Appl Microbiol Biotechnol (1996) 45:141–147

ORIGINAL PAPER

M. J. Butler · J. S. Aphale · C. Binnie
M. A. DiZonno · P. Krygsman · G. Soltes
E. Walczyk · L. T. Malek

Cloning and analysis of a gene from *Streptomyces lividans* 66 encoding a novel secreted protease exhibiting homology to subtilisin BPN′

Received: 15 May 1995/Received last revision: 2 October 1995/Accepted: 4 October 1995

Abstract Amino-terminal degradation has been observed for many of the secreted heterologous proteins produced by *S. lividans* 66. We, therefore, set out to characterize the relevant proteinases and their genes. A tripeptide chromogenic substrate was used to identify a gene that was shown to encode a secreted protein which removed tripeptides from the amino terminus of extracellular proteins (tripeptidyl aminopeptidase, Tap; Butler et al. 1995). This activity was removed by a homologous gene deletion replacement and the ability of the *S. lividans* strain to remove N-terminal tripeptides was greatly reduced, but still significant. When the *tap*-deleted strain was used as a host for the rescreening of a *S. lividans* 66 genomic DNA library, a number of other genes encoding proteases with aminopeptidase activities were discovered. One clone (P5-4) produced a 45-kDa secreted protein (Ssp), which showed activity against Ala-Pro-Ala-β-naphthylamide (APA-βNH-Nap) substrate. Further analysis of the cloned DNA showed an open-reading frame encoding a protein larger than 45 kDa. Direct Edman degradation of the secreted protein confirmed that it was encoded within the cloned DNA and probably processed from a larger precursor. Protein sequence analysis revealed a striking homology to subtilisin BPN′ in three regions around the active-site residues suggesting that the protein is a serine protease. As expected, the protease activity was inhibited by phenylmethylsulphonyl fluoride. Mutant strains with most of the *ssp* gene deleted exhibited reduced activity against APA-βNH-Nap substrate compared to their non-deleted parental strains.

M. J. Butler[1] · J. S. Aphale · C. Binnie (✉) · M. A. DiZonno
P. Krygsman · G. Soltes · E. Walczyk · L. T. Malek
Cangene Corporation, 3403 American Drive, Mississauga,
Ontario, L4V IT4 Canada.
Fax: (905) 673-5123

Present address:
[1] Strangeways Research Laboratory, Worts Causeway, Cambridge, CB1 4RN England

Introduction

The development of microbial expression systems to produce biologically active proteins requires an understanding of the gene-expression machinery and endogenous proteolytic capability of the host cells. *Streptomyces lividans* strains have been successfully used by several workers to produce soluble protein secreted directly into the culture medium. (Anné and van Mellaert 1993; Bender et al. 1990; Brawner et al. 1990, 1991; Chang and Chang 1988; Malek et al. 1990). Until recently relatively little information has been available about the endogenous proteases present in *S. lividans*; although some proteolytic activities (Aretz et al. 1989) have been observed the proteins and their respective genes have not been described. Aminopeptidase activities (Aphale and Strohl 1993) and some relevant genes have been isolated and characterised (Butler et al. 1994a, b; 1993). Recently, a previously unknown secreted tripeptidyl aminopeptidase (Tap) from *S. lividans* was described which was shown to remove tripeptides from the N-termini of recombinant proteins (Krieger et al. 1994). Inactivation of the *tap* gene by homologous recombination using a DNA fragment, in which a deletion had been made within the Tap-encoding region, reduced the Tap activity in such deletion strains (Butler et al. 1995). However, a low but significant level of Tap-like activity was still observable. Therefore, we searched for other activities similar to those of Tap, using the deletion strain MS7 as a host for a genomic library and screening for the overexpression of the ability to hydrolyse the chromogenic tripeptide substrate Ala-Pro-Ala-β-naphthylamide (APA-βNH-Nap) in an agar-plate assay (Butler et al. 1993). This work describes the isolation, characterization and deletion of another gene, *ssp*, encoding a secreted protease (Ssp) active against this chromogenic substrate.

