Posttranslational processing of the xylanase Xys1L from *Streptomyces halstedii* JM8 is carried out by secreted serine proteases.

Running title: xylanase processing

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SUMMARY

The xylanase Xys1L from *Streptomyces halstedii* JM8 is processed extracellularly, to produce a protein of 33.7 kDa, Xys1S that retains catalytic activity but not its cellulose-binding capacity (Ruiz Arribas *et al.* Appl. Environ. Microbiol. 1997, 63:2983-2988). Here, we demonstrate that at least five serine proteases isolated from *Streptomyces* have the ability to process the xylanase Xys1L. The genes of two of these extracellular serine proteases, denominated SpB and SpC, were cloned from *S. lividans* 66 (a strain commonly used as a host for protein secretion), sequenced, and overexpressed in *S. lividans*, and both purified proteases were able to process Xys1L *in vitro*. Three other previously reported *Streptomyces* purified serine proteases, -SAM-P20, SAM-P26 and SAM-P45- also processed Xys1L *in vitro*. The involvement of serine proteases in xylanase processing-degradation *in vivo* was demonstrated by co-expression of the xylanase gene (*xysA*) and the gene coding for the serine protease inhibitor (SLPI) from *S. lividans*. Co-expression prevented processing and degradation of Xys1L and resulted in a three-fold increase in the xylanase activity present in the culture supernatant. SpB and SpC have also the capacity to process other secreted proteins such as p40, a cellulose-binding protein from *S. halstedii* JM8, but do not have any clear effect on other secreted proteins such as amylase (Amy) from *S. griseus* and xylanase Xys30 from *S. avermitilis*.

INTRODUCTION

The normal habitat of actinomycetes is soil, where they develop in mycelial form. Their ability to thrive in this habitat is based on their nutritional capabilities, conferred by a plethora of hydrolytic enzymes that allow them to recycle polysaccharides, proteins and fats, and hence, essentially all animal and plant residues. Microorganisms belonging to the genus *Streptomyces* are the most abundant representatives of this group and have a complex life cycle that responds to several signals, among which nutrient limitation plays a key role. In general, all nutrient effects are observable at morphological level, in which the onset of aerial mycelium formation coincides with both the induction of the lysis of substrate hyphae by different types of enzymes and with the production of antibiotics. (Champness, 1988; Chater, 1989; Hodgson, 2000). Carbon source accessibility can repress or trigger the expression of many genes, most of them involved in the hydrolysis of polysaccharides, although others, such as those coding for proteases (Ellaiah & Srinivasulu, 1996; Sampath & Chandrakasan, 1998) and even the genes involved in antibiotic production, are also affected (Bhatnagar *et al.*, 1988; Kojima *et al.*, 1995).

Xylan, the main component of hemicellulose, is one of the most abundant polysaccharides in soil and is totally or partially degraded by a wide number of eukaryotic and prokaryotic organisms that need several types of enzymes to achieve total degradation. Endoxylanases (EC 3.2.1.8) have attracted industrial interest due to their potential applications in very different fields, such as biobleaching of Kraft paper pulps (Bajpai, 1999; Beg *et al.*, 2000) and as an additive in animal feeding (Bajpai, 1999; Beg, *et al.*, 2000). Glycoside hydrolases have been classified in 90 families by Henrissat *et al* (<http://afmb.cnrs-mrs.fr/CAZY/index.html>) and all the endoxylanases had been included in families 10 and 11 until the recent inclusion of a xylanase from *Pseudoalteromonas haloplanktis* in family 8 (Collins *et al.*, 2002). Xylanases are most often multidomain enzymes containing one or more catalytic domains and a non-catalytic domain, usually a carbohydrate-binding domain,

1 connected to the catalytic domain by a flexible linker region rich in proline, glycine and
2 hydroxyamino acids. The role of these linker sequences is unclear and some authors have
3 reported that, together with the carbohydrate-binding domain, they play an important role in
4 the activity of xylanases against complex substrates (Black *et al.*, 1997).

5 Proteolytic processing of actinomycete cellulases and xylanases has been reported (Gilkes
6 *et al.*, 1988; Lao & Wilson, 1996; Moorman *et al.*, 1993; Ruiz-Arribas *et al.*, 1997) and
7 generally, the substrate-binding domain is removed from the original protein to release a
8 functional catalytic monodomain enzyme (Gilkes, *et al.*, 1988; Ruiz-Arribas, *et al.*, 1997). A
9 putative metalloprotease has been described as being involved in the processing of *S.*
10 *reticulii* cellulase (Moorman, *et al.*, 1993) and a serine protease has been cloned and studied
11 in *Thermobifida fusca* as implicated in cellulase processing (Lao & Wilson, 1996). The latter
12 is a member of the chymotrypsin family and is synthesized as a pre-pro protein of 375
13 amino acids that is cleaved to produce the 194-residue mature protease. Although
14 proteolysis is not necessary for the activation of cellulases or xylanases, the existence of a
15 free form of the enzyme may facilitate the hydrolysis of soluble oligosaccharides.

16 The gene *xysA* from *Streptomyces halstedii* JM8 encodes a protein of 45 kDa (Xys1L) that is
17 processed extracellularly, producing a protein of 33.7 kDa (Xys1S) after removal of a
18 cellulose-binding domain at the carboxy terminus of Xys1L. However, proteins Xys1L and
19 Xys1S have similar specific activities against xylan (Ruiz-Arribas, *et al.*, 1997). Here we
20 show that this proteolysis, which occurs in all *Streptomyces* strains used as hosts, is mainly
21 carried out by serine proteases. We cloned the genes corresponding to two of these serine
22 proteases from *S. lividans* 66, a strain used in most of our expression experiments, and here
23 demonstrate the role of both proteases in the processing of Xys1L and other extracellular
24 proteins. We also demonstrate the *in vivo* role of serine proteases in protein processing by
25 co-expression of a serine protease inhibitor and *xysA* genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. lividans* 66 or its derivative *S. lividans* 3-10 (see below) were used in all cloning experiments carried out in *Streptomyces*. *Escherichia coli* DH5 was routinely used for sub-cloning and isolation of plasmids.

