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Effect of Signal Peptide Alterations and Replacement on Export of Xylanase A in *Streptomyces lividans*

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Starting from its translation initiation site, the *Streptomyces lividans* xylanase A signal peptide consists of 41 amino acids. This signal peptide was deleted and successively replaced with one of six signal peptides from other enzymes secreted by *S. lividans* and by a signal peptide from the outer membrane protein (LamB) of *Escherichia coli*. Deletion of the xylanase A signal peptide or modification of its cleavage site abolished secretion of the enzyme. Replacement with the signal peptides of either xylanase B, cellulase A, mannanase, or acetylxylan esterase produced equivalent amounts of xylanase A, while the signal peptides of cellulase B, xylanase C, and LamB secreted less enzyme than did the wild type. All the clones exhibited the same transcription levels, which indicated that the variations in xylanase production were due to the natures of the signal sequences.

Streptomyces species are gram-positive bacteria known to secrete large quantities of proteins (6). The hemicellulases are one class of secreted proteins which are used for the "biobleaching" or prebleaching of wood pulp. This industrial application has attracted considerable attention in the paper industry as an environmentally friendly alternative to the chlorine-based processes still in use. However, in order for this method to be applicable for industrial purposes the yield of secreted hemicellulase must be increased.

The mechanism of protein secretion in *Streptomyces* species is not well characterized, but signal-dependent protein secretion is likely to utilize components organized in a pathway similar to that of the general secretory pathway of *Escherichia coli*. However, some differences do exist. For example, the signal sequences of secreted proteins in streptomycetes are generally longer than the signal sequences of either eukaryotes or gram-negative bacteria. The average length of signal sequences in eukaryotes or *E. coli* is in the range of 22 to 24 amino acids, while in gram-positive bacteria the average length is around 29 to 31 amino acids (29). For *Streptomyces* species, signal sequences as long as 70 amino acids have been reported (21). In attempting to optimize the secretion of proteins by streptomycetes, two of the questions we addressed are whether the differences in signal sequence structure affect secretion efficiency and whether substitution of a native signal sequence by that of another secreted enzyme can improve production yield.

In *Streptomyces lividans*, genes encoding three xylanases (25), two cellulases (27, 30), a mannanase (1), and an acetylxylan esterase (24) have been cloned homologously with a multicopy plasmid, pIJ702. In all cases the gene product was fully secreted, reaching a 60-fold increase over the production level of the wild-type strain. Previous studies showed that xylanase A could represent up to 80% of the total proteins recovered from the culture supernatant of the recombinant strain *S. lividans* IAF18 (3). In the present study, we attempted to show whether signal peptides are interchangeable for xylanase A production by replacing the wild-type signal sequence in *S. lividans* with signal sequences from six homologous genes encoding proteins secreted in *S. lividans.*

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MATERIALS AND METHODS

Bacterial strains and media. *S. lividans* 10-164 (cellulase- and xylanase-negative mutant) was used as a host for recombinant plasmids (19). Spores were prepared according to the method of Hopwood et al. (9). The minimal growth medium M14 had the following composition: KH_2PO_4, 1 g; K_2HPO_4, 5.5 g; $(NH_4)_2SO_4$, 1.4 g; Tween 80, 2 ml; oat spelt xylan (Sigma Chemical Co.), 10 g (as a carbon source); and 1 ml of a trace metal solution ($CoCl_2 \cdot 7H_2O$, 200 mg; $FeSO_4 \cdot 7H_2O$, 500 mg; $MnSO_4 \cdot H_2O$, 160 mg; $ZnSO_4 \cdot 7H_2O$, 140 mg [all dissolved in 100 ml of distilled water]). All ingredients were dissolved in 1 liter of distilled water and autoclaved. Before use, 0.6 ml of a 5% $MgSO_4$ solution and 1 ml of a 3% $CaCl_2$ solution were added aseptically to 100 ml of the medium described above. Thiostrepton (obtained as a gift from Bristol-Myers Squibb Canada Inc., Montréal, Québec, Canada) was added when required to achieve a final concentration of 5 μg/ml. Protoplasting and transformation of the mutant 10-164 were performed as described by Hopwood et al. (9). *E. coli* CJ236 *dut-1 ung-1 thi-1 relA-1*, pCJ105 (Cm^r), and MC1061 *araD139* Δ(*araABC-leu*)*7697* Δ*lacX74 galU galK hsr hsm strA* were purchased from Bio-Rad for use in site-directed mutagenesis experiments. Phagemid pTZ19U was purchased from Pharmacia.

