Determining the functionality of putative Tat-dependent signal peptides in *Streptomyces coelicolor* A3(2) by using two different reporter proteins

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The availability of the complete genome sequence of *Streptomyces coelicolor* A3(2) has allowed the prediction of the Tat-exported proteins of this Gram-positive bacterium. To predict secreted proteins that potentially use the Tat pathway for their secretion, the TATscan program was developed. This program identified 129 putative Tat substrates. To test the validity of these predictions, nine signal sequences, including three which were not identified by existing prediction programs, were selected and fused to the structural *xlnC* gene in place of its native signal sequence. Xylanase C (XlnC) is a cofactorless enzyme which is secreted in an active form exclusively through the Tat-dependent pathway by *Streptomyces lividans*. Among the nine chosen signal sequences, seven were shown to be Tat-dependent, one was Sec-dependent and one was probably not a signal sequence. The seven Tat-dependent signal sequences comprised two lipoprotein signal sequences and three sequences not predicted by previous programs. Pulse–chase experiments showed that the precursor-processing rate in the seven transformants was generally slower than wild-type XlnC, indicating that these signal peptides were not equivalent in secretion. This suggested that there might be some incompatibility between the signal peptide and the reporter protein fused to it. To test this possibility, the signal peptides were fused to a cofactorless chitosanase (SCO0677), a Tat-dependent protein validated in this work but structurally different from XlnC. With some fluctuations, similar results were obtained with this enzyme, indicating that the type of folding of the reporter protein had little effect on the Tat secretion process.

INTRODUCTION

Many secretory proteins are initially synthesized in the cytoplasm as a precursor containing a signal peptide at the N terminus, which mediates targeting to distinct export systems. Most of these proteins are exported via the well-defined Sec machinery (Mori & Ito, 2001), whilst a subset of precursor proteins is secreted through the recently discovered twin-arginine translocation (Tat) pathway (Berks, 1996; Berks et al., 2000). The Tat-dependent proteins have a signal peptide apparently resembling the tripartite structure of Sec signal peptides, but it is generally longer and characteristically contains a conserved twin-arginine motif at the n- and h-region boundaries, a less hydrophobic h-region and a positively charged Sec avoidance signal in the c-region (Bogsch et al., 1997; Cristobal et al., 1999). In contrast to the Sec pathway, in which the substrates are translocated in a threading mechanism, the most remarkable feature of the Tat pathway is its ability to transport fully folded or even oligomeric proteins across the cytoplasmic membrane.

While the components of the Tat pathway and the Tat-dependent substrates have been well studied in *Escherichia coli*, the Tat pathway has also been found in other bacteria and archaea (Ding & Christie, 2003; Halbig et al., 1999; Hutcheon & Bolhuis, 2003; Jongbloed et al., 2000; Ochsner et al., 2002; Schaerlaekens et al., 2001). *Streptomyces* are Gram-positive, mycelia-forming soil bacteria, well known for their ability to secrete a large variety of proteins into

A complete set of 129 candidate signal sequences is available as supplementary data with the online version of this paper.
their extracellular environment. In the model streptomycete, Streptomyces lividans, a Tat pathway was identified and proven to be functional (Schaerlaeken et al., 2001).

We have shown previously that S. lividans secretes three xylanases (XlnA, XlnB and XlnC) to the extracellular environment for hydrolysing xylan into assimilable sugars for its survival (Kluepfel et al., 1992, 1990; Morosoli et al., 1986). XlnC is distinguished from the other two xylanases by bearing in its extra-long signal peptide (49 aa) the SRRGFLG sequence that is similar to the consensus motif (S/T)RRXXFLK) present in the Tat-targeting signal sequence (Berks et al., 2000). Pulse–chase experiments further revealed that the half-life of the XlnC precursor protein is approximately 11 min, characteristic for the Tat pathway, compared with no more than 2 min for proteins exported via the Sec pathway (Faury et al., 2004). Fusion of mature XlnC to the Sec-dependent XlnA signal peptide resulted in no secretion of active XlnC in the medium. When the tatC gene encoding an essential component of the Tat pathway was disrupted in S. lividans 10-164 (a cellulase- and xylanase-negative mutant; Hurtubise et al., 1995) harbouring the xlnC gene on a high-copy-number vector, no active XlnC could be detected. These results provided strong evidence that active XlnC is secreted exclusively via the Tat pathway (Faury et al., 2004).

The release of the complete genome sequence of Streptomyces coelicolor A3(2) (Bentley et al., 2002) has allowed the identification of putative Tat-dependent substrates in this strain. By using a genomic approach, Dilks et al. (2003) established a comprehensive list of putative Tat substrates for 84 diverse prokaryotes. With 145 putative Tat-dependent substrates predicted by the program TATFIND version 1.2, S. coelicolor A3(2) ranks at the top of the list. However, such genome-based predictions are of limited value without biochemical or proteomic verification of each putative Tat-dependent protein. Therefore, a reliable approach should be established to test the functionality of these putative Tat substrates.

Our own bioinformatic program, TATscan, allowed us to predict 129 putative Tat-dependent substrates in S. coelicolor A3(2). Since XlnC is an exclusive Tat-dependent protein, it was used as a reporter protein for validating nine of these putative Tat-dependent signal peptides from S. coelicolor A3(2) in S. lividans, two closely related species. Although signal peptide recognition of Tat-dependent proteins is species-specific (Blaudeck et al., 2001), this will not invalidate our results, since the tatA, B and C genes of S. coelicolor A3(2) and S. lividans exhibit more than 99.6 % identity (Schaerlaeken et al., 2001). It could be assumed that both strains have almost identical Tat machinery.

