] ATCC, American Type Culture Collection.

the chromosome of *S. lividans* (Table 1) (7). Integration occurs via a single crossover event, and thiostrepton-resistant, *xylE*$^+$ colonies are allowed to sporulate in the absence of thiostrepton. To detect excision via a second crossover, colonies derived from such spores are screened for the appearance of a thiostrepton-sensitive, *xylE*-negative phenotype and then subjected to Southern hybridization to detect true deletion mutants.

DNA sequence characterization and manipulation. DNA was isolated and manipulated as described by Henderson et al. (12). DNA and protein sequence analyses were carried out with DNA STAR software (9). Restriction enzymes were supplied by New England Biolabs Inc. Transformation-competent cells of *E. coli* HB101 were supplied by Bethesda Research Laboratories. Southern hybridization analyses were carried out with DNA fragments labelled with digoxigenin (Boehringer Mannheim), and DNA was sequenced as described previously (7). Restriction enzyme site mapping was carried out on DNA isolated from *E. coli* HB101 transformants.

Protease activity and sequence analysis. The protease phenotype encoded by the purified plasmid clones was confirmed by retransformation into *S. lividans* protoplasts followed by overlaying with a solution of the chromogenic βNA peptide substrate. Aminopeptidase assays were performed as described elsewhere (5) by using βNA substrates (Bachem Inc.) for both liquid and agar plate-based assays or paranitroanilide substrates for liquid assays. Amino-terminal amino acid sequencing was performed by the Core Facility for Protein/DNA Chemistry in the Department of Biochemistry at Queen's University (Kingston, Canada) by using proteins electroblotted onto Immobilon polyvinylidene difluoride membranes (Millipore). Catechol oxygenase agar plate assays were performed as described by Ingram et al. (14).

Construction of plasmids for expression of soluble derivatives of SlpD and SlpE proteins. To adapt the N terminus of the SlpD protein, oligonucleotides encoding the 11 amino acids of SlpD immediately downstream of the putative +1 cysteine were synthesized. An *Eco*RI cloning site at the 5′ end allowed ligation of the oligonucleotides into the *Eco*RI site contained within the polylinker of a pT7T318U-based subclone (no. 4) of the *slpD* clone p5-6. This subclone also contained a *Hin*dIII site from the vector polylinker sequence located 380 nucleotides downstream of the *slpD* stop codon. The oligonucleotides also contained at the 3′ terminus a *Bam*HI site, which was ligated to a natural *Bam*HI site within the *slpD* coding sequence. An *Nsi*I cloning site designed within the oligonucleotides allowed for ligation to the *Pst*I site of the AP6.H vector and subsequent joining of the protease B signal plus the APAAPA leader directly to the SlpD gene at the +2 serine residue. The 1,920-nucleotide *Nsi*I-to-*Hin*dIII fragment from pOSE54 containing modified *slpD* was subsequently subcloned into AP6.H to produce AP6.*slp*D. The resulting DNA and amino acid sequences at the junction of the signal peptide and modified SlpD are shown in Fig. 1.

An analogous strategy was used to adapt the N terminus of the SlpE protein with oligonucleotides encoding 35 amino acids of SlpE immediately downstream of the putative +1 cysteine. A *Pst*I-compatible site located at the 5′ end allowed for ligation into the *Pst*I site located within the polylinker of a pT7T318U-based subclone (no. 5) of the *slpE* clone P8-2. The oligonucleotides also contained at the 3′ end a *Pfl*MI site for ligation to a natural *Pfl*MI site within the *slpE* coding sequence, located 100 nucleotides downstream from the codon encoding the +1 cysteine. An *Nsi*I cloning site contained within the oligonucleotides allowed for the subsequent ligation in the correct reading frame into the *Pst*I site of AP6.H, effectively fusing the protease B signal peptide plus the APAAPA hexapeptide leader directly to SlpE at the +2 serine residue. A *Sac*I site located 238 nucleotides downstream of the *slpE* stop codon was used in conjunction with a *Hin*dIII-*Sac*I 8-mer adapter (AGCTAGCT) to join the 3′ end of the *slpE* clone to the *Hin*dIII site in the AP6.H expression plasmid. The 1,820-nucleotide *Nsi*I-to-*Sac*I fragment encoding modified SlpE was then used along with the *Hin*dIII-*Sac*I adapter in a three-way ligation into AP6.H to produce AP6.*slp*E. The resulting DNA and amino acid sequences at the junction of the signal peptide and modified SlpE are also shown in Fig. 1.

Nucleotide sequence accession numbers. The nucleotide sequences of *slpD* and *slpE* have been submitted to GenBank under accession numbers L42758 and L42759, respectively.

