Partial Characterization of the \textit{Streptomyces lividans} \textit{xlnB} Promoter and Its Use for Expression of a Thermostable Xylanase from \textit{Thermotoga maritima}

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Xylan is a major component of hemicellulose in plant cell walls. It is covalently and noncovalently attached to cellulose, lignin, pectin, and other polysaccharides to maintain cell wall integrity (18). Enzymes that degrade xylan have many useful industrial applications, including the conversion of lignocellulosic material to fuels and chemicals (9) and the processing of hemicellulose to paper (5, 12, 39). During the process of pulp bleaching, for example, xylanases have been used instead of chlorine to increase the extractability of lignin in the production of high-quality paper (10). The use of xylanase to either replace or reduce the amount of chlorine used in pulp bleaching would have a strong positive effect on the environmental impact of the process. The widespread use of xylanase for pulp bleaching, however, has been limited by the high temperature and alkaline pH of pulp-bleaching processes, since most available xylanases are not active under these conditions. Furthermore, the use of xylanase for commercial pulp bleaching requires low-cost, high-volume production of the enzyme.

\textit{Streptomyces} species are gram-positive soil bacteria that produce and export a variety of hydrolytic enzymes that enable them to utilize complex carbohydrates in their natural habitats. Compounds such as xylan, chitin, and starch, as well as cellulosic plant material, serve as the primary sources of carbon, nitrogen, and energy for these organisms. The genes for several of the enzymes involved in complex-carbohydrate utilization have been cloned and partially characterized, although relatively little is known about their regulation. Perhaps the best-studied examples, from the point of view of gene regulation, are the \textit{\theta}-amylase, chitinase, and agarase genes.

The agarase gene, \textit{dagA}, has four promoters (6) and is transcribed by at least three different RNA polymerase holoenzymes (7). At least one of the promoters has been shown to respond to carbon source regulation. Regulation of \textit{\theta}-amylase, somewhat surprisingly, varies from species to species and is dependent on the streptomycete host used for study. Expression of the \textit{Streptomyces griseus} (38) and \textit{S. venezuelae} (41) genes is induced by maltose and repressed by glucose in their native hosts. The \textit{\theta}-amylase of \textit{S. thermoviolaceus} (1) is induced by maltotriose and repressed by mannitol but not glucose in \textit{S. thermoviolaceus}. The \textit{S. limosus} \textit{\theta}-amylase gene is induced by maltose in \textit{S. limosus}, \textit{S. lividans}, and \textit{S. coelicolor} but the pattern of repression is different in the three species (40). In \textit{S. limosus}, expression is repressed by mannitol but not glucose. In \textit{S. lividans} and \textit{S. coelicolor}, expression is repressed by glucose but not mannitol.

Chitinase genes have been cloned from \textit{S. olivaceoviridis} (3), \textit{S. thermoviolaceus} (35), \textit{S. plicatus} (28, 29), \textit{S. griseus} (25), and \textit{S. lividans} (13). Delic et al. (11) examined the regulation of the \textit{S. plicatus chi63} gene in \textit{S. lividans} and showed that expression of \textit{chi63} is induced by partially hydrolyzed chitin and repressed by glucose at the level of transcription initiation. A series of cis-acting mutations within the \textit{chi63} promoter region identified bases important for regulation as well as RNA polymerase recognition (24). An operator consisting of a perfect 12-bp direct-repeat sequence was identified and shown to be the site of both glucose repression and chitin induction (24). Work describing promoter mutations, taken together with work on mutations that affect repressor proteins (11, 16, 24, 36), provides evidence that glucose repression of some catabolite-controlled genes in \textit{Streptomyces} may act through the same repressor proteins and operator sequences that are involved in substrate induction.