To test the possible incompatibility of a signal peptide with the protein fused to it, the chitosanase SCO0677, which is proven to be Tat-dependent in this work, but structurally different from XlnC, was used. In spite of some differences, the structure of both enzymes had little effect on production since the signal peptides mediating very low or no secretion for XlnC exerted the same effect on chitosanase.

**METHODS**

**Bacterial strains and media.** S. lividans 10-164 and an isogenic tatC mutant of this strain were used as hosts for recombinant plasmids (Faury et al., 2004). Spores were inoculated in Bacto tryptic soy broth medium for plasmid isolation or in a minimal medium, M14 (Page et al., 1996), slightly modified by substituting xylose for oat spelt xylan as the sole carbon source for the xylanase activity test and pulse–chase experiments. The cultures were grown at 34 °C at 240 r.p.m. R5 medium (for transformation) and MS medium (for sporulation) were used as solid media (Kieser et al., 2000). Where required, thiostrepton was added at a final concentration of 50 µg ml⁻¹ in solid medium or 5 µg ml⁻¹ in liquid medium. Protoplast preparation and transformation of the strains were performed as described by Kieser et al. (2000).

**Bioinformatic prediction.** TATscan is a C++ program that predicts Tat substrates by searching for the pattern STTRXXFLK (where x corresponds to FILMVW) in the first 70 aa of a coding sequence. It takes as input the chromosomal genome sequence and annotation file, in this case that of S. coelicolor A3(2) strain M145 (Bentley et al., 2002), and it searches the complete set of putative coding sequences for the above pattern. A maximum of two mismatches were allowed, only at the first and the last position of the pattern. The output sequences were filtered through analysis with SignalP_HMM version 3.0 (Bendtsen et al., 2004) to exclude sequences that did not fulfil the criteria of a signal peptide or that contained a predicted Tat pattern localized inside or downstream of a putative signal peptide cleavage site.

**Plasmid constructions.** Plasmid pIAF916C contains the S. lividans xlnC gene with its native signal sequence under the control of the xlnA promoter (Faury et al., 2004). Plasmid pIAF906C contains the promoter region and the signal sequence of xlnA followed by the coding sequence of the mature part of XlnC (Faury et al., 2004). The Sec-dependent signal sequence of xlnA is flanked by a HindIII and a KpnI site. A second KpnI site located elsewhere in this plasmid was eliminated, thus giving pIAF907C. The resulting plasmid allowed the insertion of the different amplified signal sequences directly between the HindIII and KpnI sites, replacing the xlnA signal sequence.

The predicted signal peptides of nine putative proteins of S. coelicolor A3(2) that could be secreted via the Tat-dependent machinery are listed in Table 1. For construction of plasmids containing the fusion genes, nine pairs of oligonucleotide primers (Table 2) were synthesized based on the nucleotide sequences of the corresponding genes. The upstream primers started several bases prior to the ribosome-binding site and contained a HindIII site, while the downstream primers started from the end of the signal peptides and contained a KpnI site. PCR amplifications of chosen signal sequences with chromosomal DNA of S. coelicolor A3(2) were performed using these primer pairs in the presence of 2–6 % propionamide in a total volume of 50 µl. Reaction mixes were subjected to 5 min at 95 °C followed by 30 cycles of 20 s at 94 °C, 20 s at 60 °C and 40 s at 72 °C. The resulting PCR fragments were digested with HindIII and KpnI and then ligated into the corresponding sites of pIAF907C, thus generating the plasmids containing the fusion genes. The ligation mixes were used directly for transformation of protoplasts of S. lividans 10-164. The transformants were selected on R5 agar plates supplemented with thiostrepton.

To construct corresponding plasmids containing the chitosanase gene, the DNA fragment corresponding to the mature part of the
Table 1. Putative Tat-dependent substrates of *S. coelicolor* A3(2)

Putative Tat signal peptides were identified by checking the first 70 residues of all annotated proteins of the *S. coelicolor* A3(2) genome sequence for the presence of the consensus sequence [ST]RRX\(\_\)K (where \(\_\) is a hydrophobic residue) with two mismatches allowed outside the RRX\(\_\) core as described in Methods. Putative Tat motifs are in bold. The hydrophobic h-regions are shaded. Hydrophobic indexes were calculated according to Kyte & Doolittle (1982). The cleavage sites of signal peptidase I were identified with the SignalP version 3.0 algorithm (Bendtsen *et al*., 2004) and are indicated by an arrow. For lipoproteins, signal peptidase II recognition sites were identified by LipoP software (http://www.cbs.dtu.dk/services/LipoP/).

