Surface plasmon resonance-based interaction studies reveal competition of *Streptomyces lividans* type I signal peptidases for binding preproteins

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Type I signal peptidases (SPases) are responsible for the cleavage of signal peptides from secretory proteins. *Streptomyces lividans* contains four different SPases, denoted SipW, SipX, SipY and SipZ, having at least some differences in their substrate specificity. In this report *in vitro* preprotein binding/processing and protein secretion in single SPase mutants was determined to gain more insight into the substrate specificity of the different SPases and the underlying molecular basis. Results indicated that preproteins do not preferentially bind to a particular SPase, suggesting SPase competition for binding preproteins. This observation, together with the fact that each SPase could process each preprotein tested with a similar efficiency in an *in vitro* assay, suggested that there is no real specificity in substrate binding and processing, and that they are all actively involved in preprotein processing *in vivo*. Although this seems to be the case for some proteins tested, high-level secretion of others was clearly dependent on only one particular SPase demonstrating clear differences in substrate preference at the *in vivo* processing level. Hence, these results strongly suggest that there are additional factors other than the cleavage requirements of the enzymes that strongly affect the substrate preference of SPases *in vivo*.

INTRODUCTION

Proteins designed for transport across the bacterial cytoplasmic membrane are generally synthesized in the cytoplasm as a precursor with a cleavable signal peptide, which targets the precursor proteins to the translocase (Fekkes & Driessen, 1999). Preproteins can be targeted to two different translocases by the Sec/SRP (signal recognition particle)-dependent pathway and the Tat (twin-arginine translocation)-dependent pathway. The most important difference for both pathways is the translocation-competent state of the preprotein. Whereas for Sec-dependent secretion, preproteins must be in an unfolded state, the Tat pathway accommodates the translocation of folded proteins (Berks et al., 2003). Upon translocation across the cytoplasmic membrane, the signal peptide is enzymically removed by a type I signal peptidase (SPase), a membrane-bound endopeptidase (reviewed by Paetzel et al., 2000 and van Roosmalen et al., 2004), thereby releasing the mature protein at the outer side of the membrane (Dalbey & Wickner, 1985).

Whereas the presence of only one chromosomal type I SPase seems to be typical for many Gram-negative bacteria, the occurrence of multiple type I SPases within a single strain is commonly observed in Gram-positive bacteria. The largest number of type I SPases has, thus far, been found in *Bacillus cereus*, which contains genes for seven paralogous enzymes of this type. *Bacillus anthracis* and *Bacillus subtilis* both contain five chromosomally encoded type I SPases. In other Gram-positive bacteria, like *Streptomyces lividans* (Schacht et al., 1998; Parro et al., 1999), *Streptomyces coelicolor*, *Clostridium perfringens* and *Bacillus anhydriliquefaciens* (Chu et al., 2002), four different chromosomally encoded type I SPases have been identified. Furthermore, *Lactococcus plantarum*, *Listeria monocytogenes* and *Staphylococcus epidermidis* have three SPase-encoding genes, whereas *Bacillus halodurans*, *Staphylococcus aureus* (*spsA* and *spsB*) (Cregg

Abbreviations: SPase, type I signal peptidase; SPR, surface plasmon resonance; Tat, twin-arginine translocation.
et al., 1996) and Staphylococcus carnosus (sipA and sipB) have two SPase-encoding genes. Lactococcus lactis, Streptococcus pneumoniae (Zhang et al., 1997), Streptococcus mitis, Mycobacterium leprae and Mycobacterium tuberculosis are examples of Gram-positive bacteria having only one SPase-encoding gene.

In bacteria that have one type I SPase, this enzyme is essential for the processing of all secretory preproteins. When multiple type I SPases are present, like in B. subtilis and S. lividans, none of the corresponding genes was found to be essential for cell viability by itself (Tjalsma et al., 1997, 1998; Palacin et al., 2002). This indicates that the paralogous enzymes can at least partly complement each other. However, clear differences in SPase substrate specificity have been shown in B. subtilis and S. lividans. In B. subtilis, depletion of both SipS and SipT resulted in a dramatic defect in preprotein processing and secretion. Both SPases serve to increase the processing of the same preproteins was investigated by in vitro preprotein processing was investigated by monitoring the accumulation of these proteins in the culture medium of S. lividans wild-type and single SPase deletion mutants, high-level secretion of several proteins was clearly dependent upon one specific SPase.