Materials and methods

Streptomyces strains were cultured and manipulated as described by Hopwood et al. (1985). The protease-deficient strains MS7 (Δ*tap*, Δ*slpA*, Δ*slpC*, Δ*pepP*1, Δ*pepP*2) and MS9 (Δ*tap*) have been described elsewhere (Butler et al. 1995) *E. coli* transformations used competent cells from Bethesda Research Laboratories, Burlington, Ontario. Genetic manipulation was according to Maniatis et al. (1982). Hybridization conditions were described previously (Butler *et al.* 1992) and used [α-^{32}P] dATP supplied by either Amersham, Oakville, Ontario, or Du Pont Canada Inc., Mississauga, Ontario. Aminopeptidase assays were as previously described (Butler et al. 1994a) using either *p* nitroanilide (-*p*NH-Np, measured at 405 nm) or β-naphthylamide (-βNH-Nap, measured at 540 nm) chromogenic peptide substrates. Sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE) analysis was as described by Laemmli (1970).

DNA sequencing and primer-extension analysis of transcription was carried out as previously described (Butler et al. 1993; Henderson et al. 1987) with data compilation using DNASTAR (Doggette and Blattner 1986), and homology searches using the BLAST program (Altschul et al. 1990). The nucleotide sequence data have been deposited in the Genbank database, accession no. L41655. Automated Edman degradation for direct amino acid sequence determination was performed by the Sequencing and Amino Acid Analysis Laboratory, at the Core Facility for Protein/DNA Chemistry, Department of Biochemistry, Queens University, Kingston, Ontario.

The Ssp protease described in this work was partially purified from the cell-free broth of an *S. lividans* MS7 (Tap$^-$) transformant harbouring the P5-4 plasmid, grown for 22 h in trypticase soy broth, representing the optimal time for expression of this protease under these conditions. Extracellular proteins from the cell-free broth were precipitated by saturating the broth to 85% (w/v) ammonium sulphate and standing on ice for 30 min. Precipitated material was recovered by centrifugation at 16 000 *g* and at 4 °C. The resulting pellet was resuspended in a minimal amount of distilled water (crude enzyme mix) and column-dialysed to 20% ammonium sulphate in 20 mM Tris/Cl, pH 6.5 using a Bio-Rad Econo-Pak 10 GD disposable column (Bio-Rad, Hercules, Calif.).

Hydrophobic-interaction chromatography (HIC) was performed using a phenyl-Superose column (HR 5/5, Pharmacia, Uppsala, Sweden) pre-equilibrated in 20% ammonium sulphate in 20 mM Tris/Cl, pH 6.5. The sample was loaded at 24 column volumes/h (cv/h) and bound proteins were eluted from the column at the same flow rate by a linear gradient of 20%–0% ammonium sulphate in Tris/Cl, pH 6.5. Fractions representing 2 column volumes each were collected for analysis. APA-*p*NH-Np degradation assays and SDS-PAGE analysis indicated that P5-4 activity and a 45-kDa protein co-eluted from the column at approximately 7%–8% ammonium sulphate concentration. Active fractions were pooled, column-dialysed to 10% ammonium sulphate and reloaded on the phenyl-Superose column re-equilibrated to the same buffer conditions. Bound protein eluted from the column at a concentration of approximately 2.5% ammonium sulphate and was used for substrate-degradation assays. The HIC step served to remove pigments and a large amount of contaminating proteins from P5-4. The protease at this stage was approximately 75% pure, as judged by SDS-PAGE analysis. This material was used for inhibitor studies described in the text.

Results

Screening an *S. lividans* 66 genomic library with APA-βNH-Nap

Protoplasts of *S. lividans* MS7 were transformed using pooled bifunctional plasmid pSS12 DNA isolated from *E. coli* transformants representing a genomic library (Butler et al. 1992) of *S. lividans* 66 DNA. Thiostrepton-resistant transformant colonies were screened directly using the APA-βNH-Nap substrate. Among 13 000 colonies tested, 11 strongly positive clones were identified. Plasmid DNA was isolated and used to transform competent cells of *E. coli* HB101, facilitating the isolation of larger quantities of relatively pure plasmid DNA for restriction-enzyme-site analysis. Four of these clones were recognised to represent the previously described *tap* locus. Four other clones were shown to represent another locus encoding a putative membrane-bound protease showing significant homology to the Tap protein (Binnie et al. 1995). Two further clones (designated P5-4 and P5-15) were shown by Southern hybridization analysis to represent overlapping fragments from another locus in the *S. lividans* 66 chromosomal DNA by virtue of a common pattern of positive signals from restriction digests of genomic DNA probed at high stringency (0.1 × SSC, 68 °C) using each clone as a hybridization probe (not shown). Finally, another clone (P5-10) was also obtained and shown to represent a chromosomal locus unlinked to P5-4/P5-15 (unpublished data).