RESULTS

Screening genomic libraries with APA-βNA. Pooled DNA isolated from a genomic library (7) prepared in a bifunctional plasmid vector from *E. coli* HB101 cells was used to transform protoplasts of the *S. lividans* MS7 strain. Thiostrepton-resistant transformants were screened for the ability to hydrolyze APA-βNA. From 13,000 transformants 11 strongly positive colonies were isolated. Four of these were reisolates of the *tap* locus. Four other isolates showed similarity by restriction enzyme site mapping. Three of these were indistinguishable, and, therefore, only one (P5-6) was further studied. The fourth clone (P5-17) was shown to represent an overlapping DNA fragment. Southern hybridization experiments (data not shown)

AP6 *slp*D ... GCG CCT GCA TCG GCC GGG ...
... ala pro ala ser ala gly ...

AP6 *slp*E ... GCG CCT GCA TCG GGC GGC ...
... ala pro ala ser gly gly ...

FIG. 1. DNA and amino acid sequences at the junction of *slpD* or *slpE* and the modified *prtB* signal peptide.

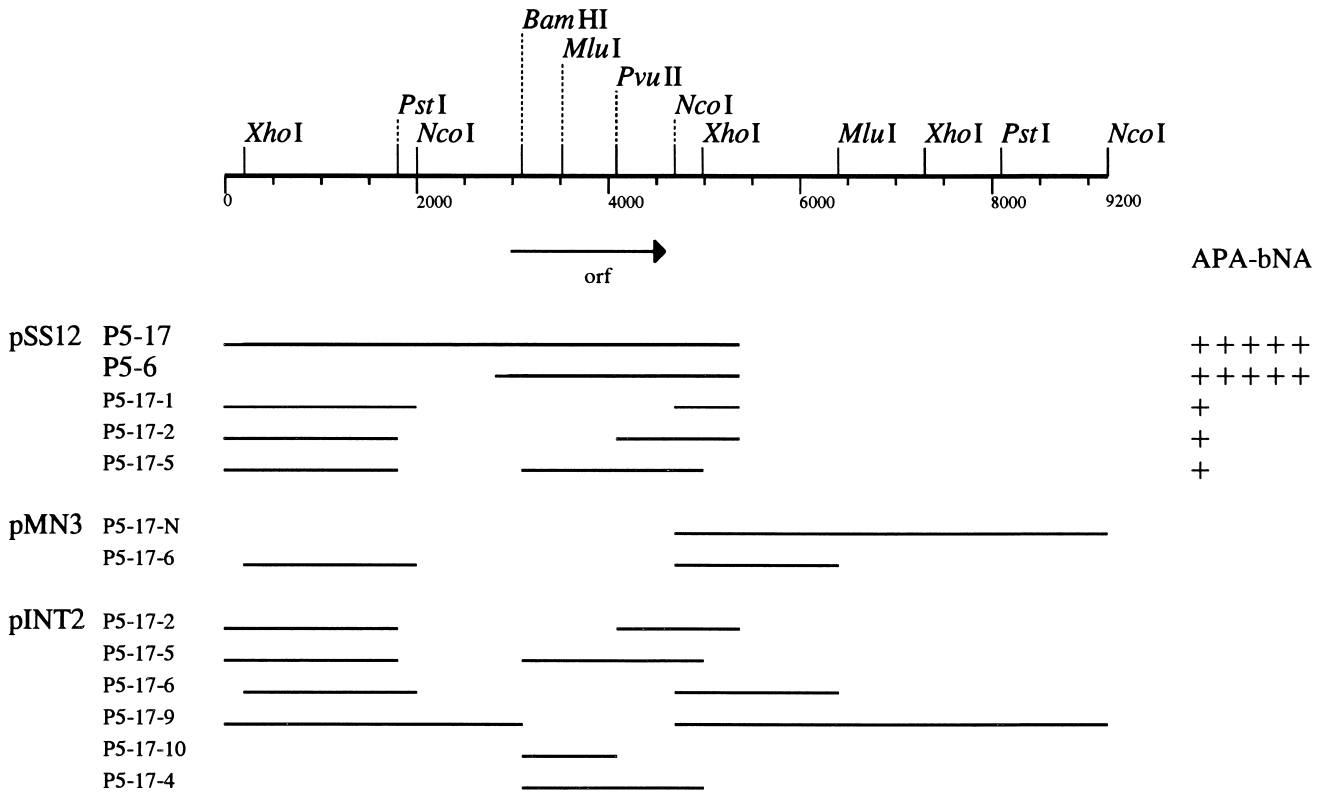

FIG. 2. Restriction map of the *slpD* locus and plasmid derivatives. The extent of substrate hydrolysis was assessed quantitatively by eye (+++++, high; +, host background level). pMN3 is a derivative of pT7T318 modified by linker addition to include *Mlu*I and *Nco*I restriction sites. The size and orientation of the *slpD* ORF are indicated.