Table 1. Oligonucleotides used in this work

Restriction endonuclease cleavage sites are in bold, 6-His-encoding sequences are in italic and cMyc epitope encoding sequences are underlined. Optimized Shine–Dalgarno sequences are underlined and in italic.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’–3’)</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>CelAHis5</td>
<td>TACATATGCAACAT-catCACCAACAAAGGCTTTTCC</td>
<td>NdeI</td>
</tr>
<tr>
<td>CelA</td>
<td>TACCATCCGACATCATTcatCACCAACAAAGGCTTTTCC</td>
<td>BamHI</td>
</tr>
<tr>
<td>CelBHis5</td>
<td>TACATATGCAACATCATTcatCACCAACAAAGGCTTTTCC</td>
<td>NdeI</td>
</tr>
<tr>
<td>CelB</td>
<td>TACATATGCAACATCATTcatCACCAACAAAGGCTTTTCC</td>
<td>NdeI</td>
</tr>
<tr>
<td>XlnBHis5</td>
<td>TACATATGCAACATCATTcatCACCAACAAAGGCTTTTCC</td>
<td>NdeI</td>
</tr>
<tr>
<td>XlnB</td>
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<td>NdeI</td>
</tr>
<tr>
<td>XlnCHis5</td>
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</tr>
<tr>
<td>XlnC</td>
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</tr>
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<td>DagAHis5</td>
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<td>NdeI</td>
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<td>DagA3</td>
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<td>NdeI</td>
</tr>
<tr>
<td>MelCHis5</td>
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<td>NdeI</td>
</tr>
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<td>MelC3</td>
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<td>NdeI</td>
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<tr>
<td>TlmHis5</td>
<td>AAGAAATCCATATGCAACATCATTcatCACCAACAAAGGCTTTTCC</td>
<td>EcoRI, NdeI</td>
</tr>
<tr>
<td>Tm3</td>
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<td>EcoRI</td>
</tr>
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<td>XlnBrbsEco</td>
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<td>EcoRI</td>
</tr>
<tr>
<td>XlnBhin</td>
<td>TACATATGCAACATCATTcatCACCAACAAAGGCTTTTCC</td>
<td>HindIII</td>
</tr>
<tr>
<td>CelArbsEco</td>
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<td>EcoRI</td>
</tr>
<tr>
<td>CelAhin</td>
<td>TACATATGCAACATCATTcatCACCAACAAAGGCTTTTCC</td>
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<tr>
<td>CelBhin</td>
<td>TACATATGCAACATCATTcatCACCAACAAAGGCTTTTCC</td>
<td>HindIII</td>
</tr>
</tbody>
</table>
Vector constructions

Plasmids used for secretory production of proteins in *S. lividans*. For the secretory production of CelA, CelB and XlnB, the genes were amplified by PCR using chromosomal DNA as template with the primers CelArbsEco/CelAHin, CelBrbsEco/CelBHin and XlnBrbsEco/XlnBHin, respectively. The upstream primer contains an optimized Shine–Dalgarno sequence and an EcoRI restriction site, while the downstream primer contains a HindIII restriction site. Next, the obtained EcoRI–HindIII DNA fragments were ligated into pBSVsi after the *vsi* promoter. The resulting pBSVsCelA, pBSVsCelB and pBSVsXlnB plasmids were digested with XbaI and HindIII to give DNA cassettes containing the *vsi* promoter and CelA/CelB/XlnB-encoding sequences that were subsequently ligated into the corresponding sites of pFD666, resulting in pFDCelA, pFDCelB and pFDXlnB, respectively.

For secretory production of *Tm*, pAX5a (Fass & Engels, 1996) was treated with EcoRl/Klenow polymerase/HindIII to give a DNA fragment containing the *ermEup* promoter and the *tm* gene. Ligation of this fragment into BamHI/Klenow polymerase-treated HindIII-digested pFD666 resulted in pFDTm.

To construct a plasmid overexpressing XlnC, pH486XlnC (Schaerlaekens et al., 2004) was digested with HindIII and BamHI to give a DNA cassette containing the *vsi* promoter and XlnC-encoding sequence. This cassette was then ligated into the corresponding sites of pFD666 to give pFDXlnC.

