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Intracellular aminopeptidases in *Streptomyces lividans* 66

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SUMMARY

We have investigated the aminopeptidase activities present in *Streptomyces lividans* strains. The majority of these activities proved to be intracellular with multiple active species. Two aminopeptidase P genes were identified to be responsible for the ability to hydrolyze amino terminal peptide bonds adjacent to proline residues. Two other broad spectrum aminopeptidases were found to display homology at both the DNA and protein levels. One showed significant homology to PepN proteins, particularly around the putative zinc-binding residues which are important for catalysis. The second broad spectrum activity was not analyzed in detail but showed a different spectrum of substrate specificity to that of PepN.

INTRODUCTION

Many commercially important therapeutic proteins can be produced in a soluble biologically-active form by direct secretion into the culture medium using *Streptomyces lividans* strains, [4,7,16]. The integrity of the protein products can be compromised by degradation at either the *N*- or *C*-terminus of the polypeptide chain or by endoproteolytic cleavage. Relatively little endoprotease activity has been detected in *S. lividans* culture broths but significant levels of aminopeptidase activity have been observed [3]. A secreted aminopeptidase has been characterized from *S. griseus* [5] but so far there has been little description of the genes encoding the aminopeptidases of *S. lividans*. We described the cloning of a gene encoding an aminopeptidase P gene [9] which is responsible for the majority of the activity in *S. lividans* 66 capable of hydrolyzing *N*-terminal amino-acyl peptide bonds adjacent to a penultimate proline residue. More recently we have cloned a gene encoding a PepN from *S. lividans* 66 [8]. Although the normal location of these aminopeptidases is intracellular, they may become significant with respect to secreted protein products particularly at later fermentation times when inevitably there is some mycelial lysis especially in the high-shear environment of a stirred tank vessel. Some of these functions have been deleted from *S. lividans* strains without any loss of yield of secreted proteins or adverse growth effects. This report describes other related intracellular aminopeptidases from *S. lividans* 66 for which we have also cloned and partially characterized the genes. Deletion of the remaining activities is the subject of ongoing investigations.

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MATERIALS AND METHODS

Streptomyces strains were cultured and manipulated as described by Hopwood et al. [13]. *E. coli* transformations used competent cells from Bethesda Research Laboratories, Burlington, Ontario. Genetic manipulation was according to Maniatis et al. [17]. Hybridization conditions were described previously [10] using [α^{32}P]dATP supplied by either Amersham, Oakville, Ontario or Du Pont Canada Inc., Mississauga, Ontario.

Aminopeptidase assays were as previously described [9] using either paranitroanilide (measured at 405 nm) or β-naphthylamide (measured at 540 nm) chromogenic peptide substrates. SDS-PAGE analysis was as described by Laemmli [14].

The substrates Gly-pNA, Lys-pNA, Glu-pNA, Ala-pro-pNA, Ser-βNA and Phe-Ala-βNA, were from Bachem (Philadelphia, PA, USA). The paranitroanilide derivatives of Ala, Leu, Met, Arg, Val, *N*-α-CBZ-Arg, *N*-α-benzoyl-Arg and the dipeptide Gly-Pro were purchased from Sigma (St Louis, MO, USA) while Pro-pNA was obtained from Star Biochemicals (Torrance, CA, USA). Enzyme assays were conducted in a disposable 96-well microtiter plate (Corning, NY, USA) in a final volume of 100 μl and color development was analyzed by a microplate autoreader (EL-309, BioTek Instruments, Mandel Scientific, Guelph, Ontario) at pre-determined time intervals.

DNA sequencing was carried out as previously described [9,10,12] with the data compilation using DNA STAR [11] and homology searches using the BLAST program [1].

Preparation of crude extract

S. lividans (containing the plasmid pCAN 100) was grown in TSB/PPG for a period of 24 h and mycelia harvested by centrifugation at 16000 × *g* for 20 min at 4 °C. The cell-

free broth was discarded and the mycelia washed once with chilled lysis buffer (50 mM HEPES-KOH, pH 7.5; 60 mM NH_4Cl; 10% v/v glycerol; 5 mM mercaptoethanol). Washed mycelia (1 g wet wt/2.5 ml lysis buffer) were disrupted by three consecutive passes through a pre-chilled cell disrupter (X-press, AB Biox, Goteborg, Sweden) as described previously [9]. Cellular debris was separated from soluble intracellular material by centrifugation at 30000 × *g* for 45 min at 4 °C. Pelleted material was discarded and the supernatant used as the source of crude enzyme.

