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Cloning and Characterization of a Gene Encoding a Secreted Tripeptidyl Aminopeptidase from *Streptomyces lividans* 66

MICHAEL J. BUTLER,† CRAIG BINNIE, MICHELE A. DIZONNO, PHYLLIS KRYGSMAN, GLENN A. SOLTES, GISELA SOOSTMEYER, EVA WALCZYK, AND LAWRENCE T. MALEK*

Cangene Corporation, Mississauga, Ontario, Canada L4V 1J7

Received 16 December 1994/Accepted 17 May 1995

The gene encoding a tripeptidyl aminopeptidase (Tap) from *Streptomyces lividans* was cloned by using a simple agar plate activity assay. Overexpression of the cloned gene results in the production of a secreted protein which has an apparent subunit molecular weight of 55,000 and is responsible for the major amino-terminal degradative activity in culture broths of *S. lividans* strains. A DNA sequence analysis revealed a potential protein-encoding region of the size expected to encode the observed protein, which contained a sequence that exhibited significant homology around a putative active site serine residue observed for lipases, esterases, and acyl transferases. Preceding the amino terminus of the secreted protein was a predicted signal peptide of 36 amino acids followed by a tripeptide, which could be autocatalytically removed from a secreted Tap precursor. The transcriptional start site for the gene was mapped by primer extension. Mutant strains of *S. lividans* lacking detectable Tap activity were able to grow and sporulate normally. Cross-species hybridization experiments showed that DNA homologs of the *tap* gene are present in most of the *Streptomyces* strains tested.

Streptomyces species have been extensively used for large-scale production of fine chemicals and antibiotics by fermentation. These bacteria are gram-positive organisms which are well suited to production of therapeutic (recombinant) proteins in which the proteins are directly secreted into the culture medium (3, 4, 6, 21). This results in a facile product purification procedure and, furthermore, yields proteins that are soluble and biologically active. However, the natural ability of *Streptomyces* strains to secrete extracellular enzymes derived from their heterotrophic existence is likely to limit the commercial exploitation of heterologous protein secretion by these microbes.

A number of endoproteases and exoproteases have been characterized from *Streptomyces lividans* TK24 (3), *S. lividans* 66 (7–10), *Streptomyces coelicolor* "Müller" (12), and *Streptomyces* sp. strain C5 (20). A neutral protease gene has been cloned from *Streptomyces cacaoi* (11) and has been shown to encode a protein that is distinctly different from the thermolysin type of enzymes. Leucine aminopeptidase activity has been observed in *S. lividans* strains (2, 3, 7). Strickler et al. (24) noted that proteins secreted by *S. lividans* strains are often found to have undergone proteolytic processing at the amino terminus. Krieger et al. (18) recently reported the isolation and characterization of a major tripeptidyl aminopeptidase (Tap) from extracellular extracts of *S. lividans* 66. In this paper we describe the isolation and characterization of the gene encoding this enzyme.