<table>
<thead>
<tr>
<th>No.</th>
<th>Gene name</th>
<th>Diff.*</th>
<th>Putative signal peptide</th>
<th>Hydrophobic index of h-region</th>
<th>Sec-avoidance signal</th>
<th>Function according to genomic annotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SCO6272</td>
<td>0</td>
<td>MTEV[SRRKL]MKGAAVSGGALPALGAPPATA↓APAAGPEDLPGPAAA↓AA</td>
<td>0.9</td>
<td>No</td>
<td>Putative secreted FAD-binding protein</td>
</tr>
<tr>
<td>7</td>
<td>SCO0766</td>
<td>1</td>
<td>MTV[TRSVLA]STAAPAAGMALAAPARA↓AD</td>
<td>1.5</td>
<td>No</td>
<td>Putative secreted (\beta)-galactosidase</td>
</tr>
<tr>
<td>15</td>
<td>SCO6557</td>
<td>1</td>
<td>MPRTRP[FWL]ALATSCLAVSSPPAHA↓RP</td>
<td>1.2</td>
<td>No</td>
<td>Putative neuramidase</td>
</tr>
<tr>
<td>21</td>
<td>SCO0624</td>
<td>1</td>
<td>MHEPHILDR[RFLK]GTAVGAALGAATAAPASA↓AP</td>
<td>1.0</td>
<td>No</td>
<td>Putative secreted protein</td>
</tr>
<tr>
<td>40</td>
<td>SCO0538</td>
<td>1</td>
<td>MDAVRRAARS[RRGVLR]AGGGAALAAAGTXTVT↓CGAGADD</td>
<td>1.1</td>
<td>No</td>
<td>Probable sugar transporter sugar-binding lipoprotein</td>
</tr>
<tr>
<td>48</td>
<td>SCO0677</td>
<td>1</td>
<td>MRHPFRDPAR[PSRTVLA]MACASLATVPALTSHAAA↓AS</td>
<td>1.6</td>
<td>Yes</td>
<td>Secreted chitosanase</td>
</tr>
<tr>
<td>113</td>
<td>SCO6665</td>
<td>2</td>
<td>MSQTSGNPPRRP[LLRALYAVIGTLGLAATVTPSAGA]↓AV</td>
<td>1.6</td>
<td>No</td>
<td>Putative secreted glucosidase</td>
</tr>
<tr>
<td>128</td>
<td>SCO2286</td>
<td>2</td>
<td>MTPANHAPSTSAPSA[PSQSHAPELRAARSL][GRRELVTG][ALFAAVN][PAAGTASA]↓AE</td>
<td>1.7</td>
<td>No</td>
<td>Putative alkaline phosphatase</td>
</tr>
<tr>
<td></td>
<td>XlnC(\dagger)</td>
<td>1</td>
<td>MQQDGTQD[RIKQSPAPLNGMS][RRFGFLG]GAGTLALTASG[LL]P[TAHA]↓AT</td>
<td>1.2</td>
<td>No</td>
<td>Secreted XlnC (<em>S. lividans</em>)</td>
</tr>
</tbody>
</table>

*Diff. represents the number of mismatch residues between the complete pattern and the corresponding sequence.

\(\dagger\)Wild-type signal sequence of XlnC from *S. lividans* present in the control plasmid pIAF916C.
protein was amplified using two oligonucleotide primers, CSNf
(5’-GGCCCGTGACCCGCGCCCGGGGCGGGGCGGATGGGTGGGTGGGG-3’) and CSNr
(5’-GGGGAGCCCTTCACCGCCAGCGCGGCGGCGGCAACCGGCGGATGGGTGGGTGGGG-3’), with
the introduction of a KpnI and a SacI site (underlined), respectively. The
resulting PCR fragment was digested with KpnI and SacI and used to
replace the xlnC gene in the chimeric plasmids obtained above.

For construction of chimeric plasmids with signal sequences fused to
the xlnA gene, pIAF906 (Page et al., 1996) was digested with KpnI and
SacI. Then, the 1-kb DNA fragment containing the xlnC gene was
isolated and used to replace the xlnC gene in the chimeric plasmids
obtained above.

**Cell fractionation and localization of precursor and mature XlnC.** After cultivation in M14 medium for 96 h, mycelia of transformants were recovered by centrifugation for 10 min at 5000 g. For each transformant, 1-2 g washed mycelia (wet wt) was resuspended in 6 ml sample buffer [10 mM Tris/HCl, pH 7.5, containing 1 mM PMSF and 1 x Complete protease inhibitor (Roche)]. Cells were broken by using a French press at 10 000 p.s.i. for three rounds. After removing the cell debris by centrifugation for 10 min at 5000 g, the homogenate was subjected to ultracentrifugation in a Beckmann L5-75 ultracentrifuge with an SW50.1 rotor at 200 000 g at 4 °C for 30 min. The supernatant represented the cytoplasmic fraction and the pellet contained the membrane fraction which was resuspended in 500 μl sample buffer. The protein concentration in the cell fractions was determined by the Lowry method. A sample (100 μg) of protein from each cell fraction was loaded onto a 12-5% SDS-PAGE gel and the localization of the precursor and mature XlnC was revealed by Western blotting using anti-XlnC serum. Intact and 1% Triton X-100 solubilized membrane fractions were used to determine xylanase activity.

**Colony PCR.** To detect the presence of the inserted signal sequences after protoplast transformation, portions of colonies of *S. lividans* 10-164, grown on solid medium, were taken and resuspended in 200 μl 10 mM Tris/HCl, pH 8-0, buffer containing 200 mg glass beads (0-10-0.11 mm diam.). The cells were broken for 45 s at a speed setting of 6-5 by using a FastPrep FP120 apparatus (Thermo Savant). After removal of cell debris by centrifugation at 13 000 r.p.m. for 5 min, 1 μl supernatant was added as a template to the PCR solution. Primers located at the 5’ end of the selected signal sequences and primers located at the 3’ end of the structural genes were used.

**Xylanase and chitosanase activity tests.** Xylanase activity was measured by using the dinitrosalicylic acid method as described by Kluepfel et al. (1990). One unit of enzyme activity is defined as the amount of enzyme required to release 1 μmol reducing sugar (expressed as xylose) from the substrate xylan in 1 min at 57 °C. Chitosanase activity was evaluated by measuring the release of reducing sugar (expressed as D-glucosamine) from the substrate chitosan as described by Lever (1972) and Fukamizo et al. (1995).