The *xysA* gene was obtained from *Streptomyces halstedii* JM8 (Ruiz-Arribas, *et al.*, 1997) and expressed in *S. lividans* 66 using plasmid pJM9 (Ruiz-Arribas *et al.*, 1995). All plasmids used are indicated in Table 1.

Media and culture conditions. *E. coli* was grown in Luria Broth (LB) at 37 °C, supplemented with ampicillin (100 µg/ml) or kanamycin (50 µg/ml) when needed. R2YE and Mannitol soya flour medium (MS) agar plates were used for *Streptomyces* growth and sporulation (Kieser *et al.*, 2000). Submerged cultures of *Streptomyces* were grown in YES medium (yeast extract 0.5 %; sucrose 10.3 %; 5 mM MgCl₂, pH 7) (Ruiz-Arribas, *et al.*, 1995) supplemented with 1 % xylose. These cultures were grown in baffled flasks with 1/10 volume of medium. 5-10 µg Thiostrepton ml⁻¹ or 15 µg Neomycin ml⁻¹ was added when necessary. Cells were grown at 28 °C and 250 rpm in an orbital shaker (Adolf Kühner AG, Birsfelden, Switzerland) for as long as required for each assay (2-5 days).

DNA manipulations and transformations. *Streptomyces* total genomic and plasmid DNA was obtained as described in Kieser *et al* (2000) *S. lividans* protoplasts were obtained from cells grown for 30 hours in YEG (1 % Yeast Extract, 1 % glucose, 10,3 % sucrose pH 7) supplemented with 5 mM MgCl₂ and 0.5 % glycine. Manipulation of *E. coli* DNA was accomplished as indicated by Sambrook *et al* (Sambrook *et al.*, 1989).

Cloning of the genes encoding the *S. lividans* proteases SpB and SpC. The complete ORFs of both protease genes were amplified by PCR using oligonucleotides based on *S. coelicolor* sequences. For the equivalent gene to *S. coelicolor* protease SCF43A.19, oligonucleotides VR017 (5'-ttttttcatATGGTCGGCAGACACGCCGCC-3') and VR018 (5'-tattatcTCGAGGACCCGCTGCCACAGGGCCGG-3') were used. For the equivalent gene to

1 *S. coelicolor* protease SCD40A.16c, oligonucleotides VR019 (5'-
2 ttttttcatATGCCCCACCGACACCGACAC-3') and VR020 (5'-
3 tattatcTCGAGCACTCGCTGCCACAGCGCCGG-3') were used. The underlined parts
4 indicate the sequences for an *NdeI* site (oligonucleotides VR017 and VR019) and a *XhoI* site
5 (oligonucleotides VR018 and VR020) that were added to facilitate the cloning steps. The
6 capital letters represent the complementary sequences to SCF43A.19 (oligonucleotides
7 VR017 and VR018) and SCD40A.16c (oligonucleotides VR019 and VR020). Amplification
8 was carried out in a MJ Research thermocycler with Taq Polymerase (Dynazyme), 1.5 mM
9 MgCl₂ and 100 ng of genomic *S. lividans* as template. Cycling conditions were: 5 min at 94
10 °C, "Hot Start" and 31 cycles of 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C; final
11 extension at 72 °C for 10 min. The PCR products (1.4 kb) were purified by agarose gel
12 electrophoresis, digested with *NdeI* and *XhoI*, and cloned in pET22b(+) (under ampicillin
13 selection and with *E. coli* DH5 as host).

14 The same amplicons were cloned under control of the *xysA* promoter and were flanked by
15 *mmrt* and *fdt* transcriptional terminators using the *E. coli*/*Streptomyces* shuttle vector
16 pN702GEM3. The plasmids obtained -pVR047 and pVR048 respectively (Table 1)- were
17 transformed into *S. lividans* 66 and *S. lividans* 3.10 for protease expression.

18 **DNA sequencing.** The DNA sequences were determined in both strands using a Perkin
19 Elmer ABI Prism 377 DNA sequencer. Several oligonucleotides designed from the DNA
20 sequences collected were used to obtain the definitive DNA sequence. Manipulation was
21 done with the Gene Construction Kit™ (GCK) and analyses were made with DNA Strider
22 (Marck, 1988). Comparisons of DNA or protein sequences were carried out on-line
23 (<http://www2.ebi.ac.uk/>) with FASTA (Pearson & Lipman, 1988) and BLAST (Altschul *et*
24 *al.*, 1997). CLUSTAL W (Thompson *et al.*, 1994) was used for sequence alignment.

25 The sequences of the genes of both proteases have been deposited in the EMBL Data Bank
26 with the following accession numbers AJ496191 and AJ496192.

Isolation of serine protease inhibitor-deficient strains. Serine protease inhibitor-deficient strains (SLPI⁻) were obtained from *S. lividans* 66 following the observations of Taguchi *et al.* in the case of *S. albogriseolus* (Taguchi *et al.*, 1995 a). This author reported that SLPI strains were obtained at high frequency after protoplast regeneration due to the gene encoding this protein being located close to the end of the linear chromosome which undergoes frequent deletions (Kuramoto *et al.*, 1996). After *S. lividans* protoplast cell wall regeneration, colonies defective in differentiation were selected and protease overproduction was studied in plates containing 0.5 % skimmed milk. The putative SLPI strains were later corroborated by immunodetection, using anti-SLPI polyclonal antibodies. One of the strains obtained was denominated *S. lividans* 3.10.

Antibody generation and immunodetection The synthetic peptide RAVTLTCAPTASGTHP derived from the *Streptomyces lividans* protease inhibitor sequence (SLPI) (Accession Number M80576) was obtained from Chiron Technologies and was inoculated into two rabbits to obtain polyclonal antibodies. Immunodetection was performed on proteins transferred to Immobilon-P membranes (Millipore) with anti-rabbit alkaline phosphatase-conjugated antibodies (Promega). A 1/25000 dilution of the anti-peptide antiserum was used.