Many of the heterologous proteins which we expressed in *S. lividans* possessed an amino-terminal alanine residue followed by a proline residue; hence, processive degradation from the NH_2 terminus required the participation of either an aminopeptidase P (to cleave the Ala-Pro bond) or a dipeptidyl aminopeptidase to remove an Ala-Pro dipeptide. However, although an aminopeptidase P activity was observed, this activity was found to be located intracellularly (9). We screened other available related chromogenic peptides (Bachem) by using colonies on agar plates and discovered, to our surprise, that the tripeptide substrate Gly-Pro-Leu-β-naphthylamide (Gly-Pro-Leu-βNA) was hydrolyzed (by a cell-free culture broth from a *S. lividans* culture) at a substantial rate (approximately 4.4 nmol min^{-1} ml^{-1}), whereas most of the mono- and diamino acid substrates were not hydrolyzed significantly; the only exception to this was Leu-paranitroanilide (rate of hydrolysis, ca. 0.7 nmol min^{-1} ml^{-1}). Therefore, we used Gly-Pro-Leu-βNA as a substrate in an agar plate assay (9) to screen a previously described genomic plasmid library of *S. lividans* DNA (10) for overproduction of the ability to hydrolyze this tripeptide substrate. We isolated two clones (clones 3 and 13) which reproducibly produced dark red colonies when they were assayed with Gly-Pro-Leu-βNA. The positive colonies were picked from the agar plates, crushed, and used to inoculate 1-ml tryptic soy broth (TSB) medium cultures in 2.5-ml screw-cap tubes. After incubation with shaking at 30°C for 2 to 3 days, the mycelium was harvested and used to isolate plasmid DNA by the method of Hopwood et al. (15). When this DNA was used to retransform protoplasts of *S. lividans* 66, all of the subsequent transformant colonies exhibited the same strong red color when they were assayed with Gly-Pro-Leu-βNA. A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (19) of the cell-free broth harvested from a liquid culture containing transformant colonies revealed a major protein band at an apparent molecular weight of 55,000 (data not shown). On the basis of Coomassie blue staining we estimated that the protein was present at a concentration of 100 μg/ml in the broth. Preparative SDS-PAGE followed by electrotransfer to a polyvinylidene difluoride (Immobilon) membrane gave sufficiently pure material to allow direct N-terminal Edman degradation to be performed. The amino acid

* Corresponding author. Mailing address: Cangene Corporation, 6280 Northwest Drive, Mississauga, Ontario, Canada, L4V 1J7. Phone: (905) 673-0200. Fax: (905) 673-0367.

† Present address: Strangeways Research Laboratory, Cambridge, CB1 4RN, England.

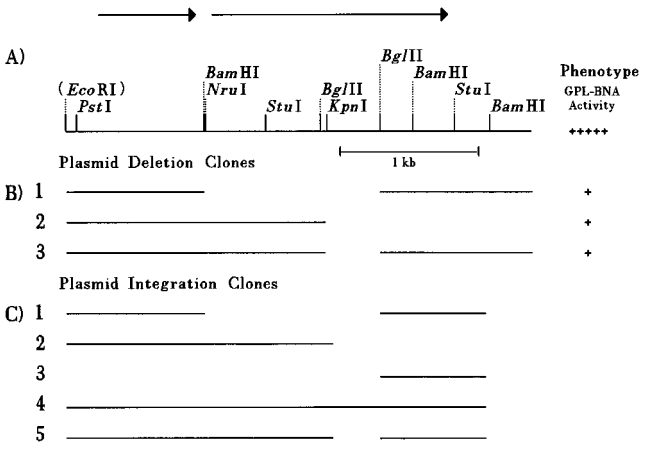

FIG. 1. Restriction enzyme site map of cloned *tap* DNA. (A) The arrows indicate the locations and directions of potential protein-encoding regions, and the larger arrow represents the *tap* gene. The phenotypes as determined by the Gly-Pro-Leu-βNA (GPL-βNA) hydrolysis agar plate assay are shown qualitatively by the number of plus signs indicating the red color that developed on the colonies. The *Eco*RI site shown in parentheses is present in the pSS12 vector adjacent to the *Bam*HI cloning site. (B) None of the three deletion clones shown produced any more red color in its colonies than the pSS12 control plasmid did, and these clones were scored + because of the background level of hydrolysis from the chromosomally encoded *tap* gene in the *S. lividans* 66 host. (C) DNA fragments subcloned into the integration plasmid and used to transform protoplasts of *S. lividans* 66 to thiostrepton resistance.

sequence observed was DGHGHGRSWDR. The cell-free broth exhibited very high activity against the Gly-Pro-Leu-βNA substrate (87 nmol min^{-1} ml^{-1}), whereas the activities against the mono- and dipeptide substrates and the NH_2-terminally blocked substrates (data not shown) were not significantly different from the activities observed in the *S. lividans* control strains (Table 1), indicating that Tap is a true aminopeptidase. Most other tripeptide β-naphthylamide substrates were readily hydrolyzed; two exceptions were Ala-Phe-Pro-β-naphthylamide and H-D-Phe-Pro-Arg-β-naphthylamide.