The screening of transformants of *E. coli* was performed on 2× TY (16 g of Bacto Tryptone, 10 g of yeast extract, and 5 g of NaCl per liter) plates containing ampicillin (100 μg/ml). DNA manipulations were performed according to the method of Sambrook et al. (22).

Plasmid constructions. The general scheme of plasmid construction is shown in Fig. 1. The xylanase A gene (*xlnA*) from *S. lividans* 1326, located on a 3.2-kb *Sph*I fragment in pIAF18 (19), was inserted in the *Sph*I site of the phagemid pTZ19U to generate pAM19.1 (20). This plasmid encodes a 43-kDa xylanase A1. One kilobase of the 3′ end of *xlnA* was deleted by digestion with *Sac*I. The resulting plasmid (pIAF801) encodes a truncated xylanase A2 (32 kDa) which is fully active but lacks its xylan binding domain (4). In order to replace the xylanase signal peptide, a restriction site was added on each side of the signal peptide-encoding sequence. This cloning cassette was constructed by site-directed mutagenesis by the method of Kunkel (13) with a commercial kit (Bio-Rad) and oligonucleotides synthesized on a Gene Assembler (Pharmacia).

Strain CJ236 harboring plasmid pIAF801 was used to prepare uracil-containing single-stranded DNA serving as a template for mutagenesis. An *Hin*dIII site was created 17 bp upstream from the translation initiation codon with the 22-mer oligonucleotide 5′-GCCTCCAAAGCTTAGTGGTCAC-3′. A *Kpn*I site was introduced 18 bp from the beginning of the mature xylanase-encoding sequence with the 22-mer oligonucleotide 5′-GCCGCCGCGGTACCGAGCGTGC-3′ (bases mutated are underlined). Finally, 0.9 kb of the 5′ end of *xlnA* was deleted by digestion with *Hin*dIII, generating the plasmid pIAF807, which was used in the signal peptide replacement experiments after *Hin*dIII-*Kpn*I digestion.

The signal peptide sequence from each gene was prepared by PCR with plasmids encoding the respective genes as templates (Gene Amp DNA Amplification Reagent Kit; Perkin-Elmer Cetus, Emeryville, Calif.). The oligonucleotides used for signal peptide amplifications are not listed. The first oligonucleotide contained the 15 nucleotides (nt) of *xlnA* sequence spanning the *Hin*dIII site