Protein purification

Intracellular material was dialyzed to 20 mM Tris · HCl by passage through a disposable desalting column (Econo-Pac 10DG, BioRad, Richmond, CA, USA) and proteins separated by passage over an anion-exchange resin (Q-Sepharose, Pharmacia, Uppsala, Sweden). Fractions indicating activity against serine-βNA were pooled and concentrated by ultrafiltration through a 30000 M_r cut off membrane (Centriprep, Amicon, Danvers, MA, USA). IEX (ion exchange) pooled proteins were further fractionated by passage through a Superose-6 (Pharmacia) gel filtration column. Fractions indicating activity against serine-βNA were concentrated in a similar way as described above and used as the source of enzyme for conducting substrate assays.

Substrate assays

Substrates were routinely dissolved in DMSO, except for Glu-pNA and Asp-pNA which were dissolved in ethanol:1 M sodium hydroxide (1:1), aliquoted and stored at −20 °C. A final substrate concentration of 0.8 mM was employed for each substrate and the release of para-nitroaniline was recorded at 405 nm. Substrate hydrolysis was monitored at 540 nm when β-napthylamide substrates were used.

Protein assay

Protein content of each sample was estimated by the dye binding assay of Bradford [6] using a commercial protein assay kit (BioRad) and bovine serum albumin as standard.

RESULTS

There are two PepP-encoding genes in S. lividans

The gene encoding the major PepP activity in *S. lividans* 66 was inactivated by homologous recombination to replace the wild type chromosomal gene with a deletion mutant gene constructed in vitro in which the *Bam*HI (10) to *Sac*I (14) DNA fragment was deleted [9]. The recombinational exchange of mutant and wild type alleles was effected using an integrational (non-replicating) vector, pINT [10]. The deletion mutant resulted in a reading frame shift with consequent inactivation of the PepP encoded at that locus. The ability of the deleted strain (designated *S. lividans* MS1) to hydrolyze Gly-Pro-paranitroanilide was reduced approximately six-fold (compared to that observed for *S. lividans* 66) [9] to an almost insignificant level. However, the ability to hydrolyze Ala-Pro-paranitroanilide was reduced by only four-fold, leaving a clearly detectable low level of activity present in cell-free extracts of the deletion strain.

This residual activity prompted us to reexamine Southern hybridization experiments using chromosomal DNA from both *S. lividans* 66 and *S. lividans* MS1. Most of the data supported the specific deletion of DNA expected at the *pepP* locus. However, the *Sph*I-digested samples showed a second hybridizing band in the *S. lividans* 66 DNA which was also present but partially obscured by the newly-formed deleted *Sph*I fragment in the MS1 DNA (lanes 10, 11, Fig. 1). The originally cloned *pepP* was contained within a 3.5-kbp *Sph*I DNA fragment and the other hybridizing *Sph*I DNA fragment was 3.0 kbp. Henceforward the 3.5 kbp-containing locus will be referred to as *pepP1* and the 3.0 kbp locus as *pepP2*.

We decided, therefore, to construct a partial DNA library from *S. lividans* 66 using size fractionated *Sph*I-digested DNA fragments (in the range 2.3–4.4 kbp) purified from an agarose gel. These fragments were ligated to *Sph*I-digested, dephosphorylated pT7T3 DNA. The ligation mixture was used to transform competent cells of *E. coli* HB101 and ampicillin-resistant colonies were screened by colony hybridization at low stringency using the 1.7-kbp *Sac*I *pepP1* DNA fragment [9]. Four positively-hybridizing clones were identified out of 1000 screened. One clone had a 3.5-kbp *Sph*I insert fragment while three others contained 3.0-kbp *Sph*I fragments. One of the latter clones was designated pOSE33 and used to make further smaller subclones for DNA sequence analysis. Using the same 1.7-kbp *pepP1* probe to screen the *Sau*3A1 library yielded 20 positively-hybridizing clones out of 2500 screened, 18 of which were identified (by *Sal*I digestion DNA fragment pattern) to be identical to the previously described *pepP1* clones [9]. Two