Identification of a *Streptomyces* subtilisin-like protease

SDS-PAGE analysis of the culture supernatant from the *S. lividans* MS7 strain retransformed with either P5-4 or P5-15 showed a major secreted protein species with an apparent subunit molecular mass of 45 kDa (Fig. 1). Preparative SDS-PAGE followed by electrotransfer of the 45-kDa protein to an Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore) allowed direct automated Edman degradation to be carried out to yield the amino acid sequence NH_2-DTGAPQVLGGEDLAAAKAASAKAEGQDPLE. Aminopeptidase activity was significantly (20-fold) increased over background levels in these culture supernates with APA-*p*NH-Np substrate (Table 1). Activity appeared to be lost on prolonged culture (42 h) of P5-4/P5-15 transformants, correlating with the disappearance of the 45-kDa band, as revealed by SDS-PAGE analysis (not shown).

Sequence analysis of the P5-4 locus

The restriction-enzyme-site map of the P5-4/P5-15 locus is shown in Fig. 2. Deletion subcloning analysis was carried out to localise the protein-encoding DNA region. Plasmid subclones were constructed in *E. coli* HB101 strains and DNA used to retransform *S. lividans* MS7 protoplasts to examine their proteolytic capability using the agar-plate APA-βNH-Nap assay. The DNA fragments and their related hydrolytic

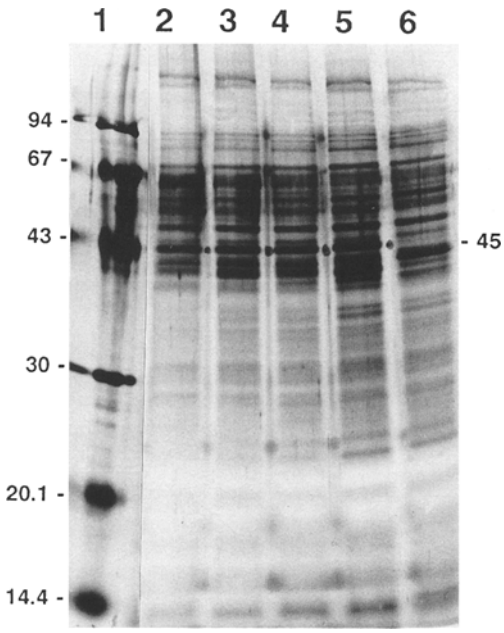

Fig. 1 SDS-PAGE analysis and silver staining of extracellular proteins (50 µl cell-free broth) from *S. lividans* MS7 transformants harbouring plasmids P5-4 and P5-15. The overproduced 45-kDa protein is indicated and is not present in *lane 6*, representing a pSS12 vector control. *Lane 2* P5-4, 23 h; *lane 3* P5-4, 26 h; *lane 4* P5-15, 23 h; *lane 5* P5-15, 26 h; *lane 6* vector pSS12, 26 h. Molecular mass markers was loaded on the same gel and their relative positions indicated as shown (*lane 1*)

Table 1 Ala-Pro-Ala-*p*-nitroanilide (*APA*p*NH-Np*) hydrolysis by 10 µl cell-free broth of *S. lividans* MS7 transformed with P5-4 and P5-15 *ssp*-containing plasmids. Background activities obtained from a pSS12-vector-only transformant are as shown. Transformants were grown in shake flasks in trypticase soy broth/0.1 M MOPS/2% glucose including thiostrepton (10 µg ml^{-1}) and sampled at 23 h and 26 h (late exponential, early stationary phase). The results are from the linear range of the assay, and uncorrected for dry cell weight, as all cultures grow essentially at the same rate

Plasmid	APA-*p*NH-Np hydrolysis			
	(ΔA_{405}, corrected for no substrate)		(ΔA_{405} ml^{-1} min^{-1})	
	23 h	26 h	23 h	26 h
P5-4	0.546	0.710	1.8	2.4
P5-15	0.729	0.991	2.4	3.3
PSS12	0.033	0.032	0.1	0.1

activities are as shown in Fig. 2. The activity data suggest the location of the active region of DNA to be around the centre of the inserted sequence in P5-4. DNA fragments from this region were, therefore, subcloned into the *E. coli* pT7T3 vector for nucleotide sequence determination. The nucleotide sequence is shown in Fig. 3. Inspection of the sequence reveals a potential protein-encoding region located between the *Nco*I(7) and