Here we report the cloning and partial characterization of a xylanase gene from \textit{S. coelicolor} that is 99\% identical to the xylanase B gene of \textit{S. lividans} (20, 32) and the use of the expression and localization signals of this gene to express and secrete a thermostable xylanase from \textit{Thermotoga maritima}. The \textit{S. lividans} xylanase B gene encodes a protein of 31 kDa and has been characterized biochemically (20). By using en-
zyme activity assays, xylanase expression was detected in S. lividans cells grown on xylan as the sole carbon source but not in cells grown on xylan plus glucose (2). From an analysis of the DNA sequence of the cloned S. lividans xlnB gene, a putative translation start site and signal sequence processing site was suggested (26, 33) based on homology to other xylanase genes and signal sequences (42). To investigate the regulation of the S. coelicolor xylan B gene, we constructed a transcriptional fusion between the promoter region and the xylE reporter gene and examined expression on various carbon sources in S. lividans. Expression of the xlnB-xylE fusion was induced by xylan and repressed by glucose, and we conclude that regulation by glucose and xylan is at the level of transcription initiation. Using both primer extension analysis and in vitro transcription assays, we identified an apparent transcription start site for xlnB that is downstream of the translation start site previously predicted (26, 33). Deletion analysis of the promoter region identified sequences between −268 and −318 to be involved in glucose repression. By using the promoter and signal sequence of the xlnB gene, an in-frame fusion was made to the coding region of the T. maritima synA gene (8). Expression of the recombinant protein was readily detectable in Streptomyces, and the activity of the enzyme was thermostable and localized extracellularly.

MATERIALS AND METHODS

Construction and screening an S. coelicolor genomic library. Chromosomal DNA was isolated from S. coelicolor A3(25) as described previously (17) and partially digested with Sau3A. DNA fragments between 2 and 7 kb in size were selected by agarose gel electrophoresis and isolated with the Geneclean II Kit (Bio 101, Inc.). A library of these fragments was constructed by ligation to Sau3A-digested pUC19 with T4 DNA ligase (Boehringer). Recombinant plasmids were introduced into Escherichia coli JM83, plated on Luria-Bertani agar plates containing 1.5 mg of 4-O-methyl-D-glucurono-D-xylan-Remazol Brilliant Blue R (Sigma) per ml and 50 μg of ampicillin (Sigma) per ml, and incubated at 37°C for 16 h. Colonies were identified for clear zones indicating xylan hydrolysis.

Subcloning and sequencing. Two plasmid-containing clones showing xylan hydrolysis, one of which, pCC76, is shown in Fig. 1, were analyzed by restriction digestion and the chromosomal fragment within them was subcloned into M13mp18 and M13mp19 for DNA sequencing. The nucleotide sequence was determined by the method of Sanger et al. (31) with the pUC19 primer and reverse primers as well as primers synthesized at the University of Georgia Molecular Genetics Instrumentation Facility, Athens. Primers were end labeled with [γ-32P]ATP (Amersham) by using polynucleotide kinase (Promega). Reactions were performed by using the fmol sequencing system (Promega) in a thermocycler (Ericomp Inc.). DNA sequences were analyzed with University of Wisconsin Genetics Computer Group software.

Construction of a xlnB promoter-xylE fusion. A DNA fragment containing the xlnB promoter was amplified from pCC76 by PCR. The primers contained additional sequence to include restriction endonuclease cleavage sites for directional cloning into plasmid pXE4 (19), which contains a promoterless copy of the xylE gene. Oligonucleotide 5' -atacctcgagccggcgtgcctgctgtaa3', which was synthesized to include a PstI recognition sequence at the 5' end (PRIMER 1 in Fig. 2), was the upstream primer, and oligonucleotide 5'-ccgggatacttgctgctgtaa3', which was synthesized to include a BamHI recognition sequence at the 3' end (PRIMER 3 in Fig. 2), was the downstream primer. PCRs were carried out with Taq DNA polymerase (Boehr-inger) and a thermocycler (Ericomp Inc.). PCR products were digested with PstI-BamHI, ligated into PstI-BamHI-digested pUC19, removed as a HindIII-BamHI fragment, and ligated into HindIII-BamHI-digested pXE4. Plasmid DNA isolated from E. coli DH5α-MCR (Bethesda Research Laboratories) was used for subsequent transformation into S. lividans 1326. The DNA sequence of the cloned fragment was determined as described above.