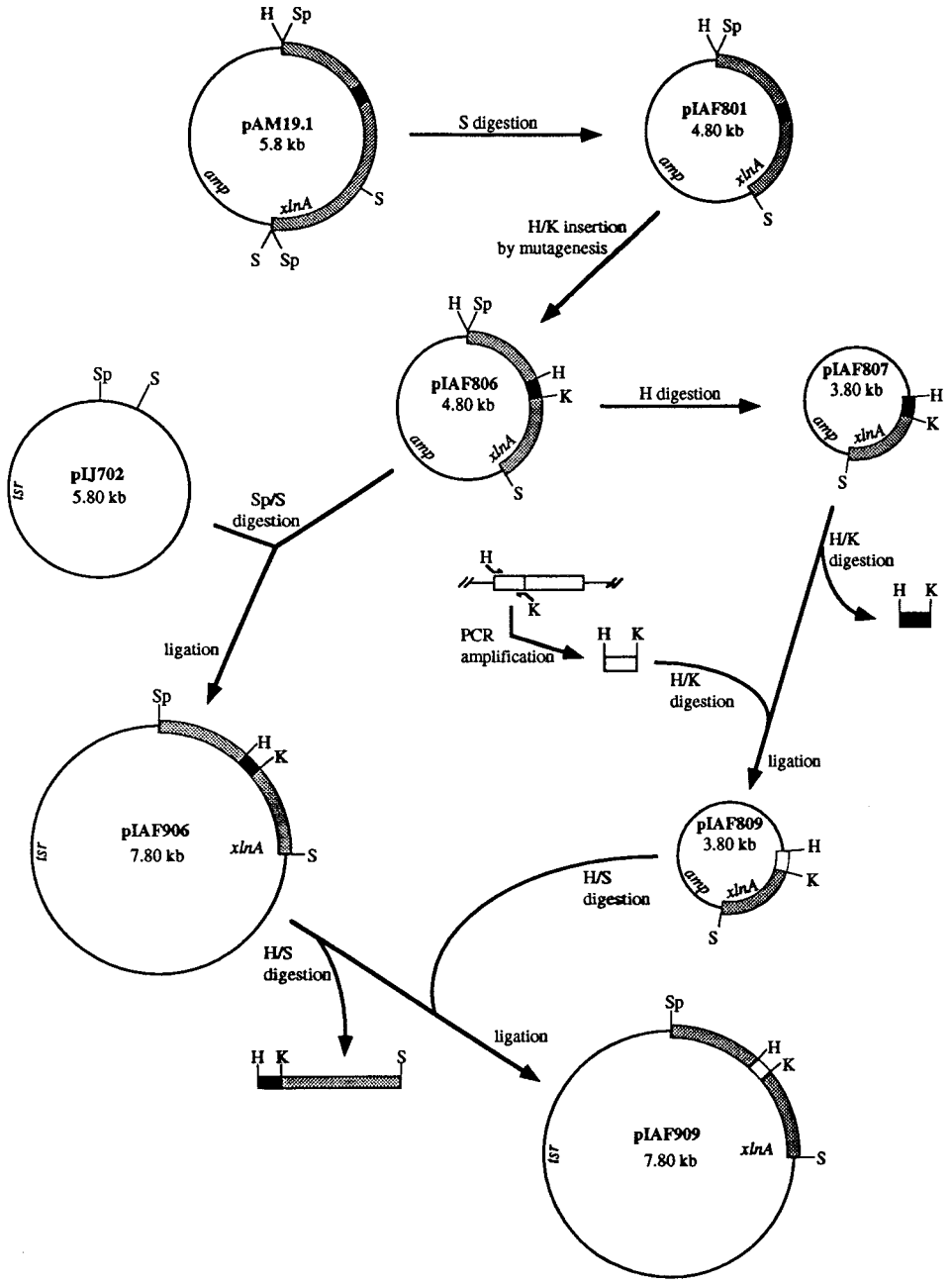

FIG. 1. Replacement of the leader peptide region of *xlnA* with mannanase. This schematic diagram shows an example of the use of a cassette for subcloning foreign leader peptide sequences. Symbols and abbreviations: grey box, *xlnA*; solid box, leader peptide sequence of *xlnA*; open box, leader sequence of *manA*; *amp*, ampicillin resistance gene; *tsr*, thiostrepton resistance gene. Abbreviations for restriction enzymes: H, *Hin*dIII; K, *Kpn*I; S, *Sac*I; Sp, *Sph*I.

and at least 15 nt of the 5′ end of the chosen signal sequence, and the second oligonucleotide contained 15 nt of the mature xylanase-encoding sequence spanning the *Kpn*I site and 15 or more nt of the 3′ end of the signal sequence. The PCR conditions were 5 min at 94°C followed by 30 cycles of 1 min at 94°C, 2 min at 40°C, and 3 min at 72°C. The reaction mixture was electrophoresed on a 2.5% agarose gel, and the amplified sequence was isolated with a MERmaid gel extraction kit (Bio 101, Inc., La Jolla, Calif.) according to the manufacturer's instructions. The purified fragment containing the desired signal sequence was digested with *Hin*dIII-*Kpn*I and then cloned into the same sites in pIAF807. This digested plasmid was prepurified with a QIAEX gel extraction kit (Qiagen Inc., Chatsworth, Calif.) to eliminate the 156-bp *Hin*dIII-*Kpn*I fragment encoding the wild-type signal sequence. The *xlnA* genes engineered in *E. coli* were inserted in pIJ702 (12) to monitor the secretion of xylanase A2 after transformation of protoplasts of *S. lividans* 10-164. For all constructions involving a signal peptide replacement, the 1-kb *Hin*dIII-*Sac*I fragment was cloned into the same sites in pIAF906, which is pIJ702 with a 2-kb *Sph*I-*Sac*I insert which also contained the two new restriction sites (*Hin*dIII and *Kpn*I). The generated plasmids are listed in Table 1.