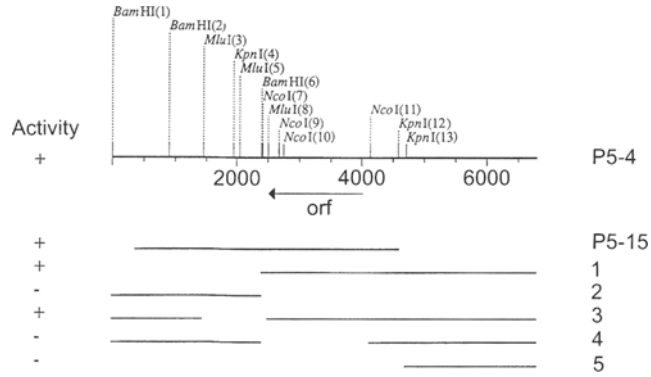

Fig. 2 Restriction-enzyme-site map of cloned *ssp* DNA. Two plasmids (P5-4 and P5-15) were isolated from a *Sau*3A1 genomic library. DNA remaining in the P5-4 plasmid deletions (Δ1 to Δ5) is shown by a *horizontal line*. The relative hydrolytic ability of strains carrying the plasmids in the agar-plate assay under Ala-Pro-Ala-β-naphthylamide is indicated at the *left-hand side* of the figure: + Strongly red colonies, − pale orange-coloured, indistinguishable from that produced by host colonies carrying only the plasmid vector. *Arrow* the location and direction of the open-reading frame (*orf*)

*Nco*I(11) sites. The predicted protein consists of 513 amino acids. A potential ribosome-binding site (GGAG) occurs 9 nucleotides upstream from the putative initiator ATG codon. The transcription start point (*tsp*) for this gene was mapped by primer-extension analysis using an oligonucleotide (5′-CGAGGGCAC GGCGGTGACGCGAGAGGGGAG) that hybridizes to a DNA sequence encoding amino acids 3–12 of the primary translation product (data not shown). The *tsp* appears to be located to a thymidine base 167 bases upstream of the translation initiation codon (Fig. 3). The hexameric sequences, TAGCTT and TTGCTG, located approximately 10 and 35 bases upstream of the *tsp* and separated by 18 bases, conform in part of consensus sequences (TAGA/GA/GT, TTGACA/G) for promoters of genes transcribed by the major form of the RNA polymerase holoenzyme ($E\sigma^{70}$) present in vegetatively growing mycelium of *Streptomyces* (Strohl 1992).

Sequence and activity comparison of P5-4 and subtilisin BPN′

The predicted N-terminal amino acids appear to resemble a typical gram-positive signal peptide with three arginine residues close to the NH_2 end. The amino acid sequence determined from the observed secreted protein was identical to that of residues 125–154 in the predicted protein sequence, indicating that the P5-4 protein is processed from a larger precursor form after secretion. When the amino acid sequence was used to screen the protein databases the most similar sequence detected was that of subtilisin BPN′, which is also processed from a larger, inactive precursor form after

Fig. 3 The nucleotide sequence of the *S. lividans* 66 *ssp* locus. The deduced amino acids in the coding region are aligned under the first nucleotide of each codon. *Asterisks* are marked above the nucleotides that constitute a potential ribosome-binding site. The translation-termination codon is indicated by a *dot* and is followed by an inverted repeat sequence ($\Delta G = -219$ kJ calculated according to the rules of Tinoco et al. 1973), which is shown by an *overline*. Amino acid residues that were observed by direct sequencing of the protein are *underlined*. The transcription start point is indicated by an *arrow* and putative -10 and -35 promoter sequences are shown in *bold* upstream of this site

secretion. Alignment of these two sequences reveals three striking regions of homology centred around each of the active-site residues characterised for subtilisin (Fig. 4).

Given the observed homology, it was of interest to examine the relative hydrolytic specificities of the two enzymes. Ideally this should be undertaken with purified proteins but since our initial objective was to identify and delete protease activities rather than exhaustively characterize host proteases, we carried out a limited study using crude fermentation broth samples of a strain containing the overproduced protein (which was designated *Streptomyces* subtilisin-like protease, Ssp) as well as the host transformed with a plasmid vector as the control. A preliminary analysis (Table 2) of the relative hydrolysis of chromogenic substrates by Ssp compared to subtilisin BPN′ (Boehringer Mannheim) suggested that the specificities of the two enzymes appear to be significantly different. The Ssp was much less effective than subtilisin BPN′ in hydrolysing azocasein and substrates in which the free NH_2-terminal group was chemically blocked or altered (SucAPA-*p*NH-Np and H-D-PFA-*p*NH-Np). Conversely, APA-*β*NH-Nap was much more rapidly hydrolysed by Ssp than by subtilisin BPN′. A further study of Ssp activity was carried out using a protein fraction enriched for APA*p*NH-Np activity, purified