**Pulse–chase experiment.** The pulse–chase experiments were carried out as described by Faury et al. (2004) with a minor modification. The mycelia were broken by using the FastPrep apparatus (see above).

**Isolation and analysis of RNA.** RNA was isolated from *S. lividans* 10-164 transformants by a CsCl gradient method as described by Page et al. (1996) with the following modification. Cells were broken by using the FastPrep apparatus (see above) for three rounds of 30 s at a speed setting of 6-5. Slot blots, containing 4 μg RNA per well, were hybridized according to Kieser et al. (2000). The xylanase probe prepared from pIAF907C was 32P-labelled by nick-translation.

**RESULTS AND DISCUSSION**

**Identification of putative Tat-exported proteins.** Using TATscan, the pattern [ST]RRXϕϕK with no more than two mismatches present outside the RRXϕϕ core was identified 399 times in the first 70 residues of a total of 7769 putative protein sequences predicted from the *S. coelicolor* A3(2) M145 genome (chromosome only, excluding plasmids SCPI and SCP2). The final number of predicted sequences consisted of 129 candidates retained after filtering for putative signal peptides. The complete set of sequences is available as supplementary data with the online version of this paper. The mean distance of the first residues of the Tat pattern from the translation start site was 9.3 ± 6.3 residues. The mean length of the predicted signal peptides was 39 ± 9 residues. This is in accordance with the mean obtained previously (Schaerlaekens et al., 2004).

The number of 129 putative Tat-dependent sequences predicted by TATscan is lower than the 143 putative Tat signal peptides (excluding two predictions localized in the plasmid sequences) found using the program TATFIND version 1.2 (Dilks et al., 2003) and the 230 Tat signal peptides obtained by a modified version of TATFIND

### Table 2. List of oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’→3’)</th>
<th>Primer name</th>
<th>Primer sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tat1F</td>
<td>ttctAGGCTacgagaggttgtgct</td>
<td>Tat1R</td>
<td>ggcgGTCACgggcctcgccgacgc</td>
</tr>
<tr>
<td>Tat7F</td>
<td>cttgAAGCTtcgtgatgaggaga</td>
<td>Tat7R</td>
<td>tgcgGTACgggccgccccggcagc</td>
</tr>
<tr>
<td>Tat15F</td>
<td>ccacAAGGTTccccagagggccagc</td>
<td>Tat15R</td>
<td>cgccgTACggggagggcagccgag</td>
</tr>
<tr>
<td>Tat21F</td>
<td>atccAACGTTaccgaggtcagcc</td>
<td>Tat21R</td>
<td>cgccgTACggggagggcagccgag</td>
</tr>
<tr>
<td>Tat28F</td>
<td>cccgAAAGCtgagatcctccgacc</td>
<td>Tat28R</td>
<td>tggcGTCACggccactggctcgc</td>
</tr>
<tr>
<td>Tat40F</td>
<td>cccgtaagCTTtgagagtaacgc</td>
<td>Tat40R</td>
<td>gacgcgGTACgccccttcccgc</td>
</tr>
<tr>
<td>Tat48F</td>
<td>cccgAACGTTggagctgctgag</td>
<td>Tat48R</td>
<td>ggcggTACgggagggctgccc</td>
</tr>
<tr>
<td>Tat113F</td>
<td>actcAAGGTTccgaggggccc</td>
<td>Tat113R</td>
<td>ggcgGTCACgggagggctgccc</td>
</tr>
<tr>
<td>Tat128F</td>
<td>tgtgcaAGTTccgaggggagcgc</td>
<td>Tat128R</td>
<td>cccgGTCACgggagggctgccc</td>
</tr>
</tbody>
</table>
(Schaerlaekens et al., 2004). A more detailed comparison (see supplementary data with the online version of this paper) showed that only 80 proteins were shared by TATscan and TATFIND version 1.2 predictions (Dilks et al., 2003), whereas 51 proteins found by TATscan were also identified using the modified TATFIND program (Schaerlaekens et al., 2004).

Among the 129 putative proteins found by our approach, only one has no annotated function or localization. Around one-fifth of the proteins are putative enzymes, participating in the hydrolysis of distinct substrates, such as polysaccharides, amides, esters, peptides and phospholipids. Other marked groups are putative substrate-binding and transport proteins or lipoproteins (30%), and putative membrane proteins (15%). In great contrast to the E. coli Tat substrates, only eight putative redox proteins could be classified as putative Tat substrates in S. coelicolor A3(2) M145.

For molecular studies, we chose nine signal peptides, varying according to the number of mismatches, the length of the n-domain, the hydrophobicity index of the h-domain of the signal peptide, the presence or absence of a Sec avoidance signal and a lipobox for lipoproteins (Table 1). The chitosanase (SCO0677) signal peptide was chosen because of the medical importance of this enzyme and our own interest in it (Fukamizo et al., 2000). Three signal peptides (from SCO0624, SCO2286 and SCO6557) were specifically chosen for further analysis because they were not predicted by the previous TATFIND programs. As shown below, all three of these predicted sequences were proven to be functional Tat signal peptides.

**XlnC production directed by putative Tat signal peptides of S. coelicolor**

Since an active XlnC is exclusively secreted by the Tat-dependent export system (Faury et al., 2004), this enzyme provides an appropriate tool to identify other potential Tat-dependent signal sequences. We tested nine different predicted signal sequences (Table 1). One signal sequence belonged to a secreted protein containing a cofactor, five are probably cofactorless secreted hydrolases, two are putative lipoproteins, but one contains a cofactor, and one is a putative secreted protein of undefined function.