Plasmid DNAs isolated from the positive clones were used to transform *Escherichia coli* HB101 to take advantage of the fact that it is faster and easier to purify bifunctional plasmid DNA from *E. coli*. Plasmid DNAs were isolated as described previously (22) from the *E. coli* transformants and were characterized by standard methods (14) in order to construct the restriction enzyme site map shown in Fig. 1A. Clones 3 and 13 appeared to contain identical DNA fragments. Deletions were made within the inserted DNA region as indicated in Fig. 1B. All three deletions resulted in the loss of the plasmid-encoded hydrolytic phenotype (scored qualitatively visually on the basis of the intensity of the red color produced in colonies in the agar plate assay; i.e., 5+ for the fragment carrying the whole cloned gene and 1+ for the background activity observed with colonies transformed with bifunctional cloning vector pSS12 and with each of the three plasmid deletion clones).

The nucleotide sequence of the *tap* gene was determined by using DNASTAR (13) and is shown in Fig. 2. Inspection of the sequence revealed a potential protein-encoding region of 1,612 nucleotides which was consistent with the molecular weight of the observed secreted protein (55,000). The putative translational initiation codon was a TTG which was preceded by a potential ribosome binding site (GGAGG). The deduced amino acid sequence contained a predicted signal peptide (28) which could be cleaved between residues 36 and 37 (ASA ↓ A). The experimentally determined amino acid sequence of the mature secreted protein was identical to the sequence encoded by residues 41 to 51 of the predicted protein sequence. This suggests that the protein is secreted as a proprotein with a three-amino-acid pro region (APA). Since the enzyme removes such tripeptides from polypeptides, it is highly likely that the removal of this APA is autocatalytic (although this was not experimentally tested).

When the deduced amino acid sequence was compared with sequences in the protein databases by using the program BLAST (1), the closest match was with sequence of the 2-hydroxy-6-oxo-2,4-heptadienoate hydrolase from *Pseudomonas putida* F1 (23). The weak homology included the GVSYG sequence which includes the serine residue potentially involved in the catalytic action of this protease. Additional amino acid sequence comparisons (data not shown) also revealed homology with the lipase-esterase-acyl transferase family of hydrolytic enzymes, in which active site serine residues occur in the motif described by Blow (5). This contrasts with the human tripeptidyl peptidase II characterized by Tomkinson and Jonsson (26), which exhibits significant homology to the bacterial serine protease subtilisin. More detailed biochemical characterization of the Tap from *S. lividans* 66 (18) confirmed that this enzyme is a serine protease since it is inhibited by phenylmethylsulfonyl fluoride but not by chelating agents; however, it is significantly different from subtilisin and appears to represent a novel class of bacterial extracellular exoproteases.

The coding sequence is flanked by two sequences, each of which contains a substantial inverted repeat (following the rules of Tinoco et al. [25]), suggesting that the *tap* gene is transcribed independently of other adjacent DNA sequences. To map the 5′ end of the *tap* mRNA, total RNAs were isolated from strains MS2 and MS7 containing pSS12 and strain MS7 containing the cloned *tap* DNA, as described by Hopwood et al. (15), by using strains that had been grown for 24 h at 32°C in shake flasks containing 100 ml of TSB, 2% glucose, and 10 μg of thiostrepton (kindly supplied by E. R. Squibb & Sons) per ml. The *tap* gene and the upstream DNA sequence were examined for secondary structures by using the PRIMER program (S & E Software). A 40-base primer (5′CGATCAGCGT GGCGGTGACCAGTGCTCCGGCCGTGCCGAA) was designed to hybridize to an RNA sequence in the predicted Tap coding region (between nucleotides 568 and 608 in Fig. 2). The primer was tested in standard double-stranded DNA sequencing reactions (by using deaza-GTP and CTP) to confirm its specificity and suitability for the primer extension analysis. Primer annealing and extension reactions were carried out as described previously (9) by Triezenberg (27) by using 50 μg of total RNA, 0.5 pmol of end-labelled primer (2×10^6 cpm), and 40 U of avian myeloblastosis virus reverse transcriptase (Seikagaku). Extension reactions were terminated with EDTA (final concentration, 20 mM), and the mixtures were incubated at 37°C for 30 min with RNase A (40 μg ml^{-1}) to prevent subsequent distortion of the sequencing gel. The primer extension reactions resulted in one major extended product in overproducing strain MS7 carrying the cloned *tap* gene (Fig. 3). A consistent result was obtained when we used a second, nonoverlapping primer (data not shown). By comparing the extended product with its sequencing ladder we identified the apparent transcription start site of the *tap* gene. The presence of a weak but complete primer extension product in RNA isolated from control strain MS7 (Fig. 3, lane 1) indicated that chromosomally expressed *tap* mRNA was still intact when a 0.3-kb in-frame deletion was generated downstream of the primer binding site. A product of the same size was generated from mRNA from a nondeleted strain; this product is not visible in Fig. 3 but was observed after prolonged autoradiographic exposure or in an independent experiment (data not