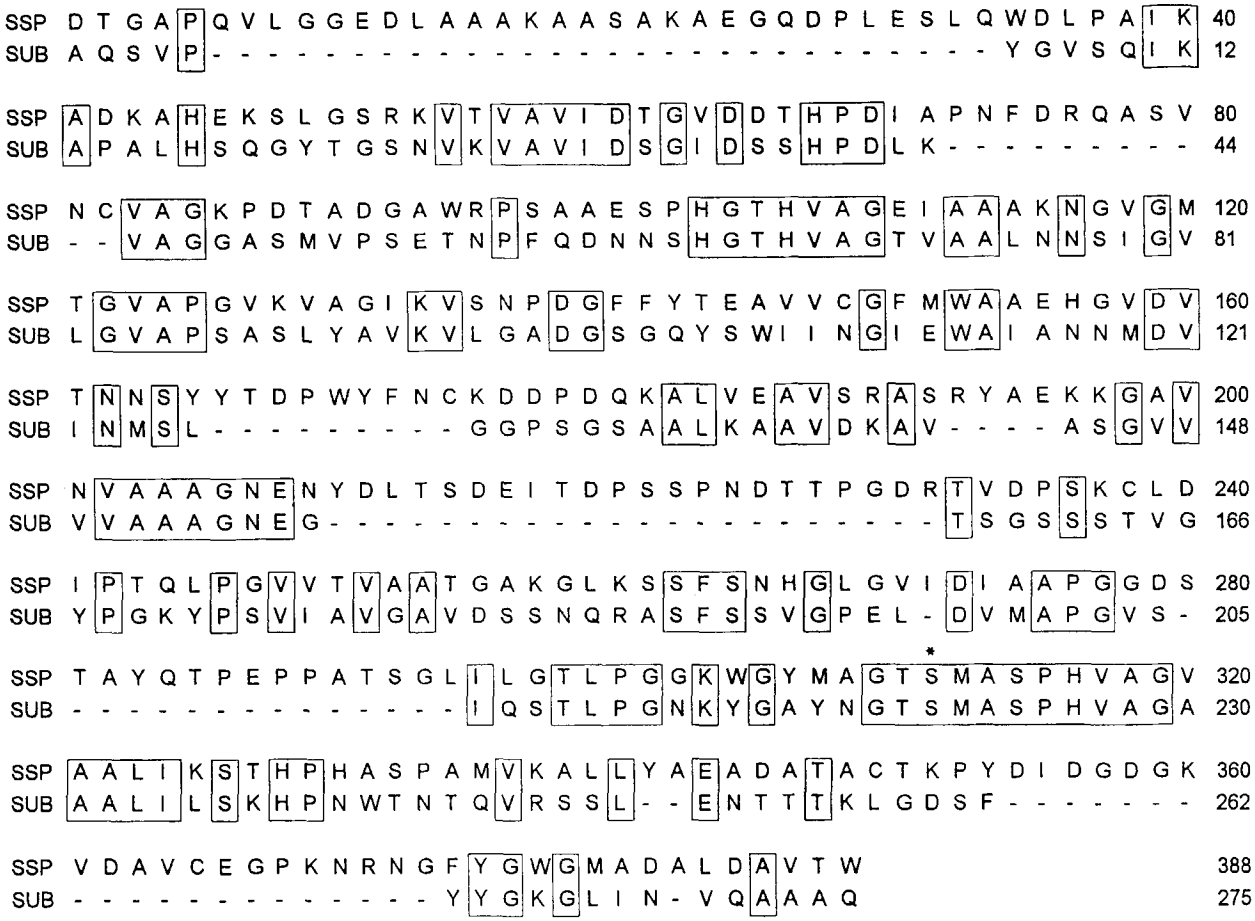

Fig. 4 Amino-acid sequence alignments between *S. lividans* Ssp and subtilisin BPN'. Amino acid numbering for each protein commences with the residue at the NH_2 terminus of the final processed product. Identical amino acids are indicated by boxes. Active- site residues in the subtilisin sequence are indicated by an *asterisk*

from an Ssp-overexpressing strain from which Tap activity had been deleted (MS7). The results showed activity to be calcium-dependent, inhibited by phenylmethylsulphonyl fluoride and EDTA (reversible with excess Ca^{2+}), and not inhibited by phenanthroline (data not shown), implying that the Ssp protease is of the serine protease type.