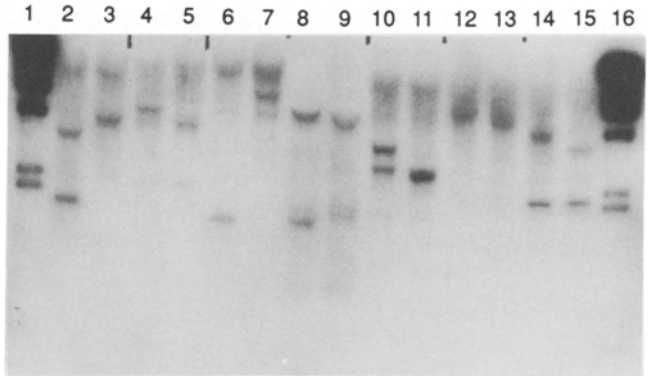

Fig. 1. Southern hybridization of the chromosomal *pepP* locus in *S. lividans* 66 and the Δ*pepP* deletion mutant strain MS1. Lanes 1 and 16 were Lambda DNA/*Hin*dIII molecular weight markers. Lanes 2, 4, 6, 8, 10, 12 and 14 contained *S. lividans* 66 DNA while lanes 3, 5, 7, 9, 11, 13 and 15 contained DNA from the MS1 deletion strain. DNA in Lanes 2 and 3 were digested with *Bam*HI; 4 and 5 with *Pst*I; 6 and 7 with *Sac*I; 8 and 9 with *Sal*I, 10 and 11 with *Sph*I, 12 and 13 with *Stu*I; 14 and 15 with *Xho*I. The nylon membrane was hybridized with a labeled probe derived from the 1.7-kbp *Sac*I (2)–*Sac*I (13) DNA fragment [9] containing the *pepP1* gene.

clones were substantially different with respect to restriction enzyme sites. Hybridization analysis showed one clone to contain co-cloned independent genomic *Sau*3A DNA fragments while the other clone (pCAN93) appeared to contain a single fragment of chromosomal DNA of length 6.9 kbp (Fig. 2). This clone contained the entire 3-kbp *Sph*I fragment with adjacent flanking DNA sequences, which were subsequently used to construct a plasmid deletion clone (in a modified pUC vector containing an *Mlu*I site within its multiple cloning site region). This deletion clone was further subcloned onto a bifunctional *Streptomyces/E. coli* plasmid vector, pSS12 [10] and assayed for Gly-Pro-β-naphthylamide hydrolytic ability. The phenotype of the deletion clone was clearly the same as the host strain (*S. lividans* 66) carrying the vector alone whereas the phenotype of transformants carrying pCAN93 clearly showed substantially more red color, indicating hydrolysis of the substrate.

The plasmid deletion clone was used to make an integration clone by subcloning using *Eco*RI and *Mlu*I into pINT. The integration clone was then used to transform protoplasts of the *S. lividans* MS1 strain and a strain (*S. lividans* MS2) was selected which contained specific deletions at both the *pepP1* and *pepP2* chromosomal loci. Southern hybridization experiments using chromosomal DNA from the *S. lividans* 66, MS1 and MS2 strains respectively confirmed that the expected specific DNA fragment deletions had occurred (data not shown).

Agar plate assays using Ala-Pro-β-naphthylamide confirmed that MS2 contained less activity than MS1 than *S. lividans* 66 (indicated by a reduction in the red color produced).

The nucleotide sequence of the 3-kbp *Sph*I DNA fragment was determined and is shown in Fig. 3. Comparison of the predicted amino acid sequence (Fig. 4) revealed a very significant homology to that of the *S. lividans* PepP previously described. The similarity is greater than to the *E. coli* PepP which is, in turn, significantly greater than to the next related sequences in the databases, i.e. *E. coli* PepQ and the human PepD which are both dipeptidases.