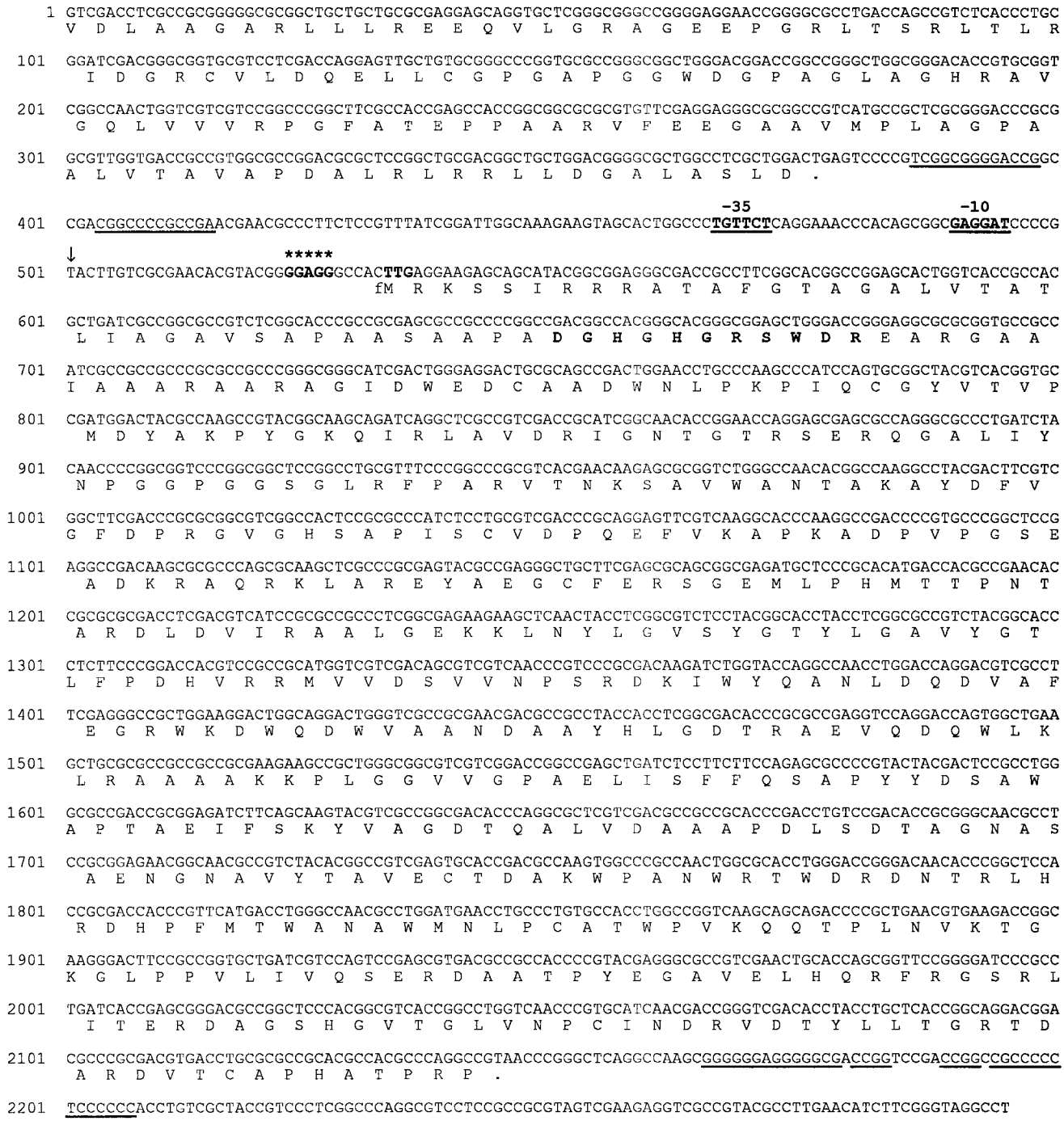

FIG. 2. Nucleotide sequence of the *S. lividans tap* gene. The deduced amino acid sequence is shown, with the first nucleotide of each codon aligned with the codon's amino acid. Inverted repeat sequences which may represent transcriptional terminators are underlined, as are the putative −35 and −10 nucleotide sequences upstream of the *tap* transcriptional start site (arrow). A putative ribosome binding site is indicated by asterisks. The translational termination codon is indicated by a dot. Amino acid residues which were determined experimentally by direct N-terminal sequencing of the mature protein are shown in boldface type.

shown). Thus, the same base (T) was identified as the transcription start site by using mRNA from cells expressing a chromosomal and/or plasmid-borne *tap* gene. A potential −10 sequence GAGGAT and a −35 sequence TGTTCT were tentatively identified. The *tap* −10 sequence is identical to the sequence of p_2 of the *ermE* promoter (17) and is in the same position with respect to the transcription start site.

The *tap* gene appears to be present in many *Streptomyces* species, since chromogenic agar plate assays performed with colonies of a variety of other *Streptomyces* species all resulted in significant levels of red color formation with the Gly-Pro-Leu-βNA substrate (data not shown). Furthermore, in Southern hybridization