Deletion of the chromosomal *ssp* gene

The phenotypic properties of the plasmid deletion clones (Fig. 2) were consistent with the nucleotide sequence data and allowed the construction of an integration plasmid (using the previously described pINT plasmid, Butler et al. 1992). The integration clone was used to create a strain containing the specific deletion at the *ssp* locus within the predicted transcription unit in both an independent *tap*-deleted strain (MS9) to create MS12 (*tap*$^-$, *ssp*$^-$) or in the multiply mutated MS7 strain, to create MS11. This independent line was established to assess the specific effect of deletion of tripeptidyl aminopeptidase genes, as the MS7 strain

Table 2 Chromogenic substrate hydrolysis comparison of P5-4 (Ssp) and Subtilisin BPN', P5-4 activity was measured from a representative transformant of *S. lividans* strains MS7 and MS9 deleted for *tap* activity and harbouring the P5-4 clone. The data shown are for a 23 h assay time. All transformants grew at the same rate. Essentially similar results were obtained from 26 h samples. The results were corrected for background activity (less than 1% of total) from a vector-only control using a cell-free broth sample. Subtilisin activity was measured using a commercial preparation (Boehringer Mannheim), and corrected for background from a no-substrate control

Substrate	Proteolytic activity ($\Delta A_{405}\,mg^{-1}\,min^{-1}$)		
	P5-4		Subtilisin BPN'
	MS7	MS9	
HD-Pro-Phe-Arg-*p*NH-Np	0.005	0.000	0.073
Succinyl-Ala-Pro-Ala-*p*NH-Np	0.009	0.000	0.374
Ala-Pro-Ala-*p*NH-NP	0.412	0.435	0.113
Leu-*p*NH-NP	0.003	0.000	0.105
Arg-*p*NH-Np	0.000	0.003	0.020
Azocasein	0.000	0.000	13.40

Table 3 APA*p*NHNp hydrolysis by cell-free broth from *S. lividans* independent strains with Tap and Ssp activity deleted, or only Tap activity deleted. A 90-µl sample of cell-free broth was tested in each case. Values are corrected for small (less than 0.5 g/l) differences in dry cell mass between strains at each assay time, and all figures quoted were from the linear range of the assay. The 16-h and 19-h sampling times reflect the optimal expression time in plasmid-free strains for this protease, occurring at the late exponential/early stationary phase of growth

Strain	APA*p*NH-Np hydrolysis ($\Delta A_{405}\,g^{-1}\,min^{-1}$)	
	16 h	19 h
S. lividans 66 wild type	130.0	144.9
S. lividans 66 MS7	13.8	17.7
S. lividans 66 MS9	12.4	16.5
S. lividans 66 MS11	7.7	11.2
S. lividans 66 MS12	7.6	8.6