established that these four isolates represented the same chromosomal locus. The remaining three positive colonies were subsequently shown to encode two other secreted aminopeptidase activities including Ssp (4). The restriction enzyme site maps for clones P5-6 and P5-17 are shown in Fig. 2. P5-17 was used to make deletions within the cloned DNA. The ability of the deleted plasmids to direct hydrolysis of APA-βNA was found to be abolished (i.e., no greater than the background from vector-only controls) in the agar plate assay with transformants of *S. lividans* MS7. The lack of activity from pSS12 deletion clones 1 and 2 suggested that the active region of DNA was at the left-hand end of the P5-6 DNA (as shown in Fig. 2). The lack of activity from pSS12 deletion clone 5 demonstrated that removal of the leftmost DNA fragment of P5-6 up to the *Bam*HI site also led to inactivation of the plasmid-encoded phenotype.

Although the P5-6 and P5-17 clones encoded significant hydrolytic capability against the APA-βNA substrate in the agar plate assay, no activity above background was observed in cell-free broth derived from cultures containing these plasmids grown in liquid tryptic soy broth media. Neither was it possible to experimentally identify the protein product of this locus. For cultures in liquid medium similar in composition to the agar medium (i.e., R2 without added phosphate or agar and containing 0.25% yeast extract instead of the usual 0.5%) APA-βNA-degrading activity was observed in the cell-free broth. However, in contrast to the previously described Tap protein, this activity was unable to hydrolyze GPL-βNA. Furthermore, no significant protein bands were detectable by silver staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins secreted into the modified R2 liquid medium by the *S. lividans* MS7 host strain carrying the cloned DNA (not shown).

*Sau*3A-generated DNA fragments from P5-6 were, therefore, subcloned into pT7T3 vectors, and the nucleotide sequence (Fig. 3) was determined. An open reading frame (ORF) which would encode a predicted protein of 539 amino acid residues was observed. There is no obviously visible conventional signal peptide sequence at the amino terminus of the predicted protein. However, there is a sequence which matches well the signal peptidase II consensus sequence, specifically, the cysteine at position 31 followed by serine at position 32. Consistent with the observations of von Heijne (18), the residues around the cysteine are TACSAGGAS. The glycine residues are consistent with those in other lipoprotein signal peptides in which turn-promoting residues occur in positions +2 to +6 (with respect to the cleavage site), whereas in nonlipoprotein signal peptides they tend to occur at positions −6 to −4. As noted for other signal peptides of gram-positive origin, the N-terminal region of the putative signal peptide is highly positively charged with seven arginine residues (and one aspartate residue). Overall, these observations are consistent with the presumed membrane-bound location of the SlpD protein.

Screening genomic Libraries with a substrate for endoproteolytic activity. Another chromogenic substrate was designed to model a sequence (NH_2-APARSPS. . .) in the amino-terminal region of human granulocyte-macrophage colony-stimulating factor which was known to be subjected to proteolytic degradation under certain conditions when expressed by *S. lividans* (unpublished observation). An endoprotease which cleaves granulocyte-macrophage colony-stimulating factor between the Arg and Ser residues to generate a −4 form has