A second broad spectrum aminopeptidase homologous to the S. lividans *66 PepN*

We recently described the cloning and characterization of a *pepN* from *S. lividans* 66 [8]. During the analysis of the *S. lividans pepN* locus, we consistently observed additional hybridizing bands in chromosomal Southern hybridization experiments with *pepN* probes. In screening for other broad spectrum aminopeptidase activities, we used Ser-β-naphthylamide to screen a genomic partial *Sau*3AI library for overexpression in *S. lividans* of the ability to hydrolyze this substrate in an agar plate assay. Thirteen positive clones were observed of which the most active were *pepN* clones (11 of which were isolated). However, two identical clones (one of which was designated pCAN 100) were clearly

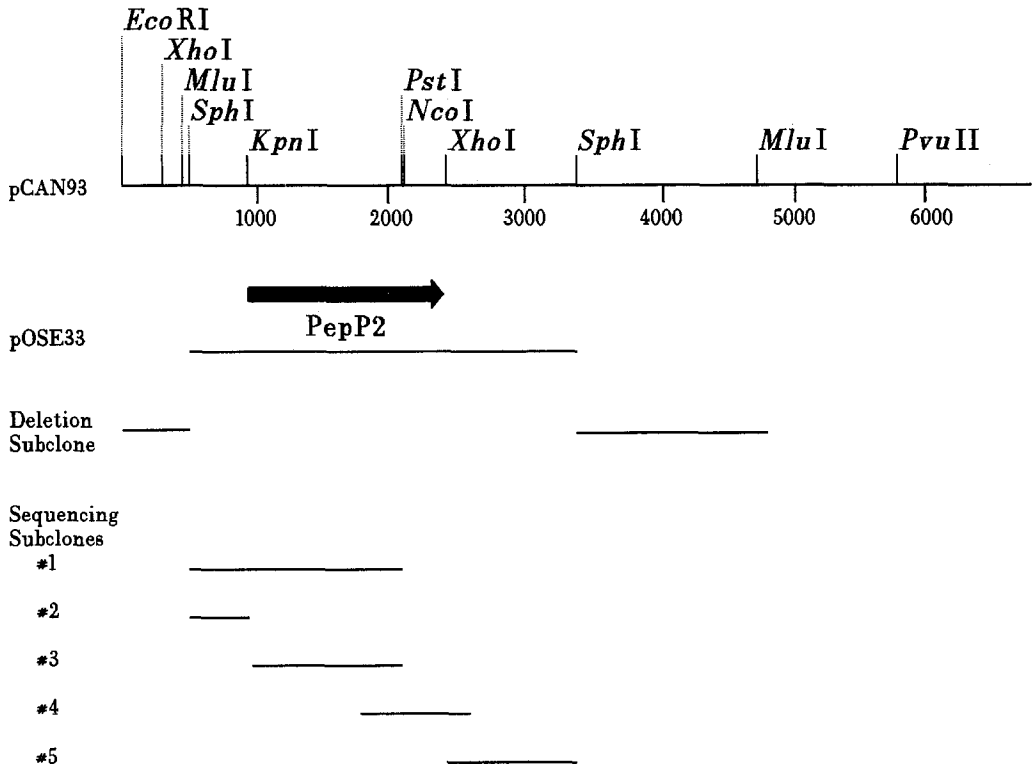

Fig. 2. Restriction enzyme site map of cloned *pepP2* DNA. pCAN93 was isolated from a *Sau*3A1 library. pOSE is a cloned chromosomal *Sph*I DNA fragment. DNA remaining in the plasmid deletion subclone is shown by a horizontal line. This plasmid showed no overproduction of aminopeptidase activity in *S. lividans*, correlating the PepP activity observed in strains containing pCAN93 to the presence of the control *Sph*I DNA fragment.

```
GGTACCGTCCGGCGGAAGTCCAGCGAAAGAAGGTGCACGACGTGTCGAACCGTCGGAAGAACAGCCTGTACCCCACGCTCTCGGCGGAGCTGTCCGCACT  100
                                                fM  S  N  R  R  K  N  S  L  Y  P  T  L  S  A  E  L  S  A  L

GATGCGCACCGGCTGGGCGGACACGGAGCGGCACGACCTCGCGCCCGCCGAGCAGGCCCCGTACGCGGCCCTCCGCCGTGCCGCGCTCTCCGCACGGTTC  200
 M  R  T  G  W  A  D  T  E  R  H  D  L  A  P  A  E  Q  A  P  Y  A  A  L  R  R  A  A  L  S  A  R  F

CCCGGCGAACGGCTGGTGGTCCCCTCGGGCAACCTCAAGGTCCGCTCGAACGACGACACCTACCCCTTCCGGTCCTACTCCGGCTACGTGCACATGACCG  300
P  G  E  R  L  V  V  P  S  G  N  L  K  V  R  S  N  D  D  T  Y  P  F  R  S  Y  S  G  Y  V  H  M  T  G

GAGACCAGGCCCGGGACGGAGCCCTGGTGCTCGAACCCCGCCCGGACGGCGGCCACGACGCCTACTGCTACCAACTGCCCCGGGACAGCCGCGACGACGA  400
  D  Q  A  R  D  G  A  L  V  L  E  P  R  P  D  G  G  H  D  A  Y  C  Y  Q  L  P  R  D  S  R  D  D  D

CGAGTTCTGGACCGGCGCCCACGCCGAACTGTGGACCGGTCGGCGCCGCTCGCTGGCCGAGTCGGAGCGGGTGCTCGGCCTGCCGTGCCGCGACGTGCGC  500