experiments in which we used genomic DNAs from a number of strains and the cloned *tap* gene as a probe we observed strongly hybridizing bands in all cases except *Streptomyces fradiae* (Fig. 4). The washes used were low-

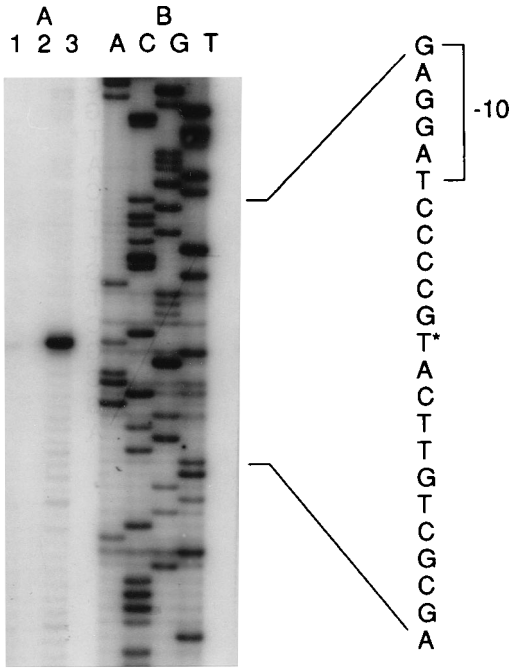

FIG. 3. (A) Mapping the 5′ end of *tap* by primer extension analysis. A primer complementary to the 5′ end of the *tap* coding region was hybridized to total RNAs from the following *S. lividans* strains: MS7 (pSS12) (chromosomal *tap* deletion, vector only) (lane 1); MS2 (pSS12) (chromosomal *tap* intact, vector only) (lane 2); and MS7 clone 3 (chromosomal *tap* deletion, plasmid-borne *tap* gene) (lane 3). (B) Primer extension products compared on a sequencing gel alongside the products of a DNA sequencing reaction obtained by using the same primer, with the sequence representing the sense strand (i.e., the complement of the bases shown). The asterisk indicates the 5′ base of *tap* mRNA in all three cases, and a potential −10 region is indicated.

stringency washes (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]); however, the background was low compared with the major hybridizing bands, indicating that there were probably related specific homologous DNA fragments in the chromosomes of the species tested. In a similar experiment a strongly hybridizing band was also observed with *Streptomyces ambofaciens* DNA (data not shown).