Plasmid pFDMelC, used for secretory production of MelC (transacti-vator protein) and MelC2 (aptoynosinase), was constructed by inserting an *EcoRv*–PstI plj1702 (Kieser et al., 2000) fragment containing the MelC promoter and coding sequence into BamHI/Klenow polymerase-treated PstI-digested pFD666.

**Plasmids used for preprotein overproduction in *E. coli*.** Using *S. lividans* chromosomal DNA as template, DNA fragments encoding preCelA, preCelB, preXlnB, preXlnC and preSti-1 were amplified by PCR with the respective primers CelAHis5/CelA3, CelBHIs5/CelB3, XlnBHIs5/XlnB, XlnCHis5/XlnC3 and StiHis5/Sti3. Next, the amplified DNA fragments were digested with *Nde*I and *BamHI* and ligated into *Nde*I/*BamHI*-treated *Pst*I3a, resulting in pCelA, pCelB, pXlnB, pXlnC and pSti, respectively. Plasmid pTm, used for pre*Tm* overproduction, was constructed by inserting an *Nde*I–EcoRl DNA fragment, amplified by PCR using pAX5a as template with the primers TmHis5/Tm3, into the corresponding sites of pET3a. For pre*DagA* overproduction, an *Nde*I–*BamHI* fragment, amplified by PCR using *S. coelicolor* chromosomal DNA as template with the primers DagAHis5/DagA3, was inserted into the corresponding sites of pET3a to give pDagA. PreMelC1 was produced in *E. coli* from preMelG, a pET3a derivative in which an *Nde*I–*BamHI* fragment was cloned that was amplified by PCR with the primers MelCHis5/ MelC3 using the plasmid plj1702 as template.

**Secretory production of target proteins in *S. lividans*.** To express *celA*, *celB*, *xlnB*, *xlnC*, *tm* and *melC1melC2* in *S. lividans* wild-type and single SPase mutants (Palacin et al., 2002), protoplasts of these strains were transformed with pFDCelA, pFDCelB, pFDXlnB, pFDMelC, pFDMelC, pFDTm and pFDMelC, respectively. Plasmid pAGAs5 (Palacin et al., 2002) was used to drive *dagA* expression. *Sti-1* was not cloned in multicopy, since the expression product could be detected when encoded from the chromosome.

All but *dagA* expression cultures were grown in NM medium. *dagA* expression cultures were grown in NMMP (2 × casein peptone) (Parro et al., 1997). After 24 h, proteins present in the supernatant were precipitated using TCA (20% final concn). To rule out variation resulting from different growth rates of *S. lividans* wild-type and single SPase mutants, the samples taken for precipitation were corrected for mycelial dry weight. Proteins were separated by SDS-PAGE as described by Laemmli (1970) and transferred onto a nitrocellulose Porablot membrane (Macherey Nagel). Target proteins were detected colorimetrically using specific polyclonal antisera and anti-rabbit immunoglobulin G conjugated to alkaline phosphatase.

**Expression and purification of preproteins.** For the expression of preproteins, *E. coli* BL21(DE3)pLysS was transformed with the respective expression plasmids and grown at 37°C to an OD600 of 0-6. Then IPTG was added to a final concentration of 1 mM to drive expression. At the same time 2 mM sodium azide was added to block ATPase activity of SecA and, hence, to prevent translocation and cleavage of the signal peptide from Sec-dependent preproteins. Next, cultures were grown at 37°C for an additional 4 h.

From a 600 ml IPTG-induced culture, the *E. coli* BL21(DE3)pLysS cells producing the desired hexahistidine-(6-His)-tagged preprotein were harvested. Preproteins accumulated mainly in the cell as insoluble inclusion bodies. Hence, they were purified from the resulting cell pellet under denaturing conditions (8 M urea) on a Ni2+-NTA column (Qiagen) as recommended by the manufacturer. The obtained fractions were screened for the presence of preprotein using SDS-PAGE and fractions containing the desired preprotein were pooled. Finally, urea in the protein samples was removed by gel filtration on a PD-10 column (GE Healthcare), which was previously equilibrated with 10 mM HEPES, pH 9-0, and 0·5% Triton X-100. Using this procedure, typically 500 µg pure preprotein could be obtained.