 E  F  W  T  G  A  H  A  E  L  W  T  G  R  R  R  S  L  A  E  S  E  R  V  L  G  L  P  C  R  D  V  R

ACGGCGGCCGCCGACCTGGCCGCCGTCAGCGAGGTGCGGACCCGGATCGTGCGCGGGATCGACCCCGCCCTGGAGGCGGCCGTCACCACCGACGAGGAGC  600
T  A  A  A  D  L  A  A  V  S  E  V  R  T  R  I  V  R  G  I  D  P  A  L  E  A  A  V  T  T  D  E  E  R

GCGACGCCGAGCTGGAGGACGCGCTGAGCGACCTGCGCCTGGTCAAGGACGCCTGGGAACTCGGGGAGCTGCGCAAGGCGGTGGACTCCACCGTGCGGGG  700
  D  A  E  L  E  D  A  L  S  D  L  R  L  V  K  D  A  W  E  L  G  E  L  R  K  A  V  D  S  T  V  R  G

CTTCACCGACGTCGTGGGCGAGTTGTCCCGGGCCGTCGCCTCGTCGGAGCGGTGGCTGGAGGGCACCTTCTTCCGCCGGGCACGCCTGGAGGGCAACGCG  800
 F  T  D  V  V  G  E  L  S  R  A  V  A  S  S  E  R  W  L  E  G  T  F  F  R  R  A  R  L  E  G  N  A

GTGGGCTACGGCACGATCTGCGCCGCCGGCGAGCACGCCACGATCATGCACTGGACGGACAACGACGGCCCGGTCCGCCCGGGAGACCTGCTGCTCCTGG  900
V  G  Y  G  T  I  C  A  A  G  E  H  A  T  I  M  H  W  T  D  N  D  G  P  V  R  P  G  D  L  L  L  L  D

ACGCCGGTGTGGAGACGCGCTCCCTCTACACCGCCGACGTCACCCGCACCCTGCCGATCAGCGGCACCTTCACGCCGCTCCAGCGCGAGGTGTACGACGC 1000
  A  G  V  E  T  R  S  L  Y  T  A  D  V  T  R  T  L  P  I  S  G  T  F  T  P  L  Q  R  E  V  Y  D  A

GGTGTACGAGGCCCAGGAGGCCGGGATCGCCACGGTCAAGCCGGGCGCCGCGTACCGCGACTTCCACGAGGCCGCCCAGCGCCACCTGGCGGCACGGCTG 1100
 V  Y  E  A  Q  E  A  G  I  A  T  V  K  P  G  A  A  Y  R  D  F  H  E  A  A  Q  R  H  L  A  A  R  L

GTGGAGTGGGGCTTCATCGAGGGCCCCGCCGAGCGGGCGTACGAACTGGGTCTGCAGCGCCGCTTCACCATGGCCGGCACCGGTCACATGCTGGGCCTGG 1200
V  E  W  G  F  I  E  G  P  A  E  R  A  Y  E  L  G  L  Q  R  R  F  T  M  A  G  T  G  H  M  L  G  L  D

ACGTCCACGACTGCGCGCGGGCCCGCACGGAGGAGTACGTCGAGGGCGTGCTGGAGCCGGGCATGTGCCTGACCGTCGAACCGGGCCTGTACTTCCAGGC 1300
  V  H  D  C  A  R  A  R  T  E  E  Y  V  E  G  V  L  E  P  G  M  C  L  T  V  E  P  G  L  Y  F  Q  A

GGACGACCTGACCGTGCCCGAGGAGTGGCGGGGGATCGGCGTCCGGATCGAGGACGACCTCGTCGTCACCGAGGACGGCCACGAGAATCTGTCCGCGGGC 1400
 D  D  L  T  V  P  E  E  W  R  G  I  G  V  R  I  E  D  D  L  V  V  T  E  D  G  H  E  N  L  S  A  G

CTGCCCCGCAGCGCCGACGAGGTCGAGGCCTGGATGGCCCGCTTCGCGGGCTGAGCCCGCGGACGGACTCCCGGACCTCGAG                1482
L  P  R  S  A  D  E  V  E  A  W  M  A  R  F  A  G
```