To assess the effect of deleting the *tap* gene from the *S. lividans* chromosome, we first constructed subclones (as shown in Fig. 1C) in pUC-based plasmid pINT, which carries *tsr* for selection in *Streptomyces* strains and *xylE* for screening in a catechol dioxygenase plate assay (16). These subclones were tested for their ability to transform *S. lividans* MS5 protoplasts to thiostrepton resistance, which presumably indicated that they were integrated by homologous recombination into the chromosomal DNA. Integration clone 3 produced no thiostrepton-resistant colonies, probably because of its relatively small homologous DNA fragment. The other three clones all produced thiostrepton-resistant colonies. Clones 2 and 4 were controls to show that integration into this region of the chromosome was possible and did not result in destabilizing effects or interference with any essential functions. Clone 5 contained a deletion of the *Bgl*II fragment internal to the *tap* gene, resulting in loss of the Tap phenotype (Fig. 1B, clone 3). Thiostrepton-resistant *xylE*$^+$ transformants derived from clone 5 were subcultured on agar medium without thiostrepton. Spores were harvested, and serial dilutions were replated and assayed directly to determine their ability to hydrolyze Gly-Pro-Leu-βNA.

Most colonies exhibited the same amount of color as the parental colonies; however, approximately 1 in every 1,000 colonies was significantly paler in this assay. Chromosomal DNAs isolated from three such colonies were analyzed by