**Surface plasmon resonance (SPR) spectroscopy.** All SPR experiments were carried out at 20°C on a Biacore2000 instrument. Covalent immobilization of *S. lividans* SPases on a self-made mixed SAM (self-assembled monolayer) surface (on a J1-chip of Biacore) was accomplished via coupling of their primary amines to the carboxylic groups of the SAM surface as described by Geuken et al. (2004). A control surface was prepared by running the activation and deactivation procedure only.

Preproteins [10 µM in 10 mM HEPES, pH 9-0, 0-5% Triton X-100 (running buffer)] were perfused over the immobilized SPase surface (concentration of the SPase is rate-determining) at a flow rate of 20 µl min⁻¹. After 300 s of association, the preprotein sample was replaced by running buffer for 300 s, allowing the complex to dissociate. Regeneration of the surface was performed by two pulses of 10 mM glycine, pH 2-2.

Binding of preproteins to an immobilized SPase was analysed by calculating the net increase in RU (resonance units) during the association phase. A ARU value of 1000 corresponds to 1 mg m⁻² (Stenberg et al., 1991; Jönsson et al., 1991). The net increase for each preprotein is the measured increase in RU minus the RU increase due to the bulk solution and minus the RU increase resulting from non-specific binding of the respective preprotein to the activated and deactivated surface. The recognition signals were corrected for the difference in SPase immobilization which was in general smaller than 8%.

Competition studies of preproteins and derived peptides were performed as follows. A biosensor surface containing immobilized SipY was perfused with running buffer containing either 5, 10 or 15 µM Sti-1-derived peptide (pepSti or pepContSti; see Table 2). As a control, the same volume of running buffer was injected. Next, preSti-1 (5 µM in running buffer) was injected. After 600 s of association, the preprotein sample was replaced by running buffer for an additional 600 s. Regeneration of the surface was performed by two pulses of 10 mM glycine, pH 2-2. The resulting association and dissociation curves of SipY-preSti-1 with and without previously added peptide were corrected for non-specific binding and plotted as a function of time.
**Table 2. Sti-1- and XlnC-derived peptides**

SPase cleavage sites are in bold.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pepSti</td>
<td>BiotNH-TAGTAQA</td>
</tr>
<tr>
<td>pepXlnC</td>
<td>BiotNH-LPQTAAHA</td>
</tr>
<tr>
<td>pepContS</td>
<td>BiotNH-AAQTGEAA</td>
</tr>
<tr>
<td>pepContX</td>
<td>BiotNH-AAGPLHTA</td>
</tr>
</tbody>
</table>

A similar protocol was followed for evaluating the competitive effect of an XlnC-derived peptide (pepXlnC or pepContX) on preXlnC binding to immobilized SipY.

**In vitro SPase assay.** Analysis of *in vitro* SPase activity was performed as described by Geukens et al. (2002) with the exception that the assay buffer was the same as the running buffer used in the *in vitro* interaction studies described above.

**RESULTS**

To investigate if preproteins preferentially interact with one or more of the *S. lividans* SPases, we first selected a set of eight secretory preproteins originating from different *Streptomyces* species. The selected preproteins were the subtilisin inhibitor (Sti-1), cellulase A (CelA), cellulase B (CelB), xylanase B (XlnB) and xylanase C (XlnC) from *S. lividans*, agarase (DagA) from *S. coelicolor*, subtilisin inhibitor (Sti-1), cellulase A (CelA), cellulase B (CelB), tyrosinase (MelC) and tendamistat (Tm) from *Streptomyces tendae* and tyrosinase (MelC= MelC1MelC2) from *Streptomyces antibioticus*. The tyrosinase is encoded by melC2, the second ORF of the melanin operon (*melC*). The upstream gene melC1, encoding a transactivator protein with a twin-arginine signal peptide, has been demonstrated to be essential for both tyrosinase activation and secretion (Chen et al., 1992; Leu et al., 1992). For *in vitro* studies, only the MelC1 subunit was used. Sti-1 has been shown to be secreted Sec-dependently (Schaerlaekens et al., 2004), whereas the export of XlnC and MelC is Tat-dependent in *S. lividans* (Schaerlaekens et al., 2001, 2004). CelA, CelB, Tm and XlnB contain typical Sec-dependent signal peptides, whereas DagA contains a twin-arginine motif and is predicted to be exported Tat-dependently.