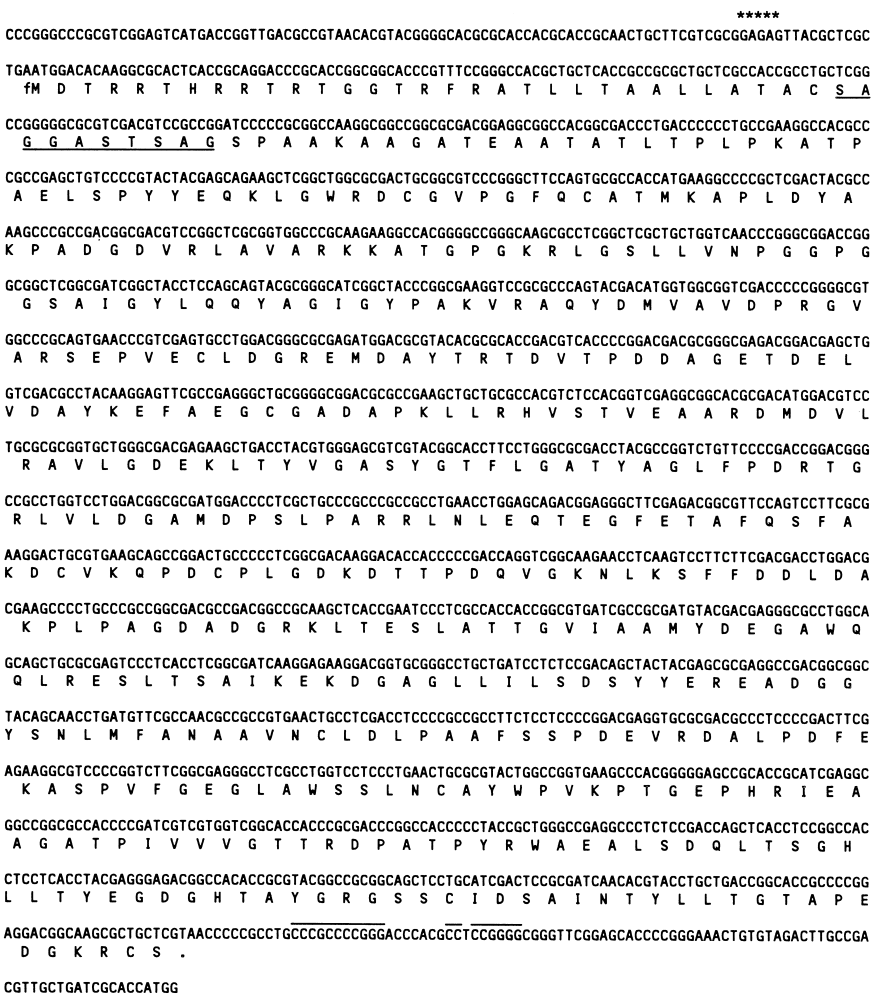

FIG. 3. Nucleotide and predicted amino acid sequences of *slpD*. The DNA sequence of the nontranscribed strand between an upstream *Sma*I site and a downstream *Nco*I site is shown. A sequence likely to form a strong ribosome binding site in *slpD* mRNA is indicated by asterisks. Underlined are the first 10 amino acids determined for the soluble SlpD derivative described in the text. A putative transcription termination region is indicated just downstream of the translation stop codon.

been identified for *S. lividans* (1a). In designing a chromogenic substrate, the amino-terminal residue was modified by the addition of a Boc group, such that proteases whose activity requires a free NH_2 group would be unable to act directly on this substrate. However, the −4 endoprotease present in the *S. lividans* host having a recognition sequence compatible with that of the substrate (specifically Boc-APARSPA-βNA) would be able to cleave and remove the Boc-linked tetrapeptide from the tripeptide-linked βNA. The later tripeptide, with a free NH_2 terminus, provides a substrate for the native Tap protease to release the chromogenic βNA moiety, which could subsequently be visualized by reaction with Fast Garnet GBC dye.

The above strategy was used to screen the *S. lividans* 66 genomic DNA library after transformation into the MS5 host strain (Tap^+). After screening of 8,000 colonies, six clones which demonstrated the ability to degrade the substrate significantly faster than the host strain alone were isolated. Surprisingly, two clones proved in restriction enzyme site analysis to be identical to P5-6, described above, and another clone was similarly shown to be the same as P5-17. Three novel clones (P8-1, -2, and -3) were isolated and shown to represent the same region of chromosomal DNA by Southern hybridization experiments. The restriction enzyme site maps are presented in Fig. 4. P8-3 contained a larger DNA fragment which was probably derived from the cocloning of noncontiguous *Sau*3A fragments in the construction of the library. P8-1 contained an inserted DNA fragment of approximately 8 kbp, while P8-2 had a smaller insert (3.6 kbp). No secreted hydrolytic activities or novel protein could be observed in liquid cultures of strains carrying these cloned DNA sequences, even when modified liquid R2 medium was used. However, the agar plate activities seen were significant; therefore, deletion mapping and DNA sequence analysis were carried out essentially as for P5-6, revealing a potential protein-coding region in the central part of the P8-2 insert (Fig. 5). Inspection of the predicted N-terminal amino acid sequence revealed another putative lipoprotein signal peptide. In this case there were fewer positively charged residues but the AGCSGGSS sequence showed a striking clustering of turn-promoting residues immediately after the cysteine, suggesting that the predicted protein may be associated with the mycelial membrane or cell wall. Comparison of the predicted protein sequence (SlpE) derived from the DNA sequence (Fig. 6) with those encoded by the *tap* and *slpD* genes showed a significant homology (35% identity) between the SlpD and SlpE proteins. Smaller but still significant levels of homology were detectable between either SlpD (25%) or SlpE