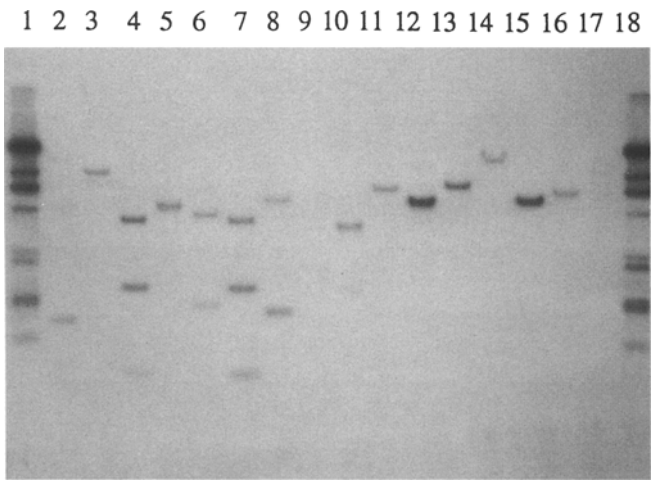

Fig. 5 Southern hybridization analysis of restriction digests of chromosomal DNA prepared from various *Streptomyces* species, probed with digoxigenin-labelled P5-4 plasmid. Hybridization was carried out at 68 °C in 6 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0), 0.18 M Na_2HPO_4, 6 mM EDTA, 1% sarkosyl, 50 µg ml^{-1} sonicated boiled calf thymus DNA, and the final wash step at 68 °C in 0.1 × SSC/0.1% SDS. Digoxigenin-labelled phage λ DNA was included as a probe to provide molecular mass markers of λ DNA digested with *Hin*dIII. *Lanes 2–9* *Nco*I-digested DNA; *lanes 10–17* *Sph*I-digested DNA. *Lanes 1, 18* λ DNA/*Hin*dIII molecular mass markers; *2, 10 S. alboniger* 504 (P. Redshaw, Austin College, Texas); *3, 11 S. ambofaciens* ATCC 23877; *4, 12 S. coelicolor* M130 (John Innes Institute, Norwich, UK); *5, 13 S. fradiae* ATCC 14544; *6, 14 S. griseus* ATCC 10137; *7, 15 S. lividans* 66 (John Innes Institute, Norwich, UK); *8, 16 S. parvulus* 2238 (John Innes Institute, Norwich, UK); *9, 17 S. rimosus* ATCC 10970

already described was also deficient in non-Tap proteases. The strains were grown in trypticase soy broth for 22 h and examined for the ability of the cell-free broth to hydrolyse APA-βNH-Nap. The results of a representative experiment (Table 3) showed a small, reproducible reduction in hydrolytic capability, with the MS11 and MS12 strains exhibiting the lowest activity. These two strains grew and sporulated in an identical manner to their parental strains when grown on minimal medium or on R2 complex solid medium (Hopwood et al. 1985), or when grown in liquid trypticase medium.

Detection of *ssp* homologues in other *Streptomyces* species

A high-stringency Southern hybridization was carried out to determine if *ssp*-like sequences were detectable in a variety of *Streptomyces* species. The results (Fig. 5) indicated that homologous sequences were detected in all species tested, the strongest signal being detected in the closely related *S. coelicolor* species.

Discussion

Genes encoding proteases that hydrolyse an APA-βNH-Nap substrate have been isolated and partially characterised. It was not surprising to identify during this search a protease related to subtilisin BPN′, since the production of a secreted subtilisin proteinaceous inhibitor (SPI) by *Streptomyces* strains has been well documented. Moreover, the modulation of protease activity by coordination of the production of a protease and its corresponding inhibitor has been widely observed in many cell types. It is, however, noteworthy that the Ssp described in this report appears to be much more active as an exo-(amino)peptidase, unlike its subtilisin homologue. Southern hybridization experiments using chromosomal DNA prepared from different *Streptomyces* species detected DNA sequences homologous to the *ssp* DNA, the homology being strongest in strains closely related to *S. lividans*, e.g. *S. coelicolor*.

A recent report (Taguchi et al. 1995) described the isolation and characterization of a 20-kDa chymotrypsin-like serine protease (SAM-P20) from a *Streptomyces albogriseolus* mutant lacking SPI. The Ssp protease described in this report, however, appears to differ significantly from SAM-P20 in its size, sequence composition and substrate specificity. The two proteases are similar to subtilisin BPN′ in that they are probably processed from larger, presumably inactive, precursor forms. However, the mechanism of activation of the Ssp protease must be different from the others as it appears to lack the required chymotrypsin-like cleavage activity necessary for self-processing, and it behaves more as a true aminopeptidase rather than an endoprotease. Activation of *S. lividans* Ssp may, therefore, require the action of a separate, so far uncharacterized, endoprotease present at the same or later stage of growth. Owing to the apparent difference between Ssp and

members of the SAM-P20 class of proteases, described above, it also appears unlikely that Ssp represents the serine protease most likely to be inhibited by the SPI native to *S. lividans* itself. Deletion of the *ssp* gene resulted in host strains in which the APA-βNH-Nap hydrolytic capability was further reduced compared to that of both the *tap*-deleted and *S. lividans* 66 strains. Detailed characterization of the specificity and properties of the Ssp will require more extensive purification of the active protein. However, we have concentrated our efforts on the elimination of this activity from the host strain, for which task this information is not essential. The remaining low level of substrate activity may be due to the presence of another aminopeptidase, P5-10, the gene for which was also uncovered during the course of isolating the *ssp* gene. Preliminary sequence analysis of the P5-10 gene and its overexpressed product has indicated that it encodes a 50-kDa extracellular serine protease of the aminopeptidase class. Further work is now underway to delete this activity from *S. lividans*. It is anticipated that a *S. lividans* mutant strain lacking all three activities (Tap, Ssp, P5-10) will be significantly better than the wild-type strain in its inability to degrade some heterologous proteins by virtue of decreased tripeptidyl aminopeptidase activity.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Anné J, Van Mellaert L, (1993) *Streptomyces lividans* as host for heterologous protein production. FEMS Microbiol Lett 114:121–128