Target proteins have signal peptides that vary in length from 27 to 49 amino acid residues and that contain a typical SPase cleavage site following the $\sim3\sim1$ rule in their C region (Table 3).

**SPase–preprotein binding experiments**

An SPR-based assay, which we previously developed and validated for the analysis of SPase–preprotein interaction (Geukens et al., 2004), was used to calculate the net SPase binding of the eight secretory preproteins. As such, a semi-quantitative comparison of the association of precursor forms of target proteins and SPase was obtained.

For this purpose, 6-His-tagged *S. lividans* SPases were overproduced in *E. coli* and purified as described previously (Geukens et al., 2002). Precursors of Sec-dependent proteins were obtained after overproduction in *E. coli* and subsequent block of translocation. To obtain precursors of Tat-dependent proteins, specific blocking of translocation was not required. All overproduced Tat substrates (XlnC, DagA and MelC1) were poorly secreted in *E. coli* and thus remained mainly in the cytoplasm as precursor (data not shown).

When these purified preproteins were perfused over the SPase surface (Fig. 1), we observed that (1) all tested preproteins interact with each of the immobilized SPases, (2) different preproteins had clear differences in SPase binding affinities, i.e. certain preproteins (Sti-1, XlnC, etc.) interact more efficiently with all SPases than others (CelA, CelB, etc.) and (3) most interestingly, a particular preprotein binds with similar efficiency to each of the SPases, suggesting SPases are able to compete for binding preproteins. As seen in Fig. 1, only small differences in SPase binding affinity could be observed for a specific preprotein.

**Specificity assay**

For testing the specificity and the physiological relevance of the measured SPase–preprotein interaction, we used a small peptide consisting of the SPase cleavage site region to see if it could compete with binding of its corresponding full-length preprotein to the SPase. Whereas a pentapeptide ranging

**Table 3. Signal peptides of target proteins**

Twin-arginine motifs are underlined.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Signal peptide</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sti-1</td>
<td>MRNTARWAATLGLTATAVCGPLAGASILSPATAPA S</td>
<td>Strickler et al. (1992)</td>
</tr>
<tr>
<td>Tm</td>
<td>MRVRALRAVLGAGGAALASPLAAGPASA D</td>
<td>Vértes et al. (1984)</td>
</tr>
<tr>
<td>XlnC</td>
<td>MQQDGTTQDRIKQSPAPLINGSRGGFLGAGTLLATASQGILLPTGAHA A</td>
<td>Shareck et al. (1991)</td>
</tr>
<tr>
<td>XlnB</td>
<td>MNLLVQPRRRRGPVLLVRSAMVLRSPIMLPQGAQA D</td>
<td>Shareck et al. (1991)</td>
</tr>
<tr>
<td>CelA</td>
<td>MRLALALLTGVSIVGLTALAGPPQA A</td>
<td>Théberge et al. (1992)</td>
</tr>
<tr>
<td>CelB</td>
<td>MRLRQPQARAPRLIALGAALGAVLALAFVSLVAAAPAQA D</td>
<td>Wittmann et al. (1994)</td>
</tr>
<tr>
<td>MelC1</td>
<td>MPELTRRRALGAAAAYVAGVPVLAPPAARA D</td>
<td>Bernan et al. (1985)</td>
</tr>
</tbody>
</table>
from $-3$ to $+2$ relative to the SPase cleavage site was found to be the minimal substrate sequence to be recognized by a SPase, Dev et al. (1990) reported that a nonapeptide ranging from $-7$ to $+2$ was a much more efficient substrate.

Therefore, the interaction of two preproteins, i.e. preSti-1 and preXlnC, to SipY was studied when, prior to the analysis of this interaction, increasing concentrations of a nonapeptide covering the proposed SPase–preprotein interaction region were added. More specifically, increasing peptide concentrations (pepSti or pepXlnC) were perfused over the SPase surface prior to the injection of an invariable amount of corresponding preprotein (preSti-1 or preXlnC, respectively). As a control, scrambled peptides (pepContS or pepContX) were used. Peptide sequences are listed in Table 2.