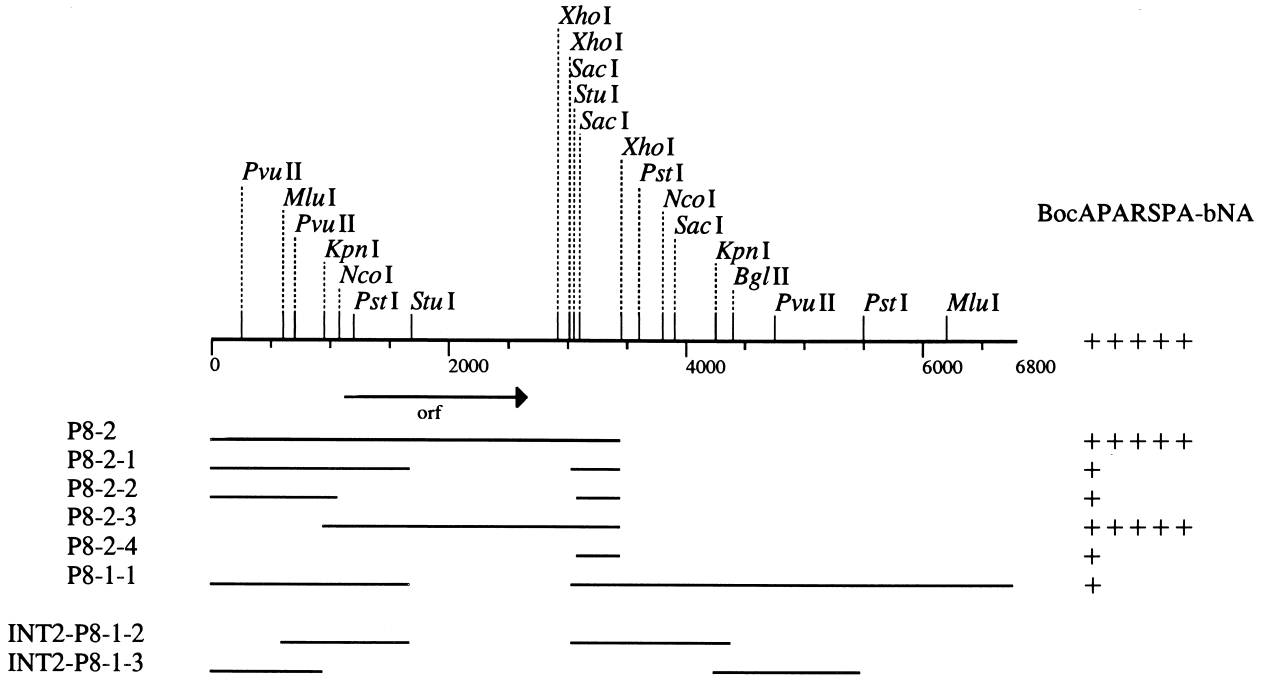

FIG. 4. Restriction map of the *slpE* locus and plasmid derivatives. Substrate activities of subclones are indicated as in Fig. 2.

(23%) and the Tap protein. The apparent conservation of amino acid sequences around the putative active-site serine residues of these proteins is consistent with their similarity to members of the lipase family of hydrolytic enzymes which are characterized by the conserved GXSXG motif (2).

Soluble secreted forms of the SlpD and SlpE proteins. In order to allow biochemical purification of the predicted proteins from culture supernatants to examine their hydrolytic capabilities (and attempt to confirm that the predicted proteins are directly responsible for these activities), the nucleotides encoding both the putative promoter/leader mRNA region and the lipoprotein signal peptide including the +1 cysteine were replaced by sequences encoding the aminoglycoside phosphotransferase (*aph*) promoter and the protease B signal peptide (12). Also, in an attempt to improve secretion efficiency, a small leader peptide (APAAPA) was added to the C terminus of the protease B signal peptide upstream of the sequences coding for the SlpD and SlpE proteins. This was accomplished by the use of oligonucleotides to adapt the encoded protease B signal peptide at its C-terminal coding region with the leader and a cloning site and to adapt the *slpD* and *slpE* genes at their 5′ ends with appropriate cloning sites.

When these plasmids were used to transform protoplasts of *S. lividans* MS11, secreted proteins for both AP6.*slp*D and AP6.*slp*E were observed at approximate molecular sizes of 55 kDa (Fig. 7). Direct automated N-terminal Edman degradation analysis of the secreted proteins produced amino acid sequences SAGGASTXAG and APAAPASGGSSDEDK for SlpD and SlpE, respectively. For SlpD, culture supernatants showed a dramatic increase in the ability to hydrolyze APA-βNA. This correlates with the N-terminal sequence data on soluble SlpD which show that it is lacking the leader peptide APAAPA, which may have been cleaved because of autocatalytic activity of the modified SlpD itself. In contrast, soluble SlpE culture supernatants showed no ability to hydrolyze Boc-APARSPA-βNA or APA-βNa, in agreement with the presence of an intact P6 leader at the N terminus of the secreted protein.

Deletion of *slpD* and *slpE* from the *S. lividans* chromosome. Several attempts were made to inactivate the *slpD* gene in *S. lividans* 66 by recombinational deletion using four different pairs of restriction fragments flanking the *slpD* ORF (Fig. 1) to direct integration and excision of an integration vector, pINT2 (7), containing paired sets. Assuming that during integration and excision the likelihood of two crossover events occurring in the same fragment of each pair is 50%, approximately half of the thiostrepton-sensitive, *xylE*-negative transformants should be of the parental type and half should exhibit a mutant phenotype. However, although integration occurred via recombination at a high frequency in all cases as judged by the appearance of thiostrepton-resistant, *xylE*$^+$ transformants, the excision event took place via a crossover event only within the same fragment, such that the only thiostrepton-sensitive, *xylE*-negative isolates obtained were of the parental type, as judged by Southern hybridization using colony blots or genomic DNA prepared from these isolates (data not shown). The observation that SlpD can be overexpressed from the P5-6 clone alone and not from other deletion derivatives of p5-17 (Fig. 2) suggests that *slpD* is transcribed as an independent unit. It appears likely, therefore, that deletion of *slpD* does not induce a deleterious effect on the expression of a downstream gene of unknown function.