Protein purification. *S. lividans* 66 transformed with pVR052 (Table 1) was used for *S. lividans* protease inhibitor (SLPI) production. *S. lividans* 3.10, a serine protease inhibitor-deficient strain transformed with pVR047 or pVR048 (Table 1), was used to obtain the proteases SpB and SpC, respectively. Two liters of YES supplemented with 1 % xylose and 15 µg/ml of neomycin were inoculated with 200 ml of a culture of the corresponding strain, which had been grown in the same medium for 36 h and subsequently divided into 500 ml aliquots in 2-liter baffled flasks. Incubation on a rotary shaker was continued at 28 °C for 5 days. The supernatants were filtered through 0.45 µm membranes, concentrated 30 times in

a Minitan cell (Millipore) equipped with a 10 kDa cut-off polysulfone membrane, and diafiltered with 20 mM Tris-HCl, pH 7.2 or with MilliQ water.

Chromatographic procedures were performed with an ÄKTA Purifier system controlled by Unicorn®-3.1 software (Amersham Pharmacia Biotech). Absorbance was monitored at 280 nm and 205 nm.

For SLPI inhibitor purification, the concentrated supernatant was equilibrated with 20 mM Tris-HCl buffer, pH 7.2, loaded into a MonoQ (HR 5/5 anion exchange) column (Pharmacia Fine Chemicals) equilibrated with the same buffer, and eluted with a 0–0.4 M NaCl gradient in the same buffer at a flow rate of 1 ml/min. The purified inhibitor eluted as a major peak at 0.11 M NaCl.

For SpB protease purification, the concentrated sample was equilibrated with 10 mM NaOAc buffer, pH 5.0, and loaded into a MonoQ column equilibrated with the same buffer.

The protein was eluted with a 0-0.84 M NaOAc gradient in the same buffer at a flow rate of 1 ml/min. Fractions from the anionic exchange column showing biological activity (eluting between 0.52-0.73 M NaOAc) were pooled, concentrated in Centricon Plus-20 devices equipped with 10 kDa Molecular Weight Cut Off (MWCO) membranes, and diafiltered with 20 mM phosphate buffer, pH 7.2. 1.7 M ammonium sulphate (final concentration) was added prior to loading into a Phenyl-Superose column (HR 5/5 hydrophobic interaction column; Pharmacia Fine Chemicals) equilibrated with the same buffer. Elution was accomplished at a flow rate of 1 ml min⁻¹, with a decreasing-step gradient from 1.7-0.85 M ammonium sulphate in 2 ml; followed by 0.85-0.68 M ammonium sulphate in 15 ml. The xylanase-processing fractions (eluting between 0.80-0.73 M) were pooled and concentrated 15-fold in Centricon Plus-20 devices equipped with 10 kDa MWCO membranes. Aliquots of 500 µl were loaded, at a flow rate of 0.1 ml/min, into a Superdex-75B column (HR 10/30 gel filtration column; Pharmacia Fine Chemicals), equilibrated in PBS, pH 6.8. The active

fractions (eluting at 11.687 mL; approx MW 29 kDa) were pooled to afford homogeneous preparations of a single protein.

For protease SpC, the concentrated supernatant was equilibrated with 20 mM Tris-HCl buffer, pH 7.7, and loaded into a MonoQ column equilibrated with the same buffer. The protease was eluted with a 0-0.3 M NaCl gradient in the same buffer. Fractions from the anionic exchange column showing biological activity (eluting between 0.15-0.22 M NaCl) were pooled, concentrated in Centricon Plus-20 devices equipped with 10 kDa MWCO membranes, and diafiltered with 20 mM Phosphate buffer pH 7.2.

The purity of all different proteins was checked in silver-stained SDS-PAGE (Morrissey, 1981). The amino-terminal end of all of the proteins was determined using an Applied Biosystems Protein Sequenator.

Protein analysis and enzyme assays. Protein concentrations were determined using the method of Peterson (Peterson, 1977). Electrophoresis in denaturing polyacrylamide gels (SDS-PAGE) was performed as described elsewhere (Ruiz-Arribas, *et al.*, 1995). Detection of proteolytic activity (zymogram analysis) was performed after electrophoresis in 15 % acrylamide SDS-PAGE containing 0.2 % gelatin (Sigma) or 0.2 % skimmed milk (Sveltesse, Nestlé) included in the separating gel. The samples were mixed with the loading buffer and heated for 15 min at 50 °C. After electrophoresis, the gel was rinsed several times in MilliQ water and washed for 30 min in 2.5 % TritonX-100 followed by a 30 min wash in 0.2 M NaPO₄, pH 7. The gel was incubated for 2-4 hours at 45 °C in the same buffer and then stained with 0.5 % Coomassie Brilliant Blue R. Clear bands corresponding to proteolytic activity were observed after destaining.

Protease activity on Xys1L was studied in an *in vitro* experiment in which Xys1L (supernatants containing either Xys1L or pure protein) was mixed with the filtered supernatant containing the protease or with pure protease solution and incubated for different periods of time at 37 °C. Typically, 4 µg of pure Xys1L and 0.25-1 µg of pure

1 proteases were tested in each assay at a final volume of 30 μ L. When total supernatants were
2 used as the source of protease, 6 μ g of total protein was used. The effect of chemical
3 protease inhibitors was tested in this type of assay at the concentrations and under the
4 conditions described in the literature. (Díaz-Rodríguez *et al.*, 1999). The inhibitors assayed
5 were: 50-150 μ M N-Acetyl-Leu-Leu-Met-al (ALLM); 50-150 μ M N-Acetyl-Leu-Leu-
6 Norleucinal (ALLN); 10-30 μ g aprotinin ml^{-1} ; 1-30 mM ethylenediaminetetraacetic acid
7 (EDTA); 1-30 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
8 (EGTA); 1-5 mM 1,10-phenanthroline; 10-50 μ M fosforamidon; 10-50 μ g leupeptin ml^{-1} ; 2-
9 10 μ M pepstatin; 1-2 mM phenylmethylsulfonyl fluoride (PMSF); 10-30 μ g soybean trypsin
10 inhibitor (STI) ml^{-1} ; 5-15 mM N -p-tosyl-L-arginine methyl ester hydrochloride (TAME);
11 10-100 μ M N -p-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK); 25-100 μ M
12 iodoacetic acid.