Aphale JS, Strohl WR (1993) Purification and properties of an extracellular aminopeptidase from *Streptomyces lividans* 1326. J Gen Microbiol 139:417–24

Aretz W, Koller K-P, Riess G (1989) Proteolytic enzymes from recombinant *Streptomyces lividans* TK24. FEMS Microbiol Lett 65:31–36

Bender E, Koller K-P, Engels JW (1990) Secretory synthesis of human inter-leukin-2 by *Streptomyces lividans*. Gene 86:227–232

Binnie C, Butler MJ, Aphale JS, Bourgault R, DiZonno MA, Krygsman P, Liao L, Walczyk E, Malek LT (1995) Isolation and characterization of two genes encoding proteases associated with the mycelium of *Streptomyces lividans* 66. J Bacteriol 177: 6033–6040

Brawner M, Taylor D, Fornwald J (1990) Expression of the soluble CD4 receptor in *Streptomyces*. J Cell Biochem Suppl 14A:103

Brawner M, Poste G, Rosenberg M, Westpheling J (1991) *Streptomyces*: a host for heterologous gene expression. Curr Opin Biotechnol 2:674–681

Butler MJ, Davey CC, Krygsman P, Walczyk E, Malek LT (1992) Cloning of genetic loci involved in endoprotease activity in *S. lividans* 66: a novel neutral protease gene with an adjacent divergent putative regulatory gene. Can J Microbiol 38:912–920

Butler MJ, Bergeron A, Soostmeyer G, Zimny T, Malek T (1993) Cloning and characterization of aminopeptidase P gene from *Streptomyces lividans*. Gene 123:115–119

Butler MJ, Aphale JS, Binnie C, DiZonno MA, Krygsman P, Soltes GA, Walczyk E, Malek LT (1994a) The aminopeptidase N-encoding *pepN* gene of *Streptomyces lividans* 66. Gene 141:115–119

Butler MJ, Aphale JS, DiZonno MA, Krygsman P, Walczyk E, Malek LT (1994b) Intracellular aminopeptidases in *Streptomyces lividans* 66. J Ind Microbiol 13:24–29

Butler MJ, Binnie C, DiZonno MA, Krygsman P, Soltes GA, Soostmeyer G, Walczyk E, Malek LT (1995) Cloning and characterization of a gene encoding a secreted tripeptidyl aminopeptidase form *Streptomyces lividans* 66. Appl Environ Microbiol 61:3145–3150

Chang S-Y, Chang S (1988) Secretion of heterologous proteins in *Streptomyces lividans*. In: Okami Y, Beppu T, Ogawara H (eds) Biology of actinomycetes '88. Japan Scientific Societies Press, Tokyo, pp 103–107

Doggette PE, Blattner FR (1986) Personal access of sequence databases on personal computers. Nucleic Acids Res 14:611–619

Henderson G, Krygsman P, Liu CJ, Davey CC, Malek LT (1987) Characterization and structure of genes for proteases A and B from *Streptomyces griseus*. J Bacteriol 169:3778–3784

Hopwood DA, Bibb MJ, Chater KF, Kieser T, Bruton CJ, Kieser HM, Lydiate DJ, Smith CP, Ward JM, Schrempf H (1985) Genetic manipulation of *Streptomyces*, a laboratory manual. John Innes Foundation, Norwich

Krieger TJ, Bartfeld D, Jenish D, Hadary D (1994) Purification and characteriztion of a novel tripeptidyl aminopeptidase from *Streptomyces lividans* 66. FEBS Lett 352:385–388

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Malek LT, Soostmeyer G, Davey CC, Krygsman P, Compton J, Gray J, Zimny T, Stewart D (1990) Secretion of granulocyte macrophage colony stimulating factor (GM-CSF) in *Streptomyces lividans*. J Cell Biochem Suppl 14A:127

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY

Strohl WR (1992) Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. Nuc Acids Res 20:961–974

Taguchi S, Odaka A, Watanabe Y, Momose H (1995) Molecular characterization of a gene encoding extracellular serine protease isolated from a subtilisin inhibitor-deficient mutant of *Streptomyces albogriseolus* S-3253. Appl Environ Microbiol 61:180–186

Tinoco I Jr, Borer PN, Dengler B, Levine MD, Uhlenbeck OC, Crothers DM, Gralla J (1973) Improved estimation of secondary structure in ribonucleic acid. Nat New Biol 246:40–41