In order to test whether inactivation of the *slpD* gene was a lethal event, an insertional mutagenesis strategy in which a 1.1-kb *Bam*HI-*Pvu*II restriction fragment internal to the *slpD* ORF was subcloned into the pINT2 vector (p5-17-10) to direct integration via a single crossover event was used. If inactivation of *slpD* is lethal, it should not be possible to obtain thiostrepton-resistant *xylE*$^+$ transformants from such an integration, whereas insertion via a 2.0-kb restriction fragment (*Bam*HI-*Nco*I) in pINT2/p5-17-4 only partially overlapping the *slpD* ORF should restore one full copy of the *slpD* gene, giving rise

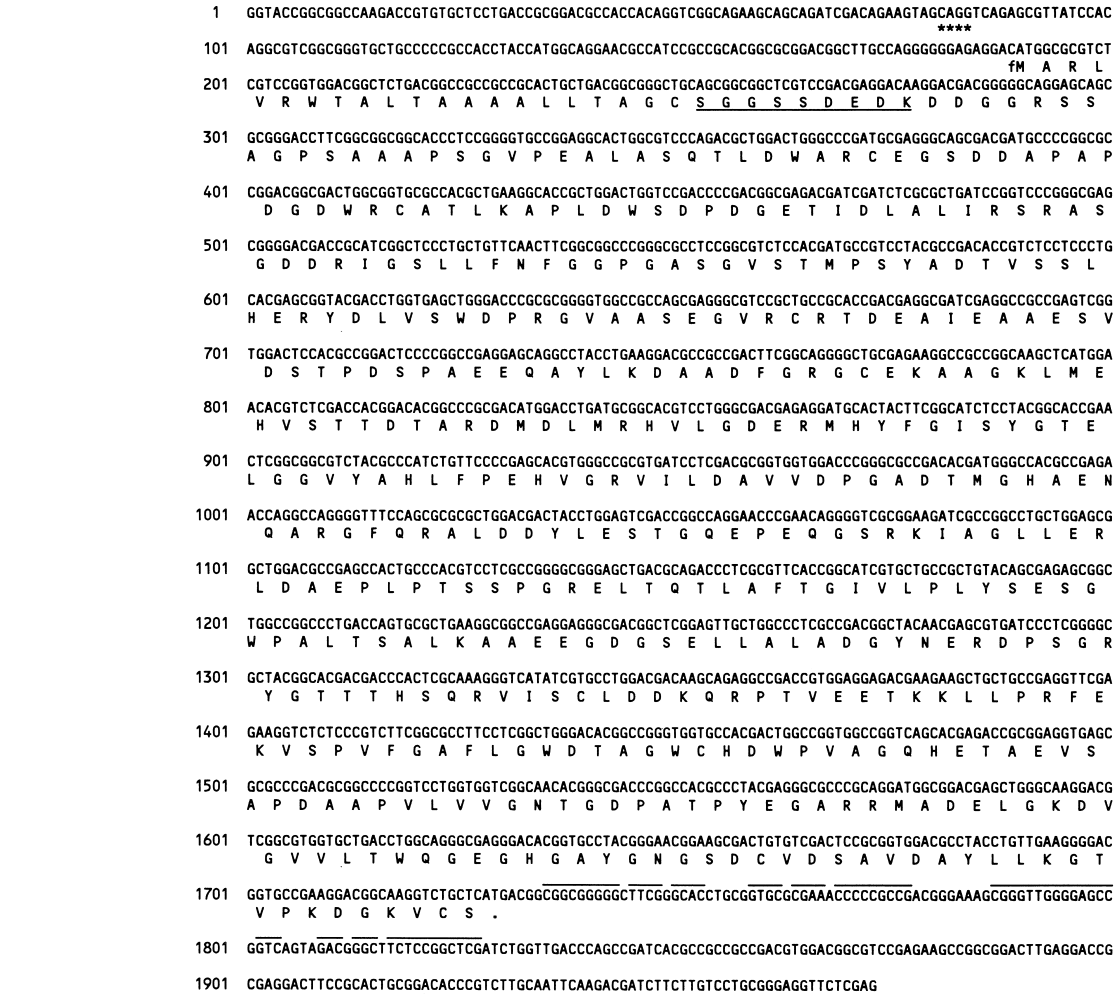

FIG. 5. Nucleotide and predicted amino acid sequences of *slpE*. The DNA sequence of the nontranscribed strand between an upstream *Kpn*I site and a downstream *Xho*I site is shown. Other sequences are indicated as in Fig. 3. A second putative transcription termination region which is probably related to a downstream convergent ORF (not shown) is shown.