13 The effect of the pure *S. lividans* proteinaceous serine protease inhibitor subtilisin type SLPI
14 was also studied at concentrations varying between 0.02 and 1 μ g.

15 Xylanase activity was measured in culture supernatants by the dinitrosalicylic acid (DNS)
16 method, using xylose as standard (Bernfeld, 1951; Biely *et al.*, 1985). One unit of enzyme
17 activity was defined as the amount of enzyme required to release 1 μ mol of reducing sugars
18 (expressed as xylose equivalents) over one minute.

19 Enzymes and reagents were purchased from Boehringer Mannheim, Promega, Bethesda
20 Research Laboratories, Pharmacia, Sigma, Merck, Panreac, Bio-Rad, Santa Cruz and
21 Ambion, and were used following the manufacturers' guidelines.

RESULTS

Extracellular serine proteases process the Xylanase Xys1L. The processing of Xys1L to produce Xys1S was mainly observed in aged cultures (older than 48 h) of the original strain *S. halstedii* JM8 and in all the *Streptomyces* strains used as heterologous hosts: *S. lividans* 66, *S. albus* J1074 and *S. parvulus* J12283 (Ruiz-Arribas, *et al.*, 1995). An *in vitro* experiment was developed as follows: the filtered supernatant from a 48 hour culture of *S. lividans* 66 bearing plasmid pJM9, which permits overexpression of xylanase Xys1L, was incubated at 37 °C for 1-3 hours in the presence of *S. halstedii* JM8 filtered supernatant. The processing of Xys1L to originate Xys1S increased with the incubation time and non-specific degradation of Xys1S was also observed after longer incubations (3 h, Fig. 1a). These processing-degradation events were not observed when the *S. halstedii* JM8 supernatant had been previously boiled for 5 minutes (Fig. 1a). The results of this experiment demonstrated the presence of extracellular thermolabile protease/s responsible for the processing-degradation observed. A similar effect was observed with all supernatants from all the *Streptomyces* strains tested (data not shown).

The effect of different chemical protease inhibitors (Materials and Methods) was studied in the same type of *in vitro* processing assay. Only PMSF, a serine protease inhibitor, elicited partial inhibition of the protease(s), (Fig. 1b). Inhibitors for other types of proteases, such as metalloproteases (EDTA, EGTA), aspartyl proteases (pepstatin) and cysteine proteases (iodoacetic acid), did not show any effect. From these results, we concluded that the main proteases involved in this process belong to the serine proteases group.

Effect of proteinaceous serine protease inhibitor (subtilisin type) on the processing of Xys1L. Since *S. lividans* 66 is a commonly used laboratory host strain for xylanase gene (*xysA*) overexpression, we studied Xys1L processing when it was produced in this host. Xylanase processing in *S. lividans* occurs at the same position (after D362) (Ruiz-Arribas, *et*

1 *al.*, 1997) as in the original *S. halstedii* strain, suggesting the presence of at least a number of
2 similar proteases in both strains. *In vitro* experiments confirmed that the four-day old filtered
3 culture of *S. lividans* 66 was also able to process Xys1L. Nevertheless, a longer incubation
4 time was required (as compared to *S. halstedii* JM8 supernatants) and only long incubation
5 times (6 h) afforded total processing of Xys1L. PMSF-induced inhibition of processing was
6 also observed, again suggesting that extracellular serine proteases were involved (data not
7 shown).

8 Since most members of the genus *Streptomyces* are a good source of proteinaceous serine
9 protease inhibitors (Taguchi *et al.*, 1993; Taguchi *et al.*, 1997), we decided to study the
10 effect of the subtilisin-type inhibitor (SLPI) from *S. lividans* on the processing of Xys1L.
11 First, twenty putative *S. lividans* SLPI-defective strains were obtained following the method
12 of Taguchi *et al.* (Materials and Methods); it was observed that most of them were also
13 subject to growth problems in complex media. Two of these strains (named 3.1 and 3.10)
14 were selected and the absence of SLPI was corroborated by Western blot analysis with anti-
15 SLPI antisera (Fig. 2a). *In vitro* experiments of Xys1L processing confirmed that the
16 supernatant from these strains processed Xys1L more efficiently than the *S. lividans* wt
17 strain, suggesting that the activity of the proteases responsible was somehow blocked by the
18 inhibitor (Fig. 2b).

19 In order to overproduce the SLPI protein, its corresponding gene (Accession Number
20 M80576) was amplified with two oligonucleotides designed to clone the complete ORF
21 (with its own RBS) under control of the xylanase *xysA* promoter (Ruiz-Arribas, *et al.*, 1997)
22 in order to obtain plasmid pVR052 (Table 1). This promoter is induced by xylose or xylan
23 and is repressed by glucose and has proved to be a very efficient promoter for homologous
24 and heterologous gene expression in *Streptomyces* (Díaz *et al.*, unpublished). The production
25 of SLPI was accomplished as indicated in Materials and Methods and the protein was
26 partially purified (up to 99 %). The effect of SLPI was studied in *in vitro* experiments in

1 which pure Xys1L (Ruiz-Arribas, *et al.*, 1997) was incubated with the filtered supernatant
2 from *S. lividans* 66 and different amounts (1-10 µg) of the purified protease inhibitor. The
3 presence of inhibitor partially reduced the processing of Xys1L (data not shown). Therefore,
4 proteinaceous subtilisin serine protease inhibitors partially inhibit the serine proteases
5 responsible for Xys1L processing.