To remove the signal peptide sequence of *xlnA*, pIAF801 was deleted by looping out 120 bp with the 30-mer oligonucleotide 5′-GAGCGTGCTCTCG GCCATGACTGTGCCTCC-3′ containing 15 nt upstream of the translation initiation codon and 15 nt encoding the beginning of the mature enzyme, generating pIAF803. In a similar manner, signal peptidase cleavage site Ala-His-Ala was replaced with Val-Asp-Ser by using the 49-mer oligonucleotide 5′-GCGC CGAGCGTGCTCTCGGCGGAGTCGACCCCCGGCGGCGCGATCAG TG-3′, generating pIAF805 (bases mutated are underlined). The 2-kb *Sph*I-*Sac*I fragment of pIAF803 (deletion of the signal peptide sequence) and pIAF805 (alteration of the signal peptidase cleavage site) were cloned at the same restriction sites in pIJ702, giving pIAF903 and pIAF905, respectively.

The accuracy of the DNA sequences of the inserted *Hin*dIII-*Kpn*I fragment

TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristic	Source or reference
S. lividans		
pIJ702	Vector containing *tsr* and *mel* markers	12
pIAF901	pIJ702 derivative containing *xlnA* from *S. lividans* as a 2-kb *Sph*I-*Sac*I fragment	This work
pIAF903	pIAF901 derivative containing a deletion of the signal peptide sequence of *xlnA*	This work
pIAF905	pIAF901 derivative carrying a mutation of the signal peptide cleavage site	This work
pIAF906	pIAF901 derivative containing an *Hin*dIII and a *Kpn*I restriction site on each side of the signal peptide sequence	This work
pIAF909	pIAF906 derivative containing the signal peptide sequence of *manA*	This work
pIAF912	pIAF906 derivative containing the signal peptide sequence of *celA*	This work
pIAF913	pIAF906 derivative containing the signal peptide sequence of *celB*	This work
pIAF914	pIAF906 derivative containing the signal peptide sequence of *axe*	This work
pIAF915	pIAF906 derivative containing the signal peptide sequence of *xlnB*	This work
pIAF916	pIAF906 derivative containing the long signal peptide sequence of *xlnC*	This work
pIAF918	pIAF906 derivative containing the signal peptide sequence of *lamB*	This work
E. coli		
pTZ19U	Phagemid carrying *bla* and part of *lacZ*	Pharmacia
pAM19.1	pTZ19 derivative containing *xlnA* as a 3.2-kb *Sph*I fragment	20
pIAF801	pAM19.1 derivative containing a 1-kb deletion at the 3′ end of *xlnA*	This work
pIAF803	pIAF801 derivative containing a deletion of the signal peptide sequence of *xlnA*	This work
pIAF805	pIAF801 derivative carrying a mutation of the signal peptide cleavage site	This work
pIAF807	pIAF801 derivative containing promoterless *xlnA* as a 1-kb fragment with the *Hin*dIII-*Kpn*I signal peptide-cloning cassette	This work

amplified by PCR and of other constructs was confirmed by sequencing by the dideoxy chain termination method (23) with the ALF DNA Sequencer (Pharmacia Biotech).

Culture conditions and sampling procedures. Recombinant constructs of *S. lividans* 10-164 were grown in 125-ml shake flasks containing 20 ml of M14 minimal medium supplemented by 1% oat spelt xylan and 5 μg of thiostrepton per ml. Medium was inoculated with 2×10^6 spores per ml and incubated at 34°C on a rotary shaker at 240 rpm. Two-milliliter samples were removed at intervals. Supernatant fraction was recovered after centrifugation of 1 ml of culture for 5 min in a microcentrifuge and used for the measurement of extracellular xylanase activity. The growth was monitored by determination of the total protein content of the culture. Mycelium was disrupted by sonication in a Sonicator Ultrasonic Processor XL (Heat System, Farmingdale, N.Y.). One milliliter of culture was subjected to three 30-s bursts interspersed with 30-s cooling periods in an ice bath.