Fig. 3. Nucleotide sequence of the *pepP2* gene. The predicted protein sequence encoded by the gene is shown with the residues likely to be involved in binding of divalent metal ions shown in bold. The translation termination codon is marked with a dot. The sequence has been deposited with GenBank under the accession number L23174.

```
                    10v        20v        30v        40v        50v        60v        70v        80v        90v       100v
SLIVPEPP1    VAEELTPENPAIPETPEETEEPIKQRKNGLYPGVSDELAENMQSGWADTELHDLEPIAQAAETAARRAALSARFPGERLVIPAGNLKTRSNDTEYSFRASV
SLIVPEPP2                        VSNRRKNSLYPTLSAELSALMRTGWADTERHDLAPAEQAPYAALRRAALSARFPGERLVVPSGNLKVRSNDDTYPFRSYS
ECOPEPP                                                                    MSEISRQEFQRRRQALVEQMQPGSAALIFAAPEVTRSADSEYPYRQNS

                   110v       120v       130v       140v       150v       160v       170v       180v       190v
SLIVPEPP1    EYAYLTGNQTEDGVLVMEPEGD-GHAATIYLLPRSDRENGEF-----WLDGQGELWVGRRHSLAEAGELYGIPASDVRELAGSLREATG-PVRVVRGFDAG
SLIVPEPP2    GYVHMTGDQARDGALVLEPRPDGGHDAYCYQLPRDSRDDDEF-----WTGAHAELWTGRRRSLAESERVLGLPCRDVRTAAADLAAVSEVRTRIVRGIDPA
ECOPEPP      DFWYFTGFNEPEAVLVLIKSDDTHNHSVLFNRVRDLTADIWFGRRLGQDAAPEKLGVDRALAFSEKNQQLTQLLNGLDVVYHAQGETATADVIVNSALEKL

                   200v       210v       220v       230v       240v       250v       260v       270v       280v       290v
SLIVPEPP1    IEAALTDKVTAERDEELRV--FLSEARLVKDEFEIGELQKAVDSTVRGFEDVVKVLDRAEATSERYIEGTFFLR-ARVEGNDVGYGSICAAGPHACTLHWV
SLIVPEPP2    LEAAVTTD--EERDAELED--ALSDLRLVKDAWELGELRKAVDSTVRGFTDVVGELSRAVASSERWLEGTFFRR-ARLEGNAVGYGTICAAGEHATIMHWT
ECOPEPP      RKGSRQNL--TAPATMIDQRPVVHEMRLFKSPEEIAVLRRAGEITAMAHTRAM-EKCRPGMFETH-LEGEIHHEFNRHGARYPSYNTIVGSGENGCILHYT

                   300v       310v       320v       330v       340v       350v       360v       370v       380v       390v
SLIVPEPP1    RNDGPVRSGDLLLLDAGVETHTYYTADVTRTLPISGTYSELQKKIYDAVYDAQEAGIAAVRPGAKYRDFHDASQRVLAERLVEWGLVEGPVERVLELGLQR
SLIVPEPP2    DNDGPVRPGDLLLLDAGVETRSLYTADVTRTLPISGTFTPLQREVYDAVYEAQEAGIATVKPGAAYRDFHEAAQRHLAARLVEWGFIEGPAERAYELGLQR
ECOPEPP      ENECEMRDGDLVLIDAGCEYKGYA-GDITRTFPVNGKFTQAQREIYDIVLESLETSLRLYRPGTSILEVTGEVVRIMVSGLVKLGILKGDVDELIAQNAKR

                  400v       410v       420v       430v       440v       450v       460v       470v       480v       490v
SLIVPEPP1    RWTLHGTGHMLGMDVHDCAAARVESYVDGTLEPGMVLTVEPGLYFQADDLTVPEEYRGIGVRIEDDILVTADGNRNLSAGLPRRSDEVEEWMAALKG
SLIVPEPP2    RFTMAGTGHMLGLDVHDCARARTEEYVEGVLEPGMCLTVEPGLYFQADDLTVPEEWRGIGVRIEDDLVVTEDGHENLSAGLPRSADEVEAWMARFAG
ECOPEPP      PFFMHGLSHWLGLDVHDVG-VYGQD-RSRILEPGMVLTVEPGLYIAPDA-EVPEQYRGIGIRIEDDIVITETGNENLTASVVKKPEEIEALMVAARIQ
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Fig. 4. Comparison of the predicted amino acid sequences from the *S. lividans pepP* genes SLIVPEPP 1 and 2 respectively with *E. coli* PepP (ECOPEPP). The amino acid sequences were compared using the AALIGN program [18] in the DNASTAR [11] software. Identical residues are shown in bold type.