To assess the functionality of these signal sequences, they were fused to the sequence encoding the mature XlnC protein. The gene was under the control of the xlnA promoter, which was shown to be more effective for XlnC production than the native xlnC promoter (Faury et al., 2004). The signal sequences of interest were amplified by PCR and inserted into the HindIII/KnpI sites. The generated plasmids were designated according to the number of the putative proteins assigned in the complete genomic sequence of S. coelicolor A3(2). For instance, pIAFC6272 contains the fusion of the structural gene of XlnC with the signal sequence from a putative secreted FAD-binding protein (SCO6272). Accordingly, the strain of S. lividans 10-164 harbouring the plasmid was designated IAFC6272.

The transformants were identified by colony PCR and further verified by DNA sequencing. As XlnC production was the first indication that the chosen signal peptides could mediate Tat-dependent export, the transformants were grown in liquid minimal medium and xylanase activity was monitored in the culture supernatants (data not shown). As the growth was similar for all the transformants and after 120 h of cultivation there was no further accumulation of XlnC (data not shown), xylanase activity was reported for this time point only (Table 3).

With the exception of IAFC0624 in which XlnC production doubled, all the other transformants showed values inferior to the control IAFC916C. Production from IAFC6272 and IAFC0677 was 78 and 50% of the wild-type control, respectively. Transformants IAFC1955, IAFC2286 and IAFC6557 produced only 19, 11 and 8% of the control, respectively. In the culture supernatants of IAFC0766, IAFC0538 and IAFC6665, the activity was below 2 IU ml⁻¹. This residual activity was inherent to the method itself. The commercial preparation of xylan used as substrate in the test contains 10–15% contaminants, mainly undefined polysaccharides, which could be partially hydrolysed by enzymes present in the supernatants of transformants. These hydrolysis products could react with the reducing-sugar test and therefore activity below 2 IU ml⁻¹ could be considered as background. At first glance, these three signal peptides might not be Tat-dependent.

Although the cloning experiments were carried out in S. lividans 10-164, a cellulase- and xylanase-negative mutant, it was important to show that the xylanase activity measured in the culture supernatants correlated with the presence of XlnC. Western blot analyses with anti-XlnC serum were performed on proteins secreted in the culture supernatant of the different transformants after 120 h of growth (data not shown). The 20 kDa protein band corresponding to XlnC was detected in the culture supernatant of transformants producing more than 3 IU XlnC ml⁻¹, below which no XlnC-corresponding band appeared, as was the case for the IAFC0766, IAFC0538 and IAFC6665 supernatants. According to the specific activity of XlnC [1100 IU (mg protein)⁻¹] (Kluepfel et al., 1992) and referring to the activity measured in the culture supernatants of these three transformants (Table 3), 13–50 ng XlnC was loaded onto the gel. These quantities should have been detected, since the detection limit of the immunoreaction was 10 ng (data not shown). Therefore, the residual activity measured in these three transformant supernatants corresponded to background activity.

The transcriptional level of xlnC for the different constructs was assessed by slot blot hybridization of total RNA isolated from 36-h-old cultures. There was no significant difference in the XlnC mRNA content of the transformants (data not shown). Therefore, the difference in the XlnC yield...
could not be attributed to variations at the transcriptional level.

To show that the signal sequences fused to XlnC were Tat-dependent, the different plasmids were used to transform protoplasts of a tatC mutant of *S. lividans* 10-164, which is unable to mediate Tat-dependent secretion (Faury *et al.*, 2004). Similar background activities were observed in the culture supernatants of IAFC0766, IAFC0538 and IAFC6665 tatC mutants compared to the same constructs in *S. lividans* 10-164. However, background activities were measured in the culture supernatants of the other six transformants, showing that XlnC production was abolished (data not shown). This indicated that these six foreign signal peptides fused to the XlnC mature part effectively targeted XlnC to the Tat-dependent pathway.

Taken together, these results indicated that among the nine chosen signal peptides of the putative Tat-dependent proteins of *S. coelicolor* A3(2), at this point six were able to export the exclusively Tat-dependent XlnC across the cytoplasmic membrane of *S. lividans*. Moreover, the large discrepancy of the yield of XlnC among the six transformants indicated that the signal peptides are not equivalent in XlnC production.

### XlnC secretion by various transformants of *S. lividans*

For further confirmation of Tat-dependent secretion, one of the six transformants producing XlnC was chosen to analyse the secretion process itself. Since the chitosanase gene was used as the second reporter gene in this study, pulse–chase experiments were carried out with IAFC0677 to show that the chitosanase signal peptide could effectively target XlnC. As shown in Fig. 1(a), IAFC0677 synthesized an XlnC precursor, which was gradually processed into the

### Table 3. Xylanase and chitosanase activities in the culture supernatants

Activities are reported after 120 h cultivation. The activity data are means of three independent cultures.