Catechol dioxygenase assays. Spores of S. lividans containing pCC88 (Table 1) were germinated for 28 h in NMMP minimal medium (17) containing 0.5% glycerol. Mycelia were collected and washed twice with NMMP and then grown in NMMP containing the indicated carbon source. To examine xlnB expression in the presence of different carbon sources, precultured mycelia of S. lividans containing various promoter-xylE fusions were grown for 18 h in NMMP medium containing the carbon source indicated at 0.5%. Cells were sampled, harvested, and washed with 20 mM potassium phosphate buffer (pH 7.2). Catechol dioxygenase activity was assayed as described previously (19) with some modifications. The cells were lysed by sonication for 1 min in 10 μl of Triton X-100 per ml and incubated on ice for 15 min. Lysates were centrifuged at 4°C for 20 min, and supernatants were assayed for catechol dioxygenase activity. The protein concentration was measured by the method of Bradford (Bio-Rad Laboratories, Inc.). For catechol dioxygenase assays, 0.5 mg of protein was suspended in a final volume of 500 μl of 100 mM phosphate buffer (pH 7.5) and then mixed with 500 μl of prewarmed (37°C) 0.4 mM catechol. The activity of dioxygenase is expressed as the rate of change in the optical density at 373 nm per minute per milligram of protein.

Primer extension analysis. Spores of S. lividans 1326 containing pCC88 were grown at 30°C for 48 h in liquid NMMP medium (17) containing 0.5% oatspelt xylan, with shaking at 245 rpm. Total cellular RNA was isolated (27) and used for primer extension reactions as previously described (11). An oligonucleotide, 5'-CC ACCCTTGCGGCCGCGTTAGGCGAC-3', complementary to the 5' end of the fragment containing the xylE gene (24) was used to prime the reaction. A DNA sequencing ladder was generated with the same primer, using pCC88 as template.