**Fig. 1.** Net increases in RU resulting from binding of 10 $\mu$M purified preproteins to each of the *S. lividans* SPases covalently immobilized on a mixed SAM chip in a 135 s period. The net increase for each preprotein is the measured increase in RU minus the RU increase due to the bulk solution and minus the RU increase resulting from non-specific binding of the respective preprotein to the activated and deactivated surface. The plotted values are the mean values of three independent measurements.
Fig. 2 shows that increasing concentrations of pepSti and pepXlnC resulted in a significant reduction in binding of the respective preprotein to SipY, suggesting that the short peptide can compete with the full-length preprotein for SPase binding. Since the effects of the scrambled peptides on preprotein binding to SipY were significantly less, we could conclude that the interaction measured in our setup mainly originates from SPase binding at the proposed cleavage site region in the preprotein.

**SPase enzymic activity assay**

To ascertain that the purified 6-His-tagged SPase enzymes used for covalent immobilization on the biosensor chip also retained their enzymic activity, their *in vitro* preprotein processing ability was monitored in parallel. Assessment of the *in vitro* activity of the purified SPase enzymes was based on the processing of purified preSti-1 (Sec-dependent substrate) and preXlnC (Tat-dependent substrate). Under similar conditions as in the binding assay, all four SPase proteins were able to process both substrates (Fig. 3), clearly demonstrating their functionality. Interestingly, as observed for the binding of preproteins to the SPases, the efficiency of *in vitro* preprotein processing by each SPase is not extremely different.

**Effect of SPase depletion on high-level secretion of target proteins**

The experiments described above demonstrate that a particular secretory preprotein is not preferentially recognized by any of the *S. lividans* SPases *in vitro*. In addition, a particular preprotein was efficiently processed by each SPase *in vitro* (only shown for preSti-1 and preXlnC) using similar conditions as for the SPR analysis. In the next step, the contribution of each SPase to *in vivo* processing of the same group of proteins was studied.

Therefore, the effect of single SPase deletions on the release of the eight selected secretory proteins into the culture medium was studied. It should be noted that these *S. lividans* single SPase mutants have been characterized in detail before and showed a reduced extracellular protease activity in comparison to that of the wild-type (about 15%, 30% and 45% for ΔX, ΔZ and ΔW mutants and a protease activity below the detection limit for the ΔY mutant; Palacin et al., 2002).

*S. lividans* wild-type and single SPase mutants (ΔW, ΔX, ΔY and ΔZ) were transformed with plasmids driving the secretory production of CelA, CelB, XlnB, XlnC, Tm, MelC or DagA. Sti-1 is the most abundantly secreted protein by *S. lividans*. Hence, there was no need to clone the gene in multicopy and the Sti-1 secretion was therefore evaluated when expressed from the native gene on the chromosome. Respective cultures were grown as described in Methods.

**Fig. 2.** Overlay plot of sensorgrams showing the association and dissociation between preSti-1 (5 μM in running buffer) and covalently immobilized SipY (a, b) and between preXlnC (5 μM in running buffer) and immobilized SipY (c, d). Prior to these interactions, the SipY surface was perfused with 15, 10, 5 or 0 μM competitive peptide [pepSti (a) or pepXlnC (c)] or similar concentrations of scrambled peptide [pepContS (b) or pepContX (d)]. Resulting sensorgrams were corrected for changes in RU due to the bulk solution and non-specific binding.

**Fig. 3.** Western blot analysis with specific polyclonal antibodies of *in vitro* preSti-1 and preXlnC cleavage by each of the SPases. Precursor (p) and mature form (m) were previously separated on a 12.5% SDS-polyacrylamide gel. Purified SPase (2-5 μM final concn) was added to 20 μM substrate and incubated for 15 h at 37 °C in 10 mM HEPES, pH 9.0, and 0-5% Triton X-100. Lanes: 1, precursor; 2, precursor + SipW; 3, precursor + SipX; 4, precursor + SipY; 5, precursor + SipZ.
and, after 24 h, the presence of target proteins in the culture medium was analysed by Western blotting. As shown in Fig. 4, secretion of Sti-1 was almost completely blocked in the SipY-depleted strain. In contrast, increased Sti-1 secretion was observed in the ΔX and ΔZ single mutants, whereas inactivation of SipW did not seem to have any effect on the appearance of Sti-1 in the extracellular medium.