Enzyme assays. Xylanase activity in supernatants and in cell extracts was measured by the dinitrosalicylic acid method (18). One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 μmol of reducing sugars (expressed as xylose) in 1 min at 60°C.

Proteins and Western blot (immunoblot) analysis. The protein content of enzyme preparations was determined using the method of Lowry et al. (16). Protein pellets were dissolved in a denaturing buffer, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (14), and then subjected to Western blotting (28). Identification of xylanase-related polypeptides was carried out with anti-xylanase A antibodies coupled to ^{125}I-labelled protein A (ICN Canada Ltd.).

Isolation and analysis of RNA. Mycelium from a 36-h-old culture (20 ml) was harvested by centrifugation for 5 min at 2,500 × *g*. The cell pellet was resuspended in 6 ml of 4 M guanidinium thiocyanate as described by Sambrook et al. (22). The mycelium was disrupted by sonication with three 30-s bursts interspersed with 30-s cooling periods in an ice bath. The lysate was centrifuged for 5 min at 23,000 × *g*. The supernatant was layered on a 5.7 M CsCl cushion and centrifuged for 24 h at 20°C at 120,000 × *g*. Total RNA dissolved in a 50% formamide–7% formaldehyde mixture was applied onto a nitrocellulose membrane. Slot blots, each containing 5 to 10 μg of RNA, were hybridized in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 70°C for 16 h according to the method of Hopwood et al. (9). The xylanase probe consisted of pIAF807 which was ^{32}P labelled by nick translation.

RESULTS AND DISCUSSION

Alteration and replacement of the sequence encoding the signal peptide of the xylanase A. The xylanase A gene was modified to enable a series of translational fusions with different signal peptide-encoding regions. An *Hin*dIII site and a *Kpn*I site were inserted on each side of the signal peptide-encoding region of the *xlnA* gene, resulting in plasmid pIAF807. The *Kpn*I insertion created a mutation in the structural gene in which alanine 7 was replaced with threonine. This replacement was located outside the catalytic domain (4) and, fortunately, did not affect the enzymatic activity of the mutant enzyme compared with that of the wild-type enzyme (data not shown). The *Hin*dIII-*Kpn*I fragment from pIAF807 was replaced with amplified *Hin*dIII-*Kpn*I fragments containing the signal-encoding regions of the other genes. The relevant region of each plasmid was sequenced to verify that the proper modification had occurred. For expression in *S. lividans*, the 1-kb fragment obtained by digestion of each plasmid with *Hin*dIII-*Sac*I was inserted at the same sites in pIAF906, giving the plasmids containing signal-encoding regions of mannanase (pIAF909), cellulase A (pIAF912), cellulase B (pIAF913), acetylxylan esterase (pIAF914), xylanase B (pIAF915), xylanase C (pIAF916), and LamB (pIAF918). The plasmids are listed in Table 1.

Effects of mutations in the signal peptide on secretion of xylanase. Initially, it was important to demonstrate that the intact signal peptide was necessary for xylanase A secretion. *S. lividans* cultures harboring plasmids pIAF903, in which the signal peptide is deleted, and pIAF905, which is mutated in the signal peptidase recognition site, were analyzed for xylanase production. These two clones did not secrete xylanase in the culture medium (Fig. 2b and c). Furthermore, no intracellular or cell-associated xylanase activity was found with either clone (data not shown). The possibility that the xylanase was produced but inactive was ruled out by immunoblot analysis (Fig. 3, lanes b and c). In addition, no reacting protein was detected in the cellular extract of either clone (data not shown). These results show that an intact signal peptide was essential for xylanase secretion and that the protein was rapidly degraded, suggesting the presence of an efficient proteolytic pathway for nonmaturable polypeptides in *S. lividans*. Opposite results were shown for *E. coli*, for which a preprotein in which the signal peptide cleavage site was altered remains trapped in the membrane and the carboxy-terminal end of the protein is exposed to the periplasmic side of the membrane (5).