6 **Coexpression of xylanase and protease inhibitor genes increase xylanase production.**

7 The effect of co-expressing the xylanase gene (*xysA*) and the gene that encodes the protease
8 inhibitor SLPI (*slpI*) was also studied. A transcriptional fusion in which the *xysA* promoter
9 controls the expression of both genes - *xysA* and *slpI*- was constructed. This fusion was
10 cloned into the bifunctional plasmid pN702GEM3 (Table 1) flanked by transcriptional
11 terminators to obtain plasmid pVR011 (Table 1 and Fig. 2c). As a control, the same vector
12 containing only the *xysA* gene (plasmid pNAMP7) was used. The supernatants from several
13 independent clones were analyzed by SDS-PAGE and their xylanase activity was measured.
14 The positive effect of co-expressing both genes as compared to the results corresponding to
15 cultures expressing only the *xysA* gene was observable at two levels: 1) a reduction in
16 xylanase Xys1L processing, and 2) an increase in the amount of xylanase produced, (Fig.
17 2d). Xylanase activity measurements revealed that the supernatants co-expressing *xysA* and
18 *slpI* displayed three-fold more xylanase activity (480 U ml⁻¹) than those expressing *xysA*
19 alone (150 U ml⁻¹). Therefore, this inhibitor has two positive effects that should yield a
20 higher total amount of xylanase, first by blocking Xys1L processing to produce Xys1S, and
21 then by reducing the proteolysis-degradation undergone by this protein.

22 **Cloning of two serine proteases from *S. lividans* involved in Xys1L processing.** The role
23 of a serine protease in the processing of cellulases has been described in *Thermomonospora*
24 *fusca* (now *Thermobifida fusca*), where the protease encoded by the *tfpA* gene cleaves its
25 cellulases into many different isoenzymes (Calza *et al.*, 1985; Lao & Wilson, 1996). A
26 BLAST search (Altschul, *et al.*, 1997) with the sequence of this protease (Accession

1 Number O86984) against the *S. coelicolor* data bank
2 (http://www.sanger.ac.uk/Projects/S_coelicolor/) detected two different ORFs with clear
3 similarity: ORF SCF43A.19 (Accession Number Q9XA96) shows 45 % identity (58 %
4 similarity), and ORF SCD40A.16c (Accession Number Q9L0J5), which has 46 % identity
5 (59 % similarity). Two oligonucleotides were designed for each gene (see Materials and
6 Methods) and both ORFs were amplified by PCR from *S. lividans* 66 genome and cloned in
7 pET22b(+). The complete DNA sequences of these ORFs were obtained and compared with
8 the *S. coelicolor* Data Bank. The proteases encoded by *S. lividans* genes were denominated
9 SpB (similar to SCF43A.19) and SpC (similar to SCD40A.16c). The SpB ORF is 458 amino
10 acids long and is almost identical (99 %) to SCF43A.19 from *S. coelicolor*, which is also
11 458 aa long. Two amino acid changes were observed: one located in residue 75 -L in
12 SCF43A.19 and P in SpB,- and another in residue 341, E in SCF43A.19 and G in SpB. Only
13 this latter change is present in the mature protein (see below). SpC is also 458 aa long and its
14 homologue (98 %) in *S. coelicolor* is SCD40A.16c (463 aa). The changes observed between
15 both ORFs are residue 66, L in SCD40A.16c and I in SpC; residue 122, I in SCD40A.16c
16 and V in SpC; residue 244, G in SCD40A.16c and D in SpC, and residue 377, E in
17 SCD40A.16c and G in SpC. The most significant change is a five amino acid deletion
18 between residues 409 and 413, corresponding to the amino acid repeat GDDGG that is
19 present three times in *S. coelicolor* SCD40A.16c and only twice in SpC from *S. lividans*.
20 These amino acids are located at a linker region that connects the protease catalytic domain
21 with a chitin-binding domain present at the C terminus of all these proteins. That domain is
22 47 amino acids long and shows similarity to chitin binding domains present in several
23 chitinases. Chitin binding domains in proteases have been previously described for protease
24 C from *S. griseus* (Sidhu *et al.*, 1994). The complete ORF of this protease shares 55 %
25 identity with proteases SpB and SpC.

1 In order to express both ORFs in *S. lividans*, they were cloned under control of the *xysA*
2 promoter in the bifunctional plasmid pN702GEM3 to afford plasmids pVR047 and pVR048
3 (Table 1), which were transformed into *S. lividans* 66 (wt strain) and *S. lividans* 3.10 (SLPI
4 strain). Protease production in both strains was studied by SDS-PAGE in supernatants from
5 several independent clones. No differential bands were detected by Coomassie Blue staining
6 when compared to transformants carrying an empty vector. However, several active protease
7 bands were detected in zymograms carried out in renatured SDS-PAGE containing gelatin or
8 skimmed milk, in which the samples were not boiled. This diversity of active bands was
9 identical in both host strains (wt and SLPI) (data not shown).

10 *In vitro* experiments using filtered supernatants from *S. lividans* 3.10 transformants
11 expressing SpB or SpC confirmed that Xys1L was more efficiently processed by them than
12 by the supernatant from the same strain transformed with the empty vector (Fig. 3a).

13 Both proteases were purified as indicated in Materials and Methods. Four milligrams of pure
14 SpB and five of SpC were obtained from 2 liters of culture. In SDS-PAGE, both proteases
15 failed to run according to the sequence-predicted sizes (458 aa), suggesting the existence of
16 pre-pro proteins, as is common for this type of protein (Fig. 3b). The N-terminus determined
17 for both of them starts in the EDLV sequence in SpB and in the YDLR sequence in SpC. In
18 both proteins, the first residue obtained corresponded to residue 207. SDS-PAGE of both
19 pure proteins revealed a single band of 31-33 kDa for protease SpB and a band of about 23-
20 25 kDa for protease SpC. Zymogram analyses of both pure proteases, in which the samples
21 were not boiled, detected several active bands for both of them (Fig. 3c). Zymograms with
22 boiled samples did not reveal protease activity (data not shown).

23 The observation of *in vitro* Xys1L processing with SpB or with SpC pure proteases
24 demonstrated that both were able to process Xys1L efficiently. However, SpB was more
25 active since 0.25 µg of protease sufficed to process 4 µg of Xys1L almost totally after 3
26 hours of incubation, while 0.50 µg of SpC were required to obtain a similar extent of

processing (Fig. 3d lane 0). Partial biochemical characterization of both proteases was accomplished using Xys1L as substrate. Both were more active at 50 °C than at 37 °C (the temperature normally used in our *in vitro* experiments) and the presence of 10 mM CaCl₂, or 10mM MgCl₂ in the reaction mixture failed to affect their activity (data not shown). The inhibitory effect of pure serine protease inhibitor SLPI (from 0.02 to 1 µg) increased with the amount of SLPI added in the experiment and 0.5 µg were sufficient to block the processing of Xys1L (Fig. 3d).