Preparation of RNA polymerase. S. lividans 1326 cells were grown in YEME medium (17) at 30°C for 46 h. Mycelia were harvested (30 g [wet weight]), washed twice with buffer A (34), and disrupted by two passages through a French pressure cell at 16,000 lb/in². The lysed cells were centrifuged at 15,000 × g for
TABLE 1. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Copy number</th>
<th>Host</th>
<th>Relevant characteristic</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCC16</td>
<td>High</td>
<td>E. coli</td>
<td>pUC19 derivative containing T. maritima synA ORF in EcoRI-HindIII site of pUC19</td>
<td>This work</td>
</tr>
<tr>
<td>pCC18mp19</td>
<td>High</td>
<td>E. coli</td>
<td>M13mp19 derivative containing T. maritima synA ORF in the BamHI site</td>
<td>This work</td>
</tr>
<tr>
<td>pCC20</td>
<td>High</td>
<td>E. coli and Streptomyces</td>
<td>pCC87 ligated with pJL702 at the SrfI site</td>
<td>This work</td>
</tr>
<tr>
<td>pCC21mp19</td>
<td>High</td>
<td>E. coli</td>
<td>pCC8mp19 derivative containing synA with changes of TTA (Leu) to CTA (Leu)</td>
<td>This work</td>
</tr>
<tr>
<td>pCC24</td>
<td>High</td>
<td>E. coli</td>
<td>pCC85 derivative containing T. maritima synA ORF from pCC21mp19</td>
<td>This work</td>
</tr>
<tr>
<td>pCC26</td>
<td>Low</td>
<td>E. coli and Streptomyces</td>
<td>pCC86 derivative containing T. maritima synA ORF from pCC21mp19</td>
<td>This work</td>
</tr>
<tr>
<td>pCC27</td>
<td>Low</td>
<td>E. coli and Streptomyces</td>
<td>pCC113 derivative containing T. maritima synA ORF from pCC21mp19</td>
<td>This work</td>
</tr>
<tr>
<td>pCC28</td>
<td>High</td>
<td>E. coli and Streptomyces</td>
<td>pCC24 ligated with pJL702 at SrfI site</td>
<td>This work</td>
</tr>
<tr>
<td>pCC76</td>
<td>High</td>
<td>E. coli</td>
<td>pCC19 derivative containing entire xbl promoter and gene from S. coelicolor</td>
<td>This work</td>
</tr>
<tr>
<td>pCC85</td>
<td>High</td>
<td>E. coli</td>
<td>pUC19 derivative containing S. coelicolor xblB promoter/signal sequence</td>
<td>This work</td>
</tr>
<tr>
<td>pCC87</td>
<td>Low</td>
<td>E. coli and Streptomyces</td>
<td>pCC85 derivative containing T. maritima synA ORF from pCC16</td>
<td>This work</td>
</tr>
<tr>
<td>pCC88</td>
<td>Low</td>
<td>E. coli and Streptomyces</td>
<td>pNE4 derivative containing S. coelicolor synB promoter/signal sequence from pCC76</td>
<td>This work</td>
</tr>
<tr>
<td>pCC113</td>
<td>Low</td>
<td>E. coli and Streptomyces</td>
<td>pX4 derivative containing deleted S. coelicolor xblB promoter/signal sequence from pCC76</td>
<td>This work</td>
</tr>
<tr>
<td>pX4</td>
<td>Low</td>
<td>E. coli and Streptomyces</td>
<td>Vector containing promoterless yIE</td>
<td>Ingram, 1989</td>
</tr>
<tr>
<td>pJL702</td>
<td>Streptomyces</td>
<td></td>
<td>Vector containing tra and mel markers</td>
<td>Hopwood, 1985</td>
</tr>
<tr>
<td>pUC19</td>
<td>High</td>
<td>E. coli</td>
<td>Vector containing ampicillin resistance gene and lacZ</td>
<td>Sambrook, 1989</td>
</tr>
<tr>
<td>M13mp19</td>
<td>High</td>
<td>E. coli</td>
<td>Bacteriophage M13 viral DNA derivative containing lacZ marker</td>
<td>Sambrook, 1989</td>
</tr>
</tbody>
</table>

RESULTS

Cloning the xylanase B gene from S. coelicolor. A chromosomal library of S. coelicolor DNA was constructed in E. coli JM83 by using pUC19 and screened for clones expressing xylanase activity on Luria-Bertani agar plates containing 4-O-methyl-D-glucurono-D-xylan-Remazol Brilliant Blue R. Xylanase activity was assayed by visual inspection of plates after incubation at 37°C for 16 h. Positive clones, detected by the presence of clear zones around colonies suggesting xylan hydrolysis, were recovered at a frequency of approximately 1 in 5,000 colonies, pCC76, shown in Fig. 1, contained a 3.3-kb DNA fragment with an ORF that was 99% identical to the xylanase B gene of S. lividans. We therefore designate this gene from S. coelicolor xblB. Also contained on this fragment was an ORF...
identical to an acetylxylan esterase, *axeA*, from *S. lividans* (32), an extended region upstream of *xlnB*, and an ORF that is identical to the RNase P gene from *S. lividans* (32).

Detection of *xlnB* promoter activity by using a transcriptional fusion to the *xylE* reporter gene. Using the information previously reported for the *xlnB* gene of *S. lividans* (26, 33), PCR was used to amplify a DNA fragment containing the sequences from −375 to +126 with respect to the putative translation start site (first bold ATG) shown in Fig. 2. This fragment was cloned upstream of a promoterless copy of a catechol dioxygenase (*xylE*) reporter gene (19), and this fusion, contained on plasmid pCC88, was used to assay *xlnB* expression. *S. lividans* cells containing pCC88 were grown in medium containing either oatspelt xylan or birchwood xylan. Expression from the *xlnB* promoter was induced by both oatspelt and birchwood xylans, but oatspelt xylan was the more efficient of the two (Fig. 5). Oatspelt xylan was used as the inducer in all subsequent experiments to examine *xlnB* promoter expression.