<table>
<thead>
<tr>
<th>Transformant</th>
<th>Xylanase activity (IU ml⁻¹)</th>
<th>Xylanase activity (%)*</th>
<th>Transformant</th>
<th>Chitosanase activity (IU ml⁻¹)</th>
<th>Chitosanase activity (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAF916C (wild-type)</td>
<td>36 ± 2</td>
<td>100</td>
<td>IAF916Csn</td>
<td>4·0 ± 0·2</td>
<td>160</td>
</tr>
<tr>
<td>IAF907C†</td>
<td>1 ± 0·1</td>
<td>3</td>
<td>IAF907Csn†</td>
<td>0·6 ± 0·1</td>
<td>24</td>
</tr>
<tr>
<td>IAFC6272</td>
<td>28 ± 2</td>
<td>78</td>
<td>IAFC6272</td>
<td>6·3 ± 0·3</td>
<td>252</td>
</tr>
<tr>
<td>IAFC0766</td>
<td>1 ± 0·1</td>
<td>3</td>
<td>IAFC0766</td>
<td>0·2 ± 0·05</td>
<td>8</td>
</tr>
<tr>
<td>IAFC6557</td>
<td>3 ± 0·2</td>
<td>8</td>
<td>IAFC6557</td>
<td>0·1 ± 0·05</td>
<td>4</td>
</tr>
<tr>
<td>IAFC6665</td>
<td>79 ± 4</td>
<td>220</td>
<td>IAFC6665</td>
<td>12 ± 0·5</td>
<td>480</td>
</tr>
<tr>
<td>IAFC1955</td>
<td>7 ± 0·6</td>
<td>19</td>
<td>IAFC1955</td>
<td>0·5 ± 0·05</td>
<td>20</td>
</tr>
<tr>
<td>IAFC0538</td>
<td>2 ± 0·2</td>
<td>6</td>
<td>IAFC0538</td>
<td>0·2 ± 0·06</td>
<td>8</td>
</tr>
<tr>
<td>IAFC0677</td>
<td>18 ± 1</td>
<td>50</td>
<td>IAFC0677 (wild-type)</td>
<td>2·5 ± 0·2</td>
<td>100</td>
</tr>
<tr>
<td>IAFC6665</td>
<td>0·5 ± 0·1</td>
<td>1</td>
<td>IAFC6665</td>
<td>0·1 ± 0·05</td>
<td>4</td>
</tr>
<tr>
<td>IAFC2286</td>
<td>4 ± 0·3</td>
<td>11</td>
<td>IAFC2286</td>
<td>1·3 ± 0·1</td>
<td>52</td>
</tr>
<tr>
<td>IAF901 (wild-type)</td>
<td>96 ± 9</td>
<td>100</td>
<td>IAF901</td>
<td>0·1 ± 0·05</td>
<td>4</td>
</tr>
<tr>
<td>IAFA0766</td>
<td>0·6 ± 0·2</td>
<td>0·6‡</td>
<td>IAFA0766</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>IAFA6665</td>
<td>16 ± 2</td>
<td>17‡</td>
<td>IAFA6665</td>
<td>2</td>
<td>100</td>
</tr>
</tbody>
</table>

*Values are reported as the percentage activity compared to that of the enzyme fused to the wild-type signal peptide.
†IAF907C and IAF907Csn contain the Sec-dependent signal sequence of XlnA and served as controls for Sec-dependent secretion.
‡IAF901 and IAFA6665 contain the structural gene of XlnA and values are reported as the percentage activity compared to that of IAF901 which contains the wild-type xlnA (Pagé *et al.*, 1996).
mature XlnC during the chase. The mean processing rate of the precursor was slightly slower than that observed previously with wild-type XlnC (Faury et al., 2004), since a small amount of precursor was still detectable after 1 h of chase. As expected, only the XlnC precursor could be observed in the tatC mutant harbouring pIAFC0677 even after a 5 h chase (Fig. 1b).

However, what gave rise to the discrepancy in the XlnC yield by the different transformants? Since differences in transcription have been excluded, at least two other factors could be involved, namely the precursor-processing rate and translation. To address this question, pulse–chase experiments were carried out for all the transformants. The relative intensity of precursor labelling allowed us to estimate the translational level of each construct, and the chase determined the precursor-processing rate. It was previously shown that the wild-type XlnC precursor disappeared completely after 1 h of chase, while precursors mutated in the Tat consensus motif persisted for 2 or even 5 h of chase, resulting in an overall low yield of XlnC (Faury et al., 2004). Therefore, labelled culture samples were collected after 2 h of chase where the presence of precursors would indicate slower processing rates. It should be pointed out that except for IAFC0538 the mature XlnC appearing in these pulse–chase experiments was always extracellularly located since it could be immunoprecipitated from the cell-free supernatants (data not shown).

As shown in Fig. 2, all the transformants synthesized a XlnC precursor of the expected size. Some differences in the labelling intensity were observed, which could partly explain the differences in XlnC yields of certain transformants. Moreover, despite precursor processing, this does not mean that XlnC was properly folded and active, since the structural gene of XlnC fused to a Sec-dependent signal sequence was effectively processed, but was inactive and rapidly degraded in the supernatant, likely because of improper folding (Faury et al., 2004). Only two transformants, IAFC0624 and IAFC0677, exhibited similar processing rates compared to the wild-type precursor (IAF916C). For transformants IAFC6272 and IAFC1955, about 60 % of precursors were still detectable, but in IAFC6272 two forms of equal intensity of the mature protein were detected. This is probably attributable to a second potential cleavage site present in the sequence of this signal peptide (see Table 1). In IAFC2286, the precursors disappeared almost completely after 2 h of chase, but little precursor was processed into mature XlnC, explaining the low XlnC yield. In IAFC0766, the precursor disappeared after 2 h without processing. In transformants IAFC6557, IAFC0538 and IAFC6665, the precursors, showing discrepancies in labeling, were slowly processed into mature XlnC, while after 2 h of chase there was still a fair amount of precursor left. These experiments showed that the six signal sequences giving rise to a low (3–7 IU ml$^{-1}$) or background activities (below 2 IU ml$^{-1}$) represented inefficient Tat signal sequences for XlnC secretion, except for IAFC0538 and IAFC1955 (see below).