The effect of other inhibitors such as 2 mM PMSF, 30 µg aprotinin ml⁻¹, 30 µg STI ml⁻¹, 100 µM TLCK, 30 µg leupeptin ml⁻¹, 10 µM pepstatin, 4.5 mM 1,10-phenanthroline and 30 mM EDTA were also studied. As expected, both proteases were totally inhibited by PMSF, although, SpB was almost totally inhibited by aprotinin while SpC was not. Among the other inhibitors studied only leupeptin shows partial inhibition of SpB and does not have any clear effect on SpC activity (at the concentration assayed) (Fig. 3e).

All these experiments demonstrated that both serine proteases are involved in the processing of Xys1L and that both respond similarly to the presence of the inhibitors SLPI and PMSF but not to the presence of other inhibitors such as aprotinin and leupeptin. These small differences in their inhibition profiles suggest that SpB and SpC are similar but not absolutely equivalent.

Other serine proteases from *Streptomyces* also process Xys1L. Now that the *S.coelicolor* genome has become available, at least 12 proteins have been identified as serine proteases by sequence similarity. Some homologues of these proteases have been cloned from different *Streptomyces* strains. Among them, SAM-P20 (Taguchi *et al.*, 1995 b), SAM-P26 (Taguchi *et al.*, 1998) and SAM-P45 (Suzuki *et al.*, 1997) from *S. albogriseolus* have been studied. Pure enzymes (a SAM-P20 homologous isolated from *S. coelicolor* Müller, SAM-P26 and SAM-P45 from *S. albogriseolus*), provided by Taguchi, were used to study the processing effects on Xys1L in *in vitro* experiments. All of them were able to process the

1 xylanase in a similar manner to SpB and SpC (Fig. 4a). Because these three serine proteases
2 interact with the subtilisin inhibitor of *S. albogriseolus*, homologous to the SLPI inhibitor
3 from *S. lividans*, we conducted studies aimed at determining whether pure SLPI might block
4 their activity on Xys1L, observing that the proteolytic activity was blocked by the presence
5 of this inhibitor.

6 Commercial serine proteases from different sources, such as subtilisin A from *Bacillus*
7 *licheniformis* (SIGMA P5380) and a serine protease from *Aspergillus* (SIGMA P4032),
8 completely and non-specifically degraded the xylanase even after short incubation periods
9 and at low protease concentrations (data not shown).

10 These results suggest that Xys1L processing is not carried out by a single enzyme but it is,
11 instead, susceptible to the action of a variety of *Streptomyces* serine proteases, which are
12 probably produced differentially, depending on the culture conditions.

13 **Effects of SpB and SpC on the processing of other extracellular proteins.** Currently, our
14 group is also studying the production of other proteins in *S. lividans* and some of them have
15 also been found to undergo post-translational modifications. We therefore tested the effects
16 of *S. halstedii* JM8 supernatant and pure SpB and SpC proteases on the following proteins:
17 p40, a protein with no known catalytic activity and with a cellulose binding domain at the
18 carboxy terminus, isolated from *S. halstedii* JM8 (Garda *et al.*, 1997) that is structurally
19 similar to the xylanase Xys1L; Amy, an α -amylase from *S. griseus* IMRU3570 (Vigal *et al.*,
20 1991), and Xys30, a xylanase from *S. avermitilis* (NCBI AAD32560). *In vitro* experiments
21 were carried out mixing *S. lividans* supernatants in which each protein of interest had been
22 overexpressed with the *S. halstedii* JM8 supernatant or with pure SpB or SpC proteases. The
23 *S. halstedii* JM8 supernatant was able to process and degrade all the proteins studied. Both
24 proteases were able to process p40, producing a protein of 35 kDa that corresponded to the
25 formerly named p35 (Garda, *et al.*, 1997). This protein had previously been observed in
26 supernatants of aged cultures (older than 48 h) overexpressing the p40 gene and corresponds

1 to a truncated form in which the CBD has been removed. A weak partial processing of the
2 amylase from *S. griseus* was observed but no effect on the xylanase Xys30 from *S.*
3 *avermitilis* was observed when pure SpB or SpC proteases were used (Fig. 4b). These results
4 clearly indicate that the serine proteases studied are active in the processing and/or
5 degradation of extracellular proteins and that their role should be considered when studying
6 the overexpression of proteins of interest in *Streptomyces* hosts.

DISCUSSION.

Proteolysis has been described as the origin of multiple isoenzyme forms in different microorganisms (Gilkes, *et al.*, 1988; Lao & Wilson, 1996; Moorman, *et al.*, 1993). In cellulases and xylanases, this process normally occurs at the linker region that joins the catalytic and the substrate-binding domains and originates free forms of the enzyme able to hydrolyse soluble oligosaccharides generated after a first attack on the substrate.

In actinomycetes, Moormann *et al* (1993) described the purification of a 36 kDa putative metalloprotease involved in the processing of an 82 kDa avicelase from *S. reticuli* (Cell1) (Moorman, *et al.*, 1993). The protease cleavage site is located at the beginning of the catalytic domain of the enzyme and generates two fragments: an amino terminal inactive fragment of 40 kDa that contains the cellulose-binding domain, and a carboxy terminal of 45 kDa that retains the catalytic activity and even exerts increased activity against *p*-nitro-phenyl- β D-cellobioside (Moorman, *et al.*, 1993). Serine proteases have been reported to be involved in the processing of glucanases in two different actinomycetes. Thus, processing of the endoglucanase CenA and the exoglucanase Cex from *Cellulomonas fimi* is carried out by the same serine protease that cuts at the linker region of both enzymes (Gilkes, *et al.*, 1988). No further investigations concerning this protease have been published and it is therefore not possible to compare it with those studied here. The role of serine proteases in the processing of cellulases has been documented in *T. fusca*, for which Lao and Wilson (Lao & Wilson, 1996) described the cloning and expression of a protease gene (*tfpA*) in *S. lividans*. This 368-amino acid protease, which might be included in the chymotrypsin family, is secreted as a pre-pro protein with 194-residues in its mature form. The two genes cloned here encode proteases sharing about 45 % identity with the catalytic domain of the *T. fusca* protease and with the *S. griseus* protease C (Sidhu, *et al.*, 1994).