Transcription from the *xlnB* promoter is strongly repressed by glucose and cellobiose. To examine the regulation of *xlnB* expression, cells containing the *xlnB-xylE* fusion were grown on a variety of carbon sources (Table 2). While expression of the *xlnB-xylE* fusion was most strongly induced by xylan, expression was also detected during growth on arabinose to a level of almost 50% of that on xylan. While some simple sugars such as mannitol, galactose, and xylose partially repressed induction by xylan, the most dramatic repression was with either glucose or cellobiose in the growth medium. These data extend the analysis of Bertrand et al. (2), who showed that xylanase activity...
was detected in cells grown on xylan but not xylan plus glucose, and indicate that regulation of xylanase expression by simple sugars is at the level of transcription initiation.

Identification of an apparent transcription start site for the \textit{xlnB} promoter by primer extension analysis. \textit{S. lividans} cells containing the \textit{xlnB} gene on plasmid pCC88 were grown in minimal liquid medium (17) containing 0.5\% oatspelt xylan with or without glucose, and RNA was isolated as previously described (17). An oligonucleotide complementary to the beginning of the fragment containing the \textit{xylE} gene (24) was used to initiate reverse transcription of \textit{xlnB}-generated RNA. As shown in Fig. 6, a primer extension product was generated in reaction mixtures containing RNA from both xylan and xylan plus glucose but the amount of product generated from xylan-glucose-grown cells was much smaller than that detected from xylan-grown cells. These results support the observations from analysis of the \textit{xlnB-xylE} fusion, indicating that expression of \textit{xlnB} is repressed by glucose and that regulation is at the level of transcription initiation.

Surprisingly, the transcription start site we observed, a G residue indicated (bold) in Fig. 2, was located within the putative leader peptide predicted from sequence analysis in previous work (32, 37). We expected to identify a transcription start site upstream of this ATG codon; however, no extension products larger than the one shown in Fig. 6 were observed.

Identification of an apparent transcription start site in \textit{in vitro} transcription assays. Given the results obtained by primer extensions analysis, a second method was used to determine the transcription start site. Two DNA fragments were prepared by PCR amplification for use as templates in \textit{in vitro} transcription assays. One contained the apparent transcription start site identified by primer extension and would generate a runoff transcript of 79 bases from this start site. The other had this site deleted and would not generate a run off transcript from the putative promoter identified by primer extension analysis. The deleted fragment would, however, probably contain a promoter upstream of the putative translation start site predicted from sequence analysis (32, 37), if one existed. RNA polymerase was prepared from \textit{S. lividans}, and \textit{in vitro} transcription assays were performed as previously described (43). As shown in Fig. 7, a run off transcript of the size predicted from the primer extension analysis was readily detected in reaction mixtures containing the DNA fragment with the intact promoter region. No transcript of the size predicted from signal sequence analysis was detected in reaction mixtures containing the DNA fragment with the apparent start site deleted, and there was no evidence of a promoter upstream of this site within the fragment. These data, taken together with the results obtained by primer extension analysis, indicate that the transcription start site of the \textit{xlnB} promoter is, in fact, downstream of the translation start site previously predicted.