Lack of active XlnC secretion by IAFC0766 and IAFC6665 could also be attributed to Sec-dependent signal peptides, which might have been erroneously predicted by TATscan. To test this possibility, the two signal sequences were fused to the xlnA structural gene, which encodes a Sec-dependent protein (Page et al., 1996), giving plasmids pIAFA0766 and pIAFA6665, and the corresponding transformants IAFA0766 and IAFA6665. As shown in Table 3, no XlnA production was detectable in IAFA0766, indicating that this predicted sequence was not a Sec-dependent signal sequence and might not be a signal sequence at all. In contrast, IAFA6665 yielded 17 % of the XlnA produced by the wild-type strain IAF901, indicating that this signal peptide was Sec-dependent, but was less efficient than the native signal peptide of XlnA.

**Localization of the precursor and mature XlnC in IAFC1955 and IAFC0538 transformants**

According to the genomic sequence of *S. coelicolor* A3(2), SCO0538 and SCO1955 are a probable sugar transporter

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**Fig. 2.** Pulse–chase experiments using *S. lividans* 10-164 transformants harbouring various plasmids. For each transformant, the left and right slots show the results after a pulse of 2 min and 2 h chase, respectively. The upper and lower bands correspond, respectively, to the precursor and the mature form of XlnC. Lanes: 1, IAF916C; 2, IAFC6272; 3, IAFC0677; 4, IAFC6557; 5, IAFC0624; 6, IAFC1955; 7, IAFC0538; 8, IAFC0677; 9, IAFC6665; 10, IAFC2286. p, Precursor; m, mature protein.
sugar-binding lipoprotein and a putative Fe–S-binding membrane lipoprotein, respectively. Since the XlnC precursors in IAFC1955 and IAFC0538 were synthesized and processed as shown by pulse–chase experiments (Fig. 2), it is important to show the cellular localization of precursors and mature products. To do so, both the cytoplasmic and membrane fractions of IAFC0538, IAFC1955, as well as the same constructs in a tatC mutant, IAF916C ΔtatC and S. lividans 10-164 were prepared and analysed by Western blotting (Fig. 3). As expected, no XlnC was detected in S. lividans 10-164 because the strain did not express xylanase genes (Hurtubise et al., 1995). Since the tatC mutant lacked an important component of the Tat secretion system (Berks et al., 2000), accumulation of precursors was observed in the cytoplasmic fraction of IAF916C ΔtatC, IAFC0538 ΔtatC and IAFC1955 ΔtatC. Moreover, no xylanase-like polypeptide was observed in the membrane fractions of tatC mutants. Also, XlnC precursors were detectable in the cytoplasmic fractions of IAFC0538 and IAFC1955, representing the de novo synthesis of the precursors. In the membrane fraction, besides the faint precursor band, two other bands were detected corresponding to the mature XlnC and a degradation product of XlnC. The presence of mature XlnC in the membrane fraction was expected since the signal peptides originated from putative membrane-associated lipoproteins. However, no xylanase activity was detected either in the intact or in the solubilized membrane fraction (data not shown), implying that XlnC attached to the membrane via the lipomodification of the cysteine residue might be improperly folded. So, it was not surprising that XlnC secretion into the culture medium was greatly diminished since XlnC remained attached to the membrane. Active XlnC was detected in the culture supernatants of IAFC1955, but not of IAF916C. This might be explained by the fact that certain lipoproteins appeared to be released from the bacterial cell by proteolytic cleavage during growth (Sutcliffe & Russell, 1995). Taken together, the results indicated that both lipoprotein signal peptides are unequivocally Tat-dependent.

**Chitosanase production directed by putative Tat signal peptides of *S. coelicolor* A3(2)**

The predicted signal sequences that mediated XlnC secretion were Tat-dependent sequences. However, variable results obtained by these protein fusions suggested that there might be some incompatibility between the signal peptide and the reporter protein fused to it, which probably reduced productive Tat-targeted secretion. To test this possibility, chitosanase SCO0677 from *S. coelicolor* A3(2) was used. Therefore, in the designation of plasmids and transformants, C for XlnC was replaced by Csn for chitosanase. The chitosanase was chosen because it was secreted through the Tat-dependent pathway as its signal peptide, when fused to the XlnC mature part, allowed secretion of the exclusively Tat-dependent XlnC (Table 3). In addition, the pulse–chase experiment showed Tat-dependent processing of the precursor protein (Fig. 1). Moreover, when pIAFCsn0677, which contained the chitosanase gene with its own signal sequence, was used to transform a tatC mutant, only a trace amount (<5%) of chitosanase activity was detected in the culture supernatant compared to the control IAFcsn0677 (Table 3). Therefore, chitosanase is indeed a true Tat-dependent enzyme, and it could not be redirected through the Sec-dependent system when the Tat machinery was impaired. Most importantly, chitosanase is composed mostly of α-helices (Marcotte et al., 1996), while XlnC contains essentially β-sheets (Törönen & Rouvinen, 1995). In addition, the catalytic mechanism of chitosanase proceeds by anomeric inversion (Fukamizo et al., 1995), whereas that of XlnC functions by anomeric retention (Gebler et al., 1992). Even though both enzymes are hydrolases and do not require a cofactor, the folding of chitosanase and XlnC is sufficiently different. Therefore, the structural chitosanase gene from *S. coelicolor* A3(2) was fused to the nine selected signal sequences, respectively.