These observations suggest that Sti-1 is preferentially processed in vivo by SipY.

As in the case of Sti-1, secretion of XlnC was dramatically reduced in the ΔY mutant. However, XlnC secretion remained unaffected by inactivation of one of the other SPases. A similar observation was made for high-level secretion of CelA and CelB by S. lividans wild-type and single SPase mutants. Unfortunately, CelA was observed to be extremely sensitive to degradation when present in the culture medium. Growth of CelA-producing S. lividans cultures in minimal medium or replacement of glucose as carbon source by xylose, which was previously reported to limit CelA degradation (Théberge et al., 1992), did not result in a significant improvement (data not shown).

In contrast, Tm secretion was not blocked by SipY depletion, but efficient secretion was found to be SipX-dependent. The appearance of Tm in the culture medium was not affected by inactivation of SipW, but increased as a result of SipY or SipZ depletion.

Whereas the five secretory proteins listed above seem to be preferred substrates for one of the four S. lividans SPases, secretion of MelC, DagA and XlnB was not blocked or dramatically reduced by single SPase inactivation. MelC secretion in S. lividans was not visibly affected in the ΔX, ΔY and ΔZ mutants, but increased in the ΔW mutant and finally, for DagA and XlnB, no significant difference of single SPase inactivation on the appearance of the mature protein in the culture medium was observed.

In contrast to the in vitro situation, in vivo processing of preSti-1, preTm, preCelA, preCelB and preXlnC was clearly dependent on one SPase, in particular SipX or SipY. preDagA, preMelC1 and preXlnB were not preferentially processed by one particular SPase in vivo, suggesting that multiple SPases could be actively involved in processing of these precursors. These data support the view of an overlapping, non-identical substrate preference of the S. lividans SPases in vivo.

**DISCUSSION**

Previously, we showed that none of the S. lividans SPases is essential for cell viability, suggesting that they can at least partly complement each other (Palacin et al., 2002). Nevertheless, clear differences in substrate specificity between SPases have also been observed (Geukens et al., 2001; Palacin et al., 2002).

Here, the substrate specificity of these SPases was analysed in more detail and its molecular basis was determined by evaluating preprotein binding to each of the SPases and high-level protein secretion in single SPase mutants. Most interestingly, we have observed that preproteins do not preferentially bind to a particular SPase, suggesting SPase competition for binding preproteins. In addition, no specificity was observed in in vitro preprotein processing.

**Fig. 4.** Western blotting analysis (12.5% SDS-polyacrylamide gel) of extracellular protein extracts prepared from 24 h cultures of wild-type S. lividans and single SPase mutants overproducing the respective target proteins. Proteins were visualized using NBT/BCIP after immunodetection with specific polyclonal antibodies.
However, analysis of the secretion of the same target proteins in *S. lividans* indicated that not all of these proteins are processed *in vivo* by each of the SPases with the same efficiency. Consequently, there appears to be no real correlation between preprotein binding and processing *in vitro* and preprotein processing *in vivo*. The most obvious examples are Sti-1, XlnC, CelB and Tm, which bind to all four SPases with a similar affinity and can be processed *in vitro* by each SPase with similar efficiency, but are preferentially processed *in vivo* by only one SPase. Concomitantly, these experiments suggest that factors other than cleavage requirements of the SPases strongly affect substrate preference *in vivo*. The same factors are most probably also responsible for the major role of SipY in the processing of exported proteins in intact cells. Indeed, our data demonstrate that the importance of SipY for efficient preprotein processing *in vivo* is not due to a more efficient binding of preproteins. Other factors seem to be involved, which are not known at this stage. It could be that preproteins are exported at defined membrane locations in the cell and that SipY is preferentially localized there. Maybe SipY is the best at processing preproteins cotranslationally during export across the membrane or SipY makes the most efficient interaction with the translocation pores. This is the subject of ongoing research.

Preprotein binding to a SPase could be specifically reduced by a peptide covering the SPase cleavage site region. This clearly demonstrated that the signal measured in our setup originates from a physiologically relevant interaction. The competitive effect of the peptide on SPase binding of its corresponding full-length preprotein was small or negligible when the increasing peptide concentrations were directly added to the preprotein solution (data not shown). This is most probably due to the fact that peptides are far less efficient substrates than full-length preproteins (Dev et al., 1990; Paetzel et al., 2000). Therefore, the peptide was perfused over the SPase surface prior to the analysis of SPase–preprotein binding. In this case, the competitive behaviour of the peptides was obvious.