different to the *pepN* clones in terms of restriction enzyme sites (see Fig. 5). After growth in TSB medium, the aminopeptidase activity was shown to be located intracellularly. SDS-PAGE showed a significantly overproduced protein band of slightly larger subunit size than that seen with *PepN* clones (data not shown). Indeed, the protein was expressed well enough to allow a single *N*-terminal amino acid sequence to be observed after electrotransfer of the protein after SDS-PAGE of a crude cell-free extract to an Immobilon™ (Millipore (Canada) Ltd, Mississauga, Ontario) membrane. The sequence obtained (and named PepG) is shown in Fig. 6, and when compared to the predicted PepN sequence, shows significant homology as well as specific differences, confirming that it is a related but different protein. Selective nucleotide sequencing of the pCAN100 DNA (reading sequence from the *Pst*I site towards the *Eco*RI site) confirms similarity to the PepN amino acid sequence. Indeed, Southern hybridization experiments using

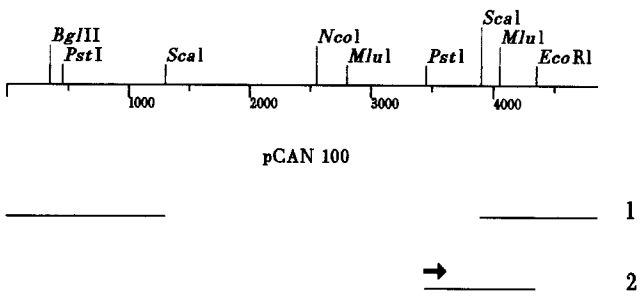

Fig. 5. Restriction enzyme site map of cloned DNA encoding the *S. lividans* PepG. The DNA remaining in a plasmid deletion clone is shown by the horizontal lines marked 1. Deletion of the *ScaI* DNA fragment was correlated with the loss of the overproduced activity to hydrolyze Ser-βnaphthylamide. The horizontal line marked 2 shows a DNA fragment which was subcloned into pT7T3 (Pharmacia) and used for DNA sequence analysis. The arrow above this fragment shows the location and direction of a potential protein encoding region predicted from this DNA sequence.

without seriously affecting the growth capabilities of such strains, provided they were supplied with sufficient quantities of amino acids and not exposed to high levels of certain peptides (which otherwise caused some toxic effects).

Little information is currently available regarding the aminopeptidases of *Streptomyces* strains, although there have been reports of leucine aminopeptidase activity in *S. lividans* [2,3] and *S.griseus* [5]. We recently described the presence of an aminopeptidase P gene and an aminopeptidase N gene in *S. lividans* [8,9]. The data presented in this paper show the presence of a second aminopeptidase P gene in *S. lividans*, which represents a minor component of the total activity in the wild type strain. This is consistent with the observation by Yoshimoto et al. [21] of a second aminopeptidase P activity in *E. coli* HB101, suggesting that multiplicity of these enzymic functions may be the rule rather than the exception.

We have also identified a second relatively broad spectrum aminopeptidase which shows significant homology to the *S. lividans* PepN. We propose the designation PepG for this