Deletion analysis of \textit{xlnB} promoter identifies sequences important for regulation and expression. To identify sequences important for the regulation of \textit{xlnB}, a series of 5‘ promoter deletions was constructed and analyzed by using transcriptional fusions to the \textit{xylE} reporter gene. As shown in Fig. 8, plasmid pCC88 contains the intact \textit{xlnB} promoter region with sequences from −430 to +79 relative to the transcription start site identified in our analysis; pCC114 contains the region

\begin{table}
\centering
\begin{tabular}{lll}
\hline
Carbon source & Catechol dioxygenase activity (mU/mg) & Relative activity (\% activity oatspelt xylan) \\
\hline
Oatspelt xylan & 27.0 & 100 \\
Arabinose & 13.1 & 48.5 \\
Cellulose & 1.6 & 5.9 \\
Galactose & 4.1 & 15.2 \\
Glucose & 2.0 & 7.4 \\
Glycerol & 2.0 & 7.4 \\
Mannitol & 3.0 & 11.1 \\
Xylose & 3.0 & 11.1 \\
Oatspelt + arabinose & 43.1 & 159.6 \\
Oatspelt + cellulose & 8.5 & 31.5 \\
Oatspelt + galactose & 17.0 & 63.0 \\
Oatspelt + glucose & 9.9 & 36.7 \\
Oatspelt + glycerol & 21.7 & 80.4 \\
Oatspelt + mannitol & 12.9 & 47.8 \\
Oatspelt + xylose & 12.2 & 45.2 \\
\hline
\end{tabular}
\caption{Expression of the \textit{xlnB-xylE} fusion in \textit{S. lividans}}
\end{table}
from −318 to +79; pCC113 contains the region from −268 to +79; and pCC112 contains the region from −216 to +79. Deletion of sequences to −318 reduced the level of expression on xylan slightly but had no effect of regulation. A deletion to −268, however, resulted in glucose-resistant expression from xlnB, suggesting that sequences between −318 and −268 relative to this apparent transcription start site contain a site required for glucose repression. A deletion to −216 severely reduced xylE expression driven by the xlnB promoter, suggesting that sequences between −268 and −216 are required for promoter activity.

The xlnB-xynA fusion protein is expressed in S. lividans and localized extracellularly. To test expression of the xlnB-xynA fusion protein, wild-type S. lividans and cells containing various xlnB-xynA fusion plasmids were grown in NMMP minimal medium containing 0.5% oatspelt xylan and the xylanase activity in crude cell extracts and culture supernatants was tested. As shown in Table 3, by using xylanase assays performed at 50°C, activity (49.61 U/mg) was detected from wild-type S. lividans cells. This strain is known to contain several xylanases that are active at this temperature. When the assays were performed at 90°C, however, no activity was detected from wild-type S. lividans cells, suggesting that the activity detected in these cells at 50°C was not stable at 90°C. Thermostable xylanase activity was detected in cells containing each of the xlnB-xynA fusion plasmids. Plasmid pCC20 contains the intact xlnB promoter (−430 to +79) driving the expression of the fusion protein on a high-copy-number plasmid, and thermostable activity was clearly detected, although at a low level (0.44 U/mg).

The T. maritima xynA gene contains two TTA codons. This codon, which specifies leucine, is rarely used in Streptomyces and is recognized by a single tRNA encoded by the bldA gene (23). By using site-directed mutagenesis, the two TTA codons, Leu21 and Leu140, were changed to CTA, which also specifies

**TABLE 3. Thermophilic xylanase activity from S. lividans**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Total protein (mg)</th>
<th>Total activity a</th>
<th>Sp act a</th>
<th>Assay temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.31</td>
<td>15.38</td>
<td>49.61</td>
<td>50°C</td>
</tr>
<tr>
<td>None</td>
<td>0.31</td>
<td>0</td>
<td>0</td>
<td>90°C</td>
</tr>
<tr>
<td>pCC20</td>
<td>0.43</td>
<td>0.19</td>
<td>0.44</td>
<td>90°C</td>
</tr>
<tr>
<td>pCC28</td>
<td>0.52</td>
<td>0.21</td>
<td>0.40</td>
<td>90°C</td>
</tr>
<tr>
<td>pCC26</td>
<td>0.46</td>
<td>0.16</td>
<td>0.35</td>
<td>90°C</td>
</tr>
<tr>
<td>pCC27</td>
<td>0.38</td>
<td>0.48</td>
<td>1.26</td>
<td>90°C</td>
</tr>
</tbody>
</table>

a One unit of xylanase activity is the amount of enzyme required to liberate 1 μmol of reducing equivalent (as xylose) per min.