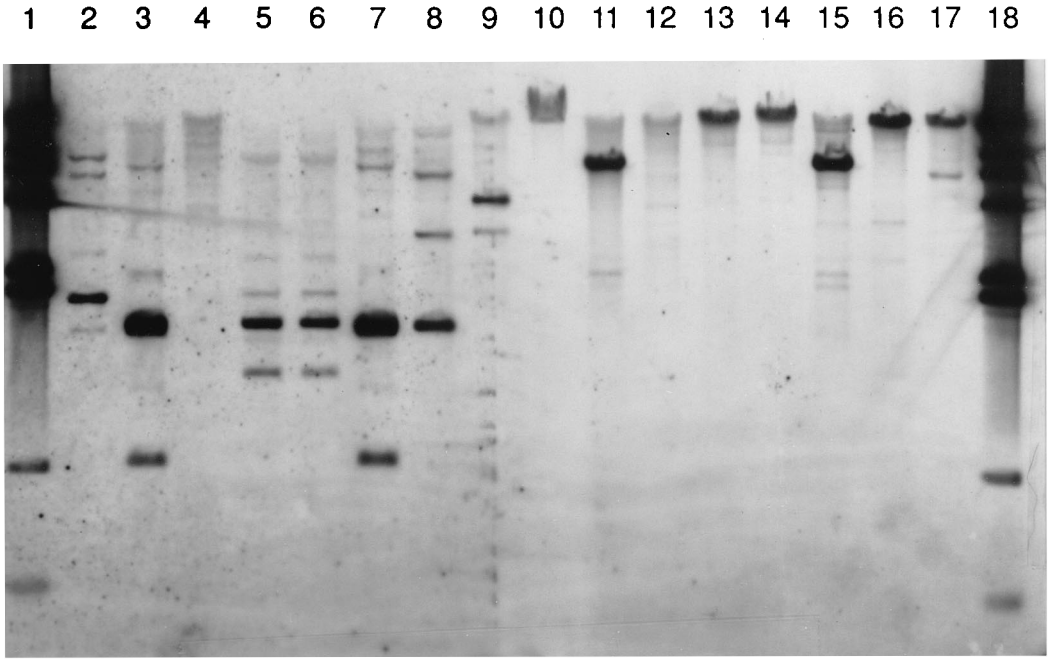

FIG. 4. Homologs of *tap* are present in many *Streptomyces* strains. Chromosomal DNA samples were digested with either *Bam*HI (lanes 2 through 9) or *Pst*I (lanes 10 through 17). Aliquots (3 μg) were separated on a 1% agarose gel and transferred to a nylon membrane. The chromosomal DNA fragment from *tap* clone 3 was excised, purified, and labelled with digoxigenin (Boehringer Mannheim) for use as a hybridization probe. Lambda DNA was also labelled and included in the hybridization buffer. Lanes 1 and 18, lambda *Hin*dIII molecular weight markers; lanes 2 and 10, *Streptomyces alboniger*; lanes 3 and 11, *S. coelicolor*; lanes 4 and 12, *S. fradiae*; lanes 5 and 13, *Streptomyces griseus* IMRU 3499; lanes 6 and 14, *S. griseus* ATCC 10137; lanes 7 and 15, *S. lividans* 66; lanes 8 and 16, *Streptomyces parvulus*; lanes 9 and 17, *Streptomyces rimosus*.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference(s)
Bacterial strains		
S. lividans 66		John Innes Institute, Norwich, United Kingdom
S. lividans MS2	Δ*pepP*	9, 29
S. lividans MS5	Δ*pepP* Δ*slpA* Δ*slpC*	9, 10, 29
S. lividans MS7	Δ*tap Bgl*II fragment	This study
S. lividans MS8	Δ*tap Bgl*II fragment	This study
S. lividans MS9	Δ*tap Bam*HI-*Bgl*II fragment	This study
S. alboniger 504		P. Redshaw, Austin College, Austin, Tex.
S. ambofaciens ATCC 23877		American Type Culture Collection
S. coelicolor M130		John Innes Institute, Norwich, United Kingdom
S. fradiae ATCC 14544		American Type Culture Collection
S. griseus ATCC 10137		American Type Culture Collection
S. griseus IMRU 3499		Waksman Institute of Microbiology, Rutgers University, Piscataway, N.J.
S. rimosus ATCC 10970		American Type Culture Collection
S. parvulus 2238		John Innes Institute, Norwich, United Kingdom
E. coli HB101		Bethesda Research Laboratories
E. coli DH5α		Bethesda Research Laboratories
Plasmids		
pUC 18/19	Ampicillin resistant	28
pT7T3.18 u	Ampicillin resistant	Pharmacia
pINT	pUC-based vector for integrative transformation into *Streptomyces* spp.	9

Southern hybridization. When we used ^{32}P-labelled probes for the *Bgl*II fragment internal to the *tap* gene and a 3.3-kbp overlapping DNA probe consisting of the chromosomal DNA insert used to make integration clone 4 (Fig. 1C), we found that only one of the three colonies contained DNA with a restriction pattern consistent with the desired specific chromosomal deletion (data not shown). This colony was designated *S. lividans* MS7 (Table 1). Similarly, *tap* integration clones 5 and 1 were used to make deletions directly in the chromosome of *S. lividans* 66, producing *tap*-deleted strains which were designated *S. lividans* MS8 and MS9, respectively (Table 1). Growth in TSB liquid medium followed by an assay to determine Gly-Pro-Leu-βNA hydrolysis confirmed that the majority of this activity had been eliminated from these strains. In liquid medium mutant strains MS7, MS8, and MS9 grew at the same rate and exhibited background levels of Tap activity, whereas the *S. lividans* 66 control had indistinguishable growth characteristics but exhibited substantial levels of Tap activity (4 to 5 nmol min^{-1} ml^{-1}).

Other proteolytic species present in *S. lividans* strains remain to be characterized, since some secreted heterologous proteins are still subject to degradation (data not shown). The removal of Tap activity as described above allowed us to discover other remaining extracellular proteolytic species, including a novel aminopeptidase that exhibits significant sequence homology to subtilisin BPN′ (7a).

Nucleotide sequence accession number. The nucleotide sequence of the *S. lividans tap* gene has been deposited in the GenBank database under accession number L27466.

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