In *in vitro* SPase processing assays, Tat-dependent preproteins were found to behave similarly to Sec-dependent preproteins (only shown here for preSti-1 and preXlnC), i.e. they are processed by each SPase with comparable efficiencies and processing takes place in a similar time course. The observation that all four SPases are capable of processing all preproteins *in vitro* together with the fact that they all bind each preprotein tested suggests they are all actively involved in preprotein processing *in vivo*. Accordingly, DagA, MelC and XlnB secretion was not severely affected by the absence of a particular SPase and multiple SPases are involved in *in vivo* processing of these precursors. In contrast, high-level secretion of Sti-1, Tm, CelA, CelB and XlnC was clearly dependent on one SPase, in particular SipX or SipY. As mentioned above, the factors that determine this SPase-dependency for *in vivo* secretion and the major role for SipY are not yet known. All together, these observations agree with an overlapping, non-identical substrate specificity of the *S. lividans* SPases. Finally, it should also be noted that all these findings are related to overproduced secretory proteins, except for Sti-1 which is already efficiently secreted under native conditions. Some of these effects may therefore become unnoticed when proteins are produced in low amounts.

Interestingly, an increase in secretion of some secretory proteins was observed in certain single SPase mutants. Because improved protein secretion in a particular single mutant was only observed for a few proteins rather than being a general issue, it is very unlikely that reduced extracellular protease activities can account for this. In addition, improved secretion is rarely observed in the *AY* mutant strain, which has the lowest extracellular protease activity. As a consequence, we favour the hypothesis that the SPase competition for binding preproteins can account for improved secretion of some target proteins in specific single SPase mutants. When the *S. lividans* SPases compete for the binding of precursor proteins, but process these preproteins with different efficiencies depending on the nature of the precursor, this may lead to improved secretion of target proteins. In particular, deletion of an SPase which cleaves a precursor efficiently is expected to result in reduced processing. However, deletion of an SPase which binds efficiently, but cleaves the substrate poorly, allows more precursor to bind to another SPase, which may cleave it more efficiently. This may result in improved secretion. Increased secretion of proteins in single SPase mutants has already described in *B. subtilis* (Van Dijl et al., 1992; Bron et al., 1998). Notably, inactivation of SipS resulted in an increased secretion of *α*-amylase in *B. subtilis*. As a consequence, SPase competition for binding precursor proteins does not seem to be restricted to *S. lividans*, but is likely to be a general feature of bacteria that contain multiple SPases. Importantly, these findings are also of biotechnological interest. In particular, the fact that SPases compete for binding preproteins combined with the fact that they show a kind of substrate preference *in vivo* indicates that one or some SPase(s) may interfere with efficient secretion of a particular protein. The obtained data can be used to construct the optimal ‘signal peptide processing machinery’ as a means to increase secretory production of a target protein. The data described here suggest that co-expression of sipY (as the major SPase) is too simple and not always the best choice. In reality, the situation is more complex. It is therefore our strategy to overexpress sip genes of SPases that process the target preprotein efficiently and delete sip genes of SPases that bind but inefficiently process the preprotein of interest.

This work also demonstrated that none of the *S. lividans* SPases is specifically dedicated to the processing of Sec- or Tat-dependent precursors and consequently these SPases act in a secretion-pathway-independent manner. This suggests that, although the preproteins are in a completely different conformational state during or after translocation across the cytoplasmic membrane, the signal peptides of Sec- and
Tat-dependent preproteins are assumed to be presented in a similar manner to the SPase.

Finally, it should also be noted that the binding affinity of proteins such as Sti-1, Tm, XlnC and DagA to each SPase was significantly higher than that of CelA, CelB, XlnB and MelC1. Interestingly, mature forms of precursors that showed a high SPase binding affinity accumulated in higher amounts than mature proteins from precursors that showed a low SPase binding affinity. It is clear that the secretion efficiency increases if proteins can efficiently interact with components of the secretion pathway. Although this certainly has to be further elaborated, this assay may be included as part of a predictive assay for the efficiency of secretion for a particular protein.

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