Proteases SpB and SpC are encoded as two proteins of 458 amino acids that have putative signal peptides at the N-terminus of 31 aa for SpB and 32 aa for SpC, followed by a

propeptide up to amino acid 207, which is eliminated in both cases to produce the mature proteins. The sequences around the residue 207 PRVL EDLV for SpB and PRTF YDLR for SpC are similar to the processing sequences of *S. griseus* serine proteases A, B, C and D. The putative catalytic triad corresponds to aminoacids H242, D270 and S351, which are included in conserved regions, but further investigations would be necessary to confirm their role as part of the active center. Both *S. lividans* proteases (SpB and SpC) and their homologues in *S. coelicolor* have a carboxy terminal domain of 47 amino acids, similar to those of chitin-binding domains present in several chitinases. The presence of this type of domain has previously been described in protease C from *S. griseus* and its putative role was explained on the basis of the coexistence of chitin and proteins in insect cuticles. It is possible that the efficient degradation of chitin would require the collaboration of proteases recruited through their chitin-binding domain (Sidhu, *et al.*, 1994).

The processing of Xys1L is not exclusive to the proteases SpB and SpC or their homologues, depending on the host. Here we observed that another three serine proteases isolated from a different strain of *Streptomyces*, SAM-P20, SAM-P26 and SAM-P45, also process Xys1L. All five proteases have homologues in the *S. coelicolor* genome, whose sequence has recently been completely determined (Bentley *et al.*, 2002). Proteases SpB and SpC have almost identical proteins encoded by ORFs SCF43A.19 and SCD40A.16c respectively. Protease SAM-P20 is 85 % identical to ORF SCI11.35c; SAM-P26 is 85 % identical to SCI11.30c, and SAM-P45 shares 43 % identity with the proteins encoded in ORFs SCF51A.10 and SCC24.17c, which have been described as putative secreted peptidases. Another eight ORFs in the *S. coelicolor* genome have been described as putative serine proteases: namely, 2SCG61.37c; SCI35.29c; SC8E4A.07; SCH17.01c; SCH5.30; SC3C3.08; SC3C3.17c, and SC10G8.13c. The multiplicity of enzymes with overlapping activities could be explained in terms of a different expression pattern that permits the expression of one or another gene, depending on the growth conditions. In the case of

1 proteases, the effect of carbon or nitrogen sources on the differential expression of the
2 corresponding genes has been described (reviewed in Hodgson, 2000). Study of the
3 expression patterns of these ORFs and their role in the processing and degradation of
4 overproduced extracellular proteins will be one of our objectives in a forthcoming work.
5 Meanwhile, co-expression of serine protease inhibitors together with target proteins might
6 be a useful approach in biotechnological applications where a target protein is processed or
7 degraded by endogenous proteases of the serine protease family.

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FIGURE LEGENDS

Figure 1. Coomassie Blue-R stained SDS-PAGE of: **a.-** *In vitro* processing of Xys1L to Xys1S by filtered supernatants of *S. halstedii* JM8 (*S.h.* JM8 and *S.h.* JM8/boiled). XC (xylanase control) is the supernatant from a *S. lividans* strain overexpressing Xys1L. This sample (XC) was incubated at 37 °C for 3 h without *S. halstedii* JM8 supernatant. 6 µg of protein were loaded in each lane. Positions of molecular weight markers are indicated at the right. **b.-** Effect of the addition of 1 mM PMSF on the *in vitro* processing of Xys1L by a filtered supernatant of *S. halstedii* JM8. XC: xylanase control. Samples were incubated at 37 °C for 2 h. 3 µg of protein were loaded in each lane.

Figure 2. a.- Immunodetection of the protease inhibitor (SLPI) in culture supernatants of *S. lividans* 66 (*S. liv.* wt), *S. lividans* 3.1 and *S. lividans* 3.10 (*S. liv.* 3.1 and *S. liv.* 3.10) are two SLPI deficient strains obtained as described in the text. **b.-** Coomassie Blue-R stained SDS-PAGE showing the *in vitro* processing of Xys1L by a filtered supernatant of the three strains to produce Xys1S. XC is the *S. lividans* supernatant overexpressing Xys1L used in the *in vitro* experiment. Three µg of total protein were loaded in each lane. Incubation was for 3 h at 37 °C. **c.-** Schematic representation of the construction present in the plasmid pVRO11 (Table 1). Both ORFs, corresponding to xylanase and inhibitor, are expressed under the control of the xylanase promoter (*xysAp*). T1 and T2 are the *mmrt* and *fdt* transcriptional terminators respectively. **d.-** Coomassie Blue-R stained SDS-PAGE showing the proteins present in 10 µl of supernatant of *S. lividans* 66 containing the plasmid pNAMP7, which only expresses the xylanase gene, and the same strain containing pVRO11, expressing the xylanase and the inhibitor.