```
A)  PepG    PGENLHRDEARN
            PG.NL R:EAR:
    PepN    PGTNLTREEARQ
                  10^

B)  PepG    LQMLHAWTNSALVHYAAPDWRETGGRLLGEGALRELRXRAPGSEQQLAWARFFASVASGEADWSCCAGLLEGT
            :Q LH  .: A: :YAAP. RE:     .E::L :LR. .:GS::QLAWAR FA:.A.. .: .  .:LL:GT
    PepN    VQSLHRQVKLAIDQYAAPTAREALLTRWTEATLAHLRAAEAGSDHQLAWARAFAATARTPEQLDLLDALLDGT
           610^      620^      630^      640^      650^      660^      670^      680^
```

Fig. 6. Comparison of the amino acid sequences of the *S. lividans* PepN and PepG proteins. The amino acid sequences were compared using the AALIGN [18] program. (A) The experimentally determined sequence from direct automated Edman degradation of the partially purified PepG protein is shown. The PepN sequence is that predicted from the DNA sequence, since the purified protein produced no sequence on Edman degradation and was presumably blocked. (B) The predicted amino acid sequence of the PepP protein was derived from the DNA sequence reading from the *PstI* site rightwards, as depicted by the arrow in Fig. 5. The PepN sequence was predicted from the DNA sequence [8].

fragments of the cloned *pepN* DNA further confirmed the homology between the two DNA species along the entire length of the protein encoding regions (data not shown).

A variety of chromogenic peptide substrates were used to examine the ability of the PepG aminopeptidase to hydrolyze different peptide bonds. The enzyme preferentially hydrolyzes the peptide bond of the *N*-terminal amino acid glycine, while slowly cleaving peptide bonds of *N*-terminal serine, alanine, leucine, methionine, arginine, lysine and phenylalanine, substrates which are preferred by the *S. lividans* PepN [8]. The *S. lividans* PepG is most prominently different from the *S. lividans* PepN in its inability to hydrolyze *N*-terminal peptide bonds of basic amino acids (Table 1).

DISCUSSION

Multiple aminopeptidases with overlapping specificities have been described in *E. coli* and *S. typhimurium* [15]. The function of these intracellular peptidases is thought to be primarily in the turnover of intracellular proteins [20]. However, it was possible to construct strains carrying deletions in the genes encoding all the aminopeptidases activity since the partially purified enzyme hydrolyzes Gly-pNA significantly faster than the other substrates tested including Ser-βNA which was used to isolate the overproducing cloned *pepG* DNA. The literature regarding leucine aminopeptidase activity in bacterial species is somewhat confusing, particularly since the designation Lap has been used to describe both leucine aminopeptidases and a lysine aminopeptidase [19]. Moreover, the leucine aminopeptidase designation has been used to describe any activity which can hydrolyze Leu-pNA [3] rather than an activity with strong specificity for leucine compared to other amino acids. However, the PepN enzyme from *S. lividans* shows a strong preference for leucine, followed by arginine and lysine residues and, therefore, this species represents the major leucine aminopeptidase activity present in *S. lividans*. Conversely, the PepG described here shows much less activity against leucine substrates but a significant preference for glycine substrates.

The construction of strains deleted for these multiple aminopeptidase activities may be useful in minimizing the degradation of heterologous proteins secreted by recombinant *Streptomyces* strains and experiments are continuing toward this end.

TABLE 1

Comparison of substrate specificity between *S. lividans* PepG and *S. lividans* PepN

Substrates	S.A.[a] × 10^{-3} (μmol min^{-1} mg^{-1})	
	PepG	PepN
Gly-pNA	121	7
Ser-βNA[b]	54	75
Ala-pNA	53	95
Leu-pNA	43	160
Met-pNA	26	88
Arg-pNA	14	140
Lys-pNA	11	146
Phe-Ala-βNA[b]	10	74
Pro-pNA	0	11
Val-pNA	0	0
Benzoyl-Arg-pNA	0	0
CBZ-Arg-pNA	0	0
Asp-βNA[b]	0	0
Glu-βNA[b]	0	0
Gly-Pro-pNA	0	0
Ala-Pro-pNA	0	5

[a]S.A. = specific activity.

[b]S.A. for β-napthylamide substrates is defined as units mg^{-1} where one unit represents a change in OD_{540} of 0.001 min^{-1}.

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