b Units per milligram of protein. Results are the average of at least duplicate experiments.
leucine. A fusion protein containing the modified xynA gene was cloned downstream of the intact xlnB promoter in either a high-copy-number (pCC28) or low-copy-number (pCC26) plasmid. As shown in Table 3, there is no significant difference in the level of activity detected between fusion proteins containing the modified xynA gene and the wild-type gene. This suggests that either the presence of TTA codons did not limit translation or the change to CTA, which still contains adenine in the third position, was not a change that improved translation efficiency.

Somewhat surprisingly, the copy number of the gene had little effect on detectable enzyme activity. Plasmid pCC28 contains the same xlnB-xynA fusion in high copy that pCC26 contains in low copy, and there is no apparent difference in expression of xylanase activity. We emphasize that we have no evidence that the amount of xlnB-xynA RNA is increased in cells containing pCC28.

Analysis of the regulation of the xlnB promoter region identified a promoter deletion that resulted in increased activity of the xylE reporter gene and therefore increased transcription from xlnB. This deletion contained sequences from −268 to +79 relative to the transcription start site, and the analysis of this deletion is shown in Fig. 8. The highest thermostable activity (1.26 U/mg) was detected from a construction that contained this deletion driving the expression of the xlnB-xynA fusion (Table 3).

**DISCUSSION**

We report the cloning of a xylanase gene from *S. coelicolor* that is 99% identical to the xlnB gene from *S. lividans* and partial characterization of its regulation. To identify the promoter of xlnB, we constructed a transcriptional fusion between the 5′ region of the xlnB gene and the xylE reporter gene (19) and defined a region of the gene that mediates transcription initiation and regulation. Analysis of this fusion suggests that expression of xlnB is efficiently induced by either oatspelt or birchwood xylan, although oatspelt xylan is apparently better, and that this induction is repressed by glucose or cellobiose. Furthermore, this analysis with a transcriptional fusion suggests that regulation by carbon source availability occurs, at least in part, at the level of transcription initiation. Interestingly, arabinose, which makes up 10% of the xylan polymer by weight, also induces expression of xlnB, although to a lesser extent. Since the effects of arabinose and xylan on induction seem to be additive, it is possible that they cause induction by different mechanisms.

A translation start site and putative signal sequence for the XlnB protein had been suggested based on comparisons of the predicted protein sequence of xlnB with other proteins (26, 33). These predictions, although based entirely on sequence analysis, were completely reasonable. In our analysis of the transcription start site of xlnB, however, we identified an apparent transcription start site within the predicted protein signal sequence. The ability to predict the transcription start site from primer extension analysis relies on the isolation of intact RNA. Primer extension products are used to measure the distance from the point of primer annealing to the 5′ end of the RNA molecule isolated, and if the mRNA is partially degraded or processed, the results would be misleading and would not identify the 5′ end of the mRNA as it is transcribed. In an attempt to eliminate artifacts that might result from mRNA processing, we used an independent method to determine the transcription start site. In vitro runoff assays rely on the use of partially purified RNA polymerase and a DNA template in an in vitro reaction. RNA polymerization is initiated within a fragment that contains the putative promoter and continues to the end of the fragment. These conditions would presumably eliminate RNA processing events, or, if processing occurred, the products would be detected in the reaction. Since in vitro transcription assays and primer extension analysis of in vivo RNA identified the same transcription start site and since there is no evidence of RNA processing, we conclude that, within the limits of the experimental techniques used, the start site is a G residue well downstream of the previously predicted translation start site. In support of this conclusion is the fact that no runoff transcript was detected when a fragment with this transcription start site deleted was used as the template. If the actual transcription start site were upstream of the translation start site predicted from sequence analysis, we should have detected it in our assays. Although the long protein signal sequence predicted from the DNA sequence appears extremely similar to known signal sequences, if our transcriptional analysis is correct, the actual signal sequence may be much shorter and the translation start site may be an ATG downstream. If this is true, xylanase B may have an 8-amino-acid leader peptide, MLPTGTAQA, followed by a signal sequence cleavage site.