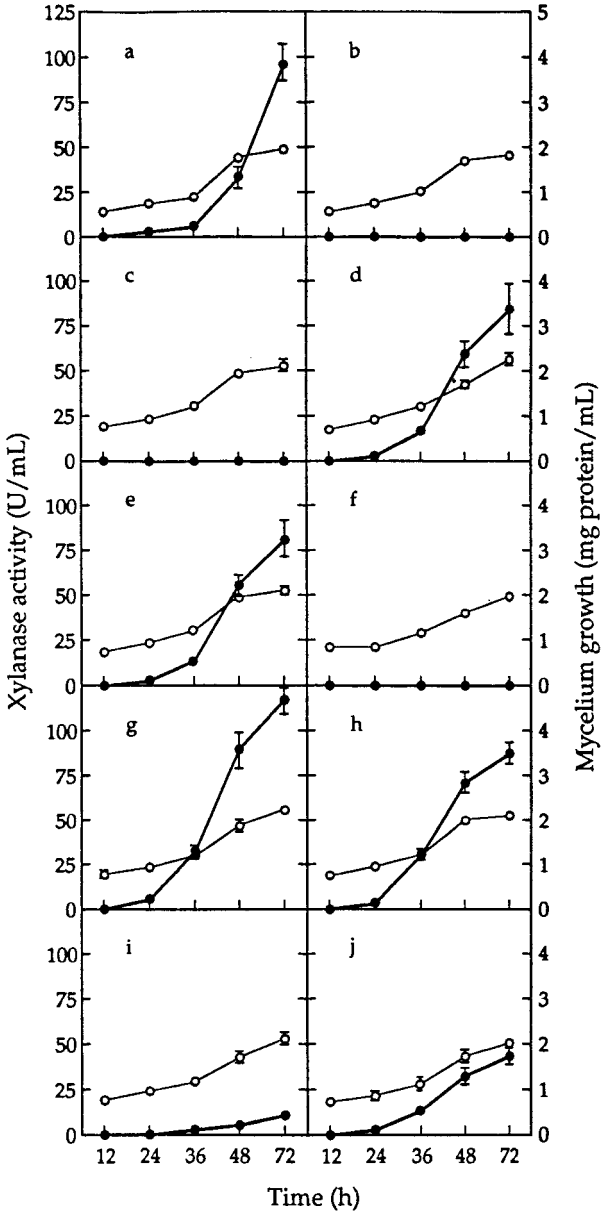

FIG. 2. Xylanase activity in extracellular fractions of *S. lividans* 10-164 transformants carrying various plasmids. (a) pIAF901 (wild type); (b) pIAF903 (deletion of the signal peptide sequence); (c) pIAF905 (mutation in the signal peptide cleavage site); (d) pIAF909 (signal peptide sequence of *manA*); (e) pIAF912 (signal peptide sequence of *celA*); (f) pIAF913 (signal peptide sequence of *celB*); (g) pIAF914 (signal peptide sequence of *axe*); (h) pIAF915 (signal peptide sequence of *xlnB*); (i) pIAF916 (signal peptide sequence of *xlnC*); (j) pIAF918 (signal peptide sequence of *lamB*). The cells were cultured at 34°C in M14 medium containing 1% xylan as carbon source. The cells were removed by centrifugation at 5,000 × *g* for 10 min. Symbols: ●, xylanase activity; ○, mycelium growth.

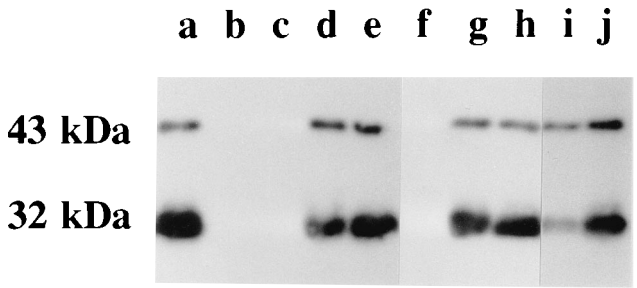

FIG. 3. Western blot analysis of extracellular xylanase produced by *S. lividans* 10-164 carrying the following plasmids: lane a, pIAF901; lane b, pIAF903; lane c, pIAF905; lane d, pIAF909; lane e, pIAF912; lane f, pIAF913; lane g, pIAF914; lane h, pIAF915; lane i, pIAF916; lane j, pIAF918. Samples of supernatant (10 μl) after 72 h of cultivation were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and probed with antixylanase antibodies coupled with ^{125}I-labelled protein A.

Effect of signal peptide replacement on secretion of xylanase A. The signal peptide of the xylanase A of *S. lividans* was substituted by six homologous signal peptides belonging to proteins secreted by *S. lividans* and by a heterologous signal peptide of LamB of *E. coli* (Table 2). The extracellular xylanase activity was measured throughout the growth cycle until stationary phase (Fig. 2). The addition, by site-directed mutagenesis, of a restriction site on each side of the signal peptide sequence (IAF906) had no effect on the level of XlnA production, which was similar to that of the wild type (IAF901) (data not shown). Compared with the control (IAF901), the clones IAF909, IAF914, and IAF915 secreted almost the same amount of enzyme (Fig. 2a, d, g, and h). Interestingly, IAF914 reached the maximal enzyme levels 24 h earlier than did the control (Fig. 2g). While further investigation of this result remains to be performed, the time savings could be an important factor in any industrial production.

The production level with the xylanase C signal sequence (clone IAF916) was particularly low and represented only 1/10 of the wild-type levels (Fig. 2i). This signal sequence (Table 2) does not have the characteristics of signal peptides (29) and contains two Asp residues in the N domain which are known to reduce the efficiency of the protein secretion (11). All these observations suggest that this long, atypical signal peptide is probably not efficiently used. This experiment showed that the signal peptide of xylanase A is not interchangeable with that of xylanase C as it was with the signal peptide of xylanase B. *S. lividans* secretes three xylanases, of which xylanases A and B are equally well produced whereas xylanase C, under standard fermentation conditions, contributes no more than 10% of the total xylanase activity of the wild-type strain (2). This suggests that the signal peptide might be one element, among others, involved in the regulation of xylanase C.

Replacement of the signal peptide of xylanase A with that of cellulase B, clone IAF913, gave negative results (Fig. 2f). This was expected, since the putative signal peptide sequence of cellulase B contained two TTA codons (30). These Leu codons are generally found in genes which are expressed during the stationary phase, in which secondary metabolism occurs. These genes are under the control of *bldA*, which encodes a tRNA recognizing UUA codons (15). Thus, after 72 h of cultivation some xylanase activity should be detected in the culture medium, but this did not happen. It is possible that all the resources of the cells are mobilized to achieve their morphological differentiation and antibiotic production and that therefore the secretion of nonessential proteins is reduced.

The putative signal peptide sequence of cellulase A contains the initiation codon TTG (27). Clone IAF912 constructed with this signal peptide sequence produced almost the same amount of XlnA as did the control (Fig. 2e), indicating that the TTG initiation codon is efficiently used.

Clone IAF918, containing the signal peptide of LamB, produced half the enzyme of the control (Fig. 2j), indicating that signal peptides from gram-negative bacteria are not efficiently

TABLE 2. Amino acid sequences of signal peptides

Protein	Signal peptide sequence[a]	Reference
Xylanase A	MGSYALPRSGVRRSIRVLLLALVVGVLGTATALIAPPGAHA↑ A	24
Xylanase B	MNLLVQPRRRRRGPVTLLVRSAWAVALAALAALMLPGTAQA↑ D	24
Xylanase C	MQQDGTQQDRIKQSPAPLNGMSRRGFLGGAGTLALATASGLLLPGTAHA↑ A	24
Mannanase	MRNARSTLITTAGMAFAVLGLLFALAGPSAGRAEA↑ A	1
Cellulase A	LKRLLALLATGVSIVGLTALAGPPAQA↑ A	27
Cellulase B	MRTLRPQARAPRGLLAALGAVLAAFALVSSLVTAAAPAQA↑ D	30
Acetylxylanesterase	MRTSTGPRASSRTLRTLASGVAVTALAAAGTVAAGAAPAQA↑ A	25
LamB	MMITLRKLPLAVAVAAGVMSAQAMA↑ V	8

[a] The signal peptide cleavage site in each sequence is indicated by an arrow.

used in *Streptomyces* species. Similar data were reported for *Bacillus* spp. by Simonen and Palva (26).