Figure 3.- a.- Coomassie Blue-R stained SDS-PAGE showing the *in vitro* processing of Xys1L by filtered supernatant from the three *S. lividans* 3.10 transformed with vector

pN702GEM3 (V), with pVRO47 (SpB) or pVRO48 (SpC). XC is the *S. lividans* supernatant overexpressing Xys1L used in the *in vitro* experiment. Incubation was carried out at 37 °C for 1h. **b.-** Coomassie Blue-R stained SDS-PAGE showing the purity of the proteases SpB and SpC after the FPLC purification indicated in the text. **c.-** Protease zymogram on gelatin with pure SpB and SpC (sample preparation was not completely denaturing and the activity bands give no indication of real molecular weight). **d.-** Coomassie Blue-R stained SDS-PAGE showing the effect of different amounts of SLPI protease inhibitor (from 0 to 1 µg) on the *in vitro* processing of pure xylanase Xys1L (Xys1L) by pure SpB (0.25 µg/assay) or pure SpC (0.5 µg/assay). Incubation was carried out for 3 h at 37 °C. **e.-** Coomassie Blue-R stained SDS-PAGE showing the effect of different protease inhibitors on the processing of pure Xys1L by pure proteases SpB (0.25 µg/assay) or SpC (0.5 µg/assay). Control (0) is the processing obtained with these proteases without any inhibitor. The inhibitor concentrations used were: 2 mM PMSF; 30 µg aprotinin ml⁻¹; 30 µg STI ml⁻¹; 100 µM TLCK; 50 µg leupeptin ml⁻¹; 10 µM pepstatin; 4.5 mM 1,10-phenanthroline and 30 mM EDTA. Incubation was carried out at 37 °C for 3 h.

Figure 4. a.- Coomassie Blue-R stained SDS-PAGE showing the *in vitro* processing of Xys1L by different *Streptomyces* proteases. XC is the xylanase control. *S.h.* JM8 shows the processing by the filtered supernatant of the *S. halstedii* JM8 strain; 3 µg of total protein were used. SpB: pure protease from *S. lividans*. SAM-P20: protease isolated from *S. coelicolor* Müller homologous to SAM-P20 from *S. albogriseolus*; SAM-P26: protease from *S. albogriseolus* (Taguchi, *et al.*, 1998); SAM-P45: protease from *S. albogriseolus* (Suzuki, *et al.*, 1997). 0.25 µg of the corresponding pure proteases were used in each assay. Incubation was carried out for 3 h at 37 °C. **b.-** Coomassie Blue-R stained SDS-PAGE showing the *in vitro* processing-degradation of different secreted proteins, p40 (Garda, *et al.*, 1997), Amy (Vigal, *et al.*, 1991) and Xys30 (NCBI AAD32560), by *S. lividans* SpB and

1 SpC proteases. XC is the *S. lividans* supernatant overexpressing the corresponding protein
2 used in the *in vitro* experiment. *S.h.* JM8, is the processing originated by the filtered
3 supernatant from this strain, used as a positive control; 3 µg of total protein were used. SpB
4 and SpC indicate the processing obtained with 0.25 µg of pure SpB or with 0.5 µg of pure
5 SpC. Incubation was carried out for 3 h at 37 °C.
6

Table 1: Plasmids used.

Plasmid	Description	Reference
pET22b(+)	<i>E. coli</i> expression vector, with T7 promoter control and poly-His tag at the C-terminus of ORFs cloned on it.	Novagen
pJM9	pIJ702 derivative containing the <i>xysA</i> gene as originally cloned in <i>S. halstedii</i> JM8	(Ruiz-Arribas, <i>et al.</i> , 1995)
pN702GEM3	<i>E. coli-Streptomyces</i> shuttle vector. It contains the pIJ702 origin of replication (for <i>Streptomyces</i>), the bifunctional neo/kan resist marker from Tn5 (for both <i>E. coli</i> and <i>Streptomyces</i>), the pUC origin of replication (for <i>E. coli</i>) and a polylinker derived from pGEM3Z (Promega).	This Work
pNAMP7	pN702GEM3 derivative containing the <i>xysA</i> (from <i>S. halstedii</i> JM8) expressed from its own promoter (<i>xysAp</i>) and flanked by transcriptional terminators (<i>mmrT</i> and <i>fdt</i>).	This Work
pVR011	pN702GEM3 derivative containing the <i>xysA</i> (from <i>S. halstedii</i> JM8) and <i>slpI</i> (from <i>S. lividans</i> 66) genes as a transcriptional fusion under the <i>xysAp</i> control. The <i>slpI</i> gene retains its ribosomal binding site. The expression cassette is flanked by transcriptional terminators (<i>mmrT</i> and <i>fdt</i>).	This Work
pVR033	pET22b(+) derivative containing the <i>S. lividans</i> protease <i>spB</i> gene obtained by PCR with the oligonucleotides VR017 and VR018. The oligonucleotides incorporate a <i>NdeI</i> and a <i>XhoI</i> restriction sites at ATG and C-terminus respectively to facilitate cloning in pET22b under T7 promoter control and with an in-frame poly-Histidine tag.	This Work
pVR034	pET22b(+) derivative containing the <i>S. lividans</i> protease <i>spC</i> gene obtained by PCR with the oligonucleotides VR019 and VR020. The oligonucleotides incorporate a <i>NdeI</i> and a <i>XhoI</i> restriction sites at ATG and C-terminus respectively to facilitate cloning in pET22b under T7 promoter control and with an in-frame poly-Histidine tag.	This Work
pVR047	pN702GEM3 derivative with the ORF corresponding to <i>spB</i> from <i>S. lividans</i> under the control of <i>xysA</i> promoter and flanked by transcriptional terminators (<i>mmrT</i> and <i>fdt</i>). The <i>SpB</i> protease is produced with a poly-Histidine tag at the C-terminus.	This Work
pVR048	pN702GEM3 derivative with the ORF corresponding to <i>spC</i> from <i>S. lividans</i> under the control of <i>xysA</i> promoter and flanked by transcriptional terminators (<i>mmrT</i> and <i>fdt</i>). The <i>SpC</i> protease is produced with a poly-Histidine tag at the C-terminus.	This Work
pVR052	pN702GEM3 derivative. It contains the <i>slpI</i> gene for the <i>S. lividans</i> subtilisin-like inhibitor (SLPI) expressed from the <i>xysA</i> promoter. The gene was amplified with oligonucleotides flanking the RBS coding region of the gene and put under the control of <i>xysA</i> promoter flanked by transcriptional terminators (<i>mmrT</i> and <i>fdt</i>).	This Work

Figure 1. Fernández-Abalos et al.

Figure 2. Fernández-Abalos et al.

S. lividans 3.10

Figure 3. Fernández-Abalos et al.

Figure 4. Fernández-Abalos et al.