While our data for the transcription start site of xlnB are not consistent with the predicted signal sequence proposed previously (26, 33), the results of our experiments are unambiguous and internally consistent. In spite of this, we report them with some caution. The homology between the putative signal sequence of xylanase B and known signal sequences is compelling. We point out that there are no sequences at positions −10 or −35 upstream of the start site we identified that resemble known RNA polymerase recognition sequences, but, given the large number of promoter classes already identified in *Streptomyces*, this may simply be a new one. Attempts to determine the N-terminal amino acid sequence of XlnB have so far been unsuccessful. Further analysis of the protein sequence of XlnB and identification of cis-acting sequences involved in RNA polymerase recognition and regulation should resolve the questions raised by our analysis.

To define the regulatory region of the promoter of xlnB, deletions of the promoter region were constructed and examined by using transcriptional fusions to the xylE reporter gene. Sequences between −268 and −318 relative to the transcription start site were shown to be required for glucose repression of xylan induction. There are several sequence motifs within this region, including a palindromic sequence, CTTCGAAATTTCCGGG, and TTCCGCG. Giannotta et al. (14) identified a region of the xlnB promoter that formed complexes with proteins from crude cell extracts in electrophoretic mobility shift assays. This region includes the sequence GAAA-TTTC, which is similar to sequences within the region of the xlnB promoter required for glucose repression. We emphasize that we have no direct evidence that these sequences are important for regulation, and they are implicated only by inspection of the xlnB promoter sequence. Although experiments to elucidate the possible role of these sequences in xlnB regulation are currently in progress in our laboratory, we have not identified bases within the CTTCGAAATTTCCGAG sequence that affect expression or regulation. While this analysis is at an early stage, some base changes in the three direct repeat sequences resulted in decreased levels of xlnB expression. A thorough analysis of these motifs may identify sequences important for regulation.

The use of transcription and localization signals for heterologous gene expression in *Streptomyces* is also at an early stage of investigation compared with other systems. *Streptomyces* is a potentially useful host for heterologous expression, especially for the expression of enzymes such as xylanase. *Streptomyces* is...
a soil organism that produces and exports a large number of complex-carbohydrate-degrading enzymes, and the use of the expression and localization signals from these enzymes for the production of similar enzymes from other sources is potentially powerful. Xylanase A from _T. maritima_ is an example. The thermostable and pH-stable activity of this enzyme make it well suited for processes such as pulp bleaching, but production of the enzyme from its host, an obligate anaerobe with fastidious growth requirements, would be difficult at best. Our investigation of the expression of this xylanase in _Streptomyces_ clearly shows that the enzyme is expressed and localized and that the activity is thermostable. Our efforts to optimize the system for high-level production, however, are at an early stage. The fact that a promoter deletion that resulted in higher transcription also resulted in higher expression suggests that increased transcription will improve production. Translation of a protein that has a 46% G+C content in an organism that has an overall G+C content of 73% is also likely to present difficulties in translation efficiency due to differences in codon usage. While changing the two TTA codons to CTA had no apparent effect on translation efficiency, the presence of an adenine in the third position (which is 96% guanine or cytosine for most _Streptomyces_ coding regions) may not have been an optimal change. The _xynA_ gene contains several TTT codons, which appear infrequently in _Streptomyces_. It is clear that a thorough dissection of the rate-limiting steps in production of xylanase A in _Streptomyces_ is needed to address issues of high-level expression.