to thiostrepton-resistant, *xylE*$^+$ transformants. The results of a transformation experiment involving these two integration plasmids (8 and 1 *S. lividans* 66 and 19 and 0 *S. lividans* MS11 thiostrepton-resistant, *xylE*$^+$ transformants for pINT2/p5-17-4 and pINT2/p5-17-10, respectively) appeared to verify that viable *xylE*$^+$ transformants could be obtained only when integration resulted in restoration of an uninterrupted copy of *slpD*, implying that its inactivation is lethal. Southern analysis of chromosomal DNA prepared from a sample of the integrants obtained in this experiment indicated that site-specific recombination of the entire integration plasmids occurred only for pINT2/p5-17-4 (not shown). One single thiostrepton-resistant, *xylE*$^+$ transformant was obtained after transformation with pINT2/p5-17-10, but integration appeared to occur at a locus unlinked to *slpD* (not shown).

In contrast to *slpD*, recombinational deletion of *slpE* was successful with *S. lividans* 66 as a parental strain, giving rise to strain MS20. In this case a 1.5-kb *Stu*I fragment was effectively deleted from the chromosome by using the integration plasmid pINT2/p8-1-2 (Fig. 4). However, *slpE* deletion strains, although viable, grow slowly on complex media and failed to grow on minimal medium (containing glucose, NH_4^+ salts) supplemented with all 20 natural amino acids or with alternative carbon sources such as sucrose, arabinose, glycerol, or starch. Restoration of growth on minimal medium could be obtained only when a complex nitrogen source such as yeast extract was added. The mutant phenotype appeared to be a result of inactivation of *slpE*, as growth of *S. lividans* MS20 on minimal medium could also be restored by transformation with plasmids p8-1 and p8-2 but not by transformation with p8-1-1 or a vector control (Fig. 4).

Homologs of *slpD* and *slpE* are present in other *Streptomyces* species. Cross-species hybridization experiments were performed at high stringency by using digoxigenin-labelled probes and chromosomal DNA prepared from a variety of *Streptomyces* species, including *S. coelicolor* A3(2), *S. alboniger*, *S. ambofaciens*, *S. fradiae*, *S. griseus*, *S. parvulus*, and *S. rimosus* (not shown). In all cases specific hybridization signals were obtained with the 2.25-kb *Kpn*I-*Sac*I fragment of p8-2 (*slpE*) as a probe. The strongest signal was obtained for *S. coelicolor* DNA, and the weakest signal was observed with *S. rimosus* DNA. A similar experiment was performed with the 2.0-kb *Bam*HI-*Xho*I fragment of p5-17 (*slpD*) as a probe. Again, all species exhibited homology, with *S. coelicolor* DNA giving the strongest signal and, in this case, *S. fradiae* giving the weakest. Both hybridizations also included digests of the cloned *tap*, *slpD*, and

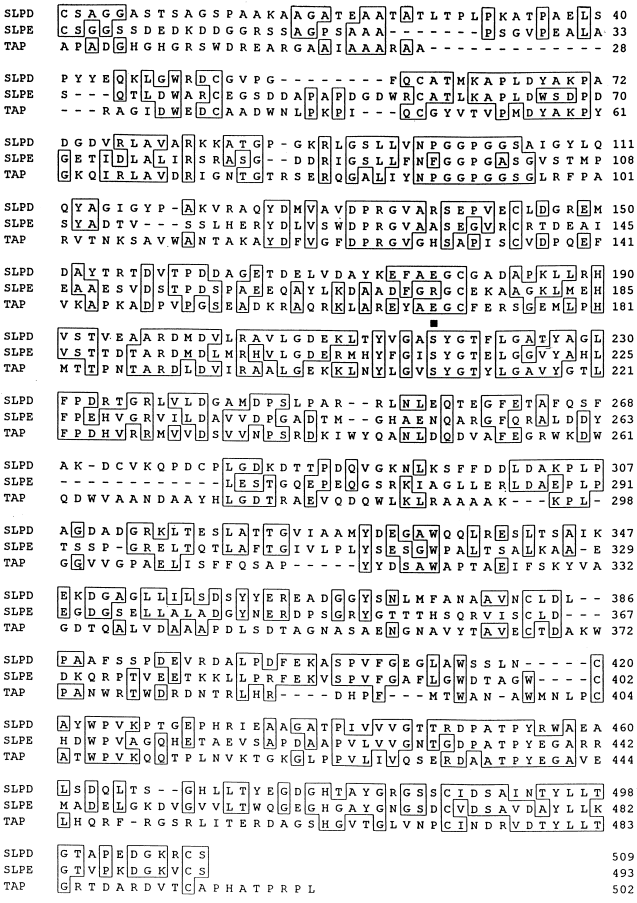

FIG. 6. Amino acid sequence alignment of SlpD and SlpE with Tap. A putative active-site Ser residue is indicated (■). Putative signal peptide sequences are included in this comparison.