Considering that xylan was the sole carbon source in these experiments, it was expected that high levels of xylanase would support better growth. However, the growth curve was similar for all clones, irrespective of the quantity of xylanase secreted in the culture medium. There is probably a lower limit beyond which xylanase production influences the growth rate. As far as we know, wild-type *S. lividans* growing on xylan as a carbon source produces a maximum of 15 IU of xylanase per ml and grows perfectly well under these conditions (2). Another reason accounts for the growth of clones secreting no xylanase, such as IAF903 and IAF905 (Fig. 2b and c). Commercial preparations of xylan contain 15% oligomeric glucose, and *S. lividans* 10-164 expressed β-glucosidase and β-xylosidase activity associated with its cell surface (10). Thus, these enzymes could hydrolyze oligoglucosides and oligoxylosides and allow growth.

In order to verify that the level of enzyme activity correlated with the amount of secreted protein, immunoblot analysis with an antixylanase serum was performed on extracellular fractions of the different clones after 72 h of growth (Fig. 3). The major 32-kDa protein band corresponding to the truncated xylanase A2 and a minor 43-kDa protein band were immunodetected in the culture supernatant of all xylanase-producing clones. The 43-kDa band showed approximately the same intensity for each clone, independently of the production of the 32-kDa xylanase expressing clones. This band corresponds to native xylanase A and results from induction of the chromosomal *xlnA*. This was an unexpected result, since the host strain *S. lividans* 10-164 is a xylanase-negative mutant. However, xylanase overproduction by strain 10-164 when transformed with a multicopy plasmid containing *xlnA* leads to the rapid hydrolysis of xylan to xylobiose, the end product of xylan hydrolysis. Recently, we have shown that the active transport of xylobiose was defective in 10-164 (10), causing the accumulation of xylobiose in the culture supernatant. Therefore, it is probable that small amounts of xylobiose, the natural inducer of *xlnA* (2), penetrate the cell by passive permeation and induce the production of chromosomal *xlnA*.

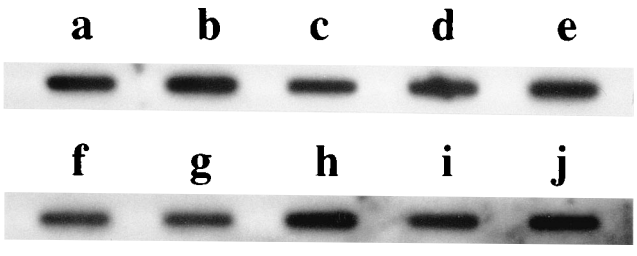

FIG. 4. Slot blot analysis after 36 h of growth of total RNA extracted from clones carrying the following plasmids: slot a, pIAF901; slot b, pIAF903; slot c, pIAF905; slot d, pIAF909; slot e, pIAF912; slot f, pIAF913; slot g, pIAF914; slot h, pIAF915; slot i, pIAF916; slot j, pIAF918. Ten micrograms of total RNA was spotted onto nitrocellulose membrane. The plasmid pIAF807 was used to probe the mRNA of xylanase A.

The variations of xylanase A production after replacement of its signal peptide could be due to a variety of factors, such as the rates of transcription, translation, and secretion of the various fusions. The transcription level of *xlnA* is assumed to be invariant because each version contains the same promoter. This was proven by slot blot hybridization of total RNA isolated from 36-h cultures. There was no significant difference in the xylanase A mRNA content of the clones (Fig. 4). Many aspects of the translation initiation process are uncertain (7), and the effects of mRNA secondary structure on translation initiation cannot be accurately predicted (17). The secondary structure of each mRNA sequence between nt −40 from the translation initiation codon and the peptide cleavage site was predicted by computer analysis with the program Squiggles (Genetics Computer Group Software). Few differences in the three major stem-loop structures were observed, and a similar ΔG (−60 kcal [ca. −250 kJ]) was calculated for each construct.

In conclusion, changes in the signal peptide affect the level of xylanase A production. Signal peptides are not interchangeable in a predictable manner, and further investigation will probably identify features that will allow the combination of a signal peptide with a given enzyme, leading to overproduction of this enzyme in *Streptomyces* species.

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