As shown in Table 3, the activities of XlnC and chitosanase are not of the same order of magnitude. To facilitate comparison of the activities of reporter enzymes, values are

![Fig. 3. Localization of the precursor and mature XlnC in various transformants by Western blot analysis using anti-XlnC serum. Lanes: 1, purified XlnC; 2, IAFC1955; 3, IAFC0538; 4, IAFC1955 ΔtatC; 5, IAFC0538 ΔtatC; 6, IAF916C ΔtatC; 7, S. lividans 10-164. C, Cytoplasmic fraction; M, membrane fraction; p, precursor; m, mature protein; d, degradation product.](https://www.microbiologyresearch.org/4606114.png)
expressed as a percentage activity compared to the reporter fused to its native signal sequence, which was fixed at 100%. As shown in Table 3, when the signal peptide of chitosanase was fused to XlnC there was a 50% decrease in the XlnC yield, while chitosanase production was increased by 160% when the XlnC signal peptide was fused to chitosanase. With IAFC6272, there was a 22% decrease in XlnC yield, while the same signal peptide in IAFCsn6272 enhanced chitosanase production by 250%. In IAFC2286, the XlnC yield decreased by 90%, while the same signal sequence in IAFCsn2286 only resulted in a 50% decrease in chitosanase production. The signal sequence in IAFC0624 and IAFCsn0624 increased by 220 and 480% of the production of the respective enzymes over their controls, and therefore represented the best signal sequence identified in this study. So, the nature of the protein fused with a given signal peptide exerted some effects on the Tat-secretion, but the overall yields of the two enzymes were rather similar.

Three putative signal peptides resulted in no XlnC production. When the same three signal sequences were fused to the chitosanase structural gene, giving the transformants IAFCsn0766, IAFCsn6665 and IAFCsn0538, very low chitosanase activities were measured in their culture supernatants, and values were at the limit of detection (Table 3). These results were expected because one was not a signal peptide, one was a Sec-dependent signal peptide, and one was a lipoprotein signal peptide. In IAFC0538 and IAFC1955, it was already shown that XlnC was located in the membrane fraction. Therefore, it is also likely that the chitosanase in IAFCsn0538 and IAFCsn1955 is located in the membrane too.

When a Sec-dependent signal peptide was fused to XlnC, the enzyme was secreted, but was inactive and rapidly degraded in the supernatant (Faury et al., 2004). In contrast, when the Sec signal peptide was fused to chitosanase (IAF907Csn), the chitosanase yield represented 25% of production compared to the wild-type precursor (Table 3). This indicated that chitosanase, albeit bearing a Tat-dependent signal peptide, could still be secreted to some extent by the Sec-dependent system when fused to a Sec-dependent signal peptide. So, some activity should have been detected in the culture supernatant of IAFCsn6665 where csn was fused to a Sec-dependent signal sequence. However, this signal peptide was not very efficient, thus resulting in very low chitosanase activity.

Among the nine signal sequences chosen for this work, seven represent types of putative Tat-dependent proteins found in S. coelicolor A3(2) by our TATscan program. The best signal sequence for the expression of the fusion proteins identified in this work is derived from a putative secreted protein with undefined function (SCO0624). The signal peptide of the putative alkaline phosphatase (SCO2286) consists of 62 aa and may represent the longest Tat-dependent signal peptide found thus far. Intriguingly, these two proteins, and another one (SCO6557) identified by TATscan, were not identified by using TATFIND version 1.2 (Dilks et al., 2003), nor were they present in the cited predictions of the modified TATFIND program (Schaerlaekens et al., 2004). In contrast, the two signal sequences (SCO0766 and SCO6665) yielding no production of the reporter proteins were proposed as putative Tat signal sequences in both previous searches (Dilks et al., 2003; Schaerlaekens et al., 2004). Our experiments show that the SCO0766 signal peptide was neither Tat- nor Sec-dependent, whereas the SCO6665 signal peptide was a low-efficiency Sec-dependent one. This implies that the diverse criteria so far employed for prediction of putative Tat substrates still awaits further modification upon the accumulation of more experimentally identified Tat signal peptides.

This work is the second experimental study on putative Tat-dependent proteins predicted by computational genome analyses. The first experimental study was carried out by Jongbloed et al. (2002) on the Tat system in Bacillus subtilis using proteomic techniques. Out of the 14 proteins bearing the [RK]R motif and detected in the extracellular proteome, only the phosphodiesterase PhoD was shown to be secreted in a strictly Tat-dependent manner (Jongbloed et al., 2000). This could be expected if the stringent criteria for the prediction of Tat-dependent substrates used in our work apply to the B. subtilis genome. Out of the 14 proteins, only PhoD meets these criteria, even though KR in the consensus motif is accepted. In fact, the program TATFIND version 1.2 predicted only seven putative Tat substrates in the whole B. subtilis genome (Dilks et al., 2003). In contrast, 145 putative Tat substrates were predicted for S. coelicolor A3(2), representing the organism in which the highest number of Tat substrates were found among the 84 prokaryotes examined. Moreover, the Tat pathway might play a wider role in this organism in the secretion of diverse proteins, ranging from various hydrolases to some substrate-binding and transport proteins. As the criteria for prediction of Tat substrates used in our work applied only to the proteins bearing an RR motif, proteins with a KR motif might still account for a portion of the whole pool of the Tat-dependent proteins in S. coelicolor A3(2). The Tat pathway is, therefore, after the general Sec pathway, another important protein secretion system in this organism. However, the computer predictions have to be interpreted with caution until they can be validated experimentally.

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