slpE genes, and the results appeared to indicate weak but significant homology between *tap* and *slpE* and between *slpD* and *slpE* but not between *tap* and *slpD.*

DISCUSSION

The two genes described in this report were isolated by the strategy of modelling synthetic peptide substrates on the basis of the amino-terminal sequences of proteins known to be processed after secretion in *S. lividans*. As expected, this approach had previously uncovered genes encoding extracellular aminopeptidases which contribute to such processing. The additional discovery of genes encoding cell membrane-associated proteinases by this approach was unexpected and may indicate that other unidentified activities could be uncovered by extension of the approach. It is of interest to note that amino-terminal processing of the extracellular serine protease inhibitor native to *S. lividans* still occurs in the absence of Tap and SlpE, suggesting the existence of as yet undiscovered activities in this species.

Another unexpected result of this work was that use of the NH_2-terminally blocked heptapeptide substrate failed to isolate a clone expressing endoprotease activity. In addition, no clones representing the previously identified *tap* gene were obtained, suggesting that the route to chromophore release from this substrate by virtue of cloning of the *slpE* gene in *S. lividans* occurred in an unexpected manner. It is also of interest to note that the *slpE* gene was independently isolated during the course of this work in a screen using a similarly blocked substrate (Boc-VRSSSRA-βNA) modelled on but not identical to the amino-terminal sequence of human tumor necrosis factor alpha, which is also cleaved to a −4 form after secretion in *S. lividans* (8). As the sequences of the two blocked synthetic substrates are so dissimilar, it is not possible to identify by inspection the likely cleavage site for SlpE, and further work would be required to identify its site of action.

The genes described in this report encode related but different protease capabilities of *S. lividans*. It is not clear why the organism should require so many active extracellular peptidases or what is the functional role of the cell-bound enzymes, although it might be argued that the possession of enzymes of overlapping specificity might confer a selective survival advantage, particularly in the relatively competitive natural soil environment, where both chemical inhibitors and mutagenic events are likely to be encountered. It is clear from the results of this work that these two cell-associated proteases are important for normal growth, unlike the true extracellular aminopeptidases, of which Tap is an example, which appear to be dispensable under normal laboratory conditions. The role of cell membrane-associated proteases in streptomycetes is unclear, but other gram-positive bacteria, such as *Lactococcus lactis*, harbor enzymes which are involved in casein degradation but which appear to be initially anchored to the cell membrane via a C-terminal hydrophobic domain (19), in contrast to the amino-terminal association likely to be involved in anchoring SlpD and SlpE to the cell membrane in *Streptomyces* spp. *L. lactis* contains another cell-associated lipoprotein, PrtM, which may be involved in activating the other caseinolytic enzymes by facilitating the autocatalytic removal of a pro- region from inactive precursor forms (11). It is possible that *Streptomyces* SlpD and/or SlpE could fulfil such a role, especially in those species which possess many hydrolytic enzymes, such as *S. griseus*. However, the deleterious effect of inactivating SlpD or SlpE in *S. lividans* would appear to suggest alternative roles for these proteinases during the process of vegetative growth, possibly indirect involvement in amino acid or oligopeptide trans-

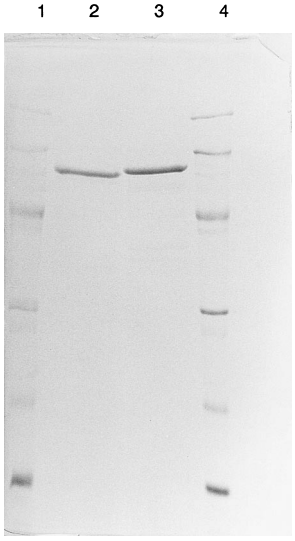

FIG. 7. SDS-PAGE analysis of cell-free broth from *S. lividans* transformed with the AP6.*slp*D and AP6.*slp*E plasmids. One milliliter of trichloroacetic acid-precipitated material was loaded in each case. Lanes 1 and 4, molecular weight markers; lane 2, modified SlpD; lane 3, modified SlpE.

port. It is difficult to envisage a direct role for a proteinase in such a system. However, the production of soluble forms of these proteases should facilitate further characterization and ultimately lead to new insights into their normal physiological role(s).

ACKNOWLEDGMENTS

We thank D. Bartfeld and T. Krieger for communication of unpublished results and L. Jamieson for typing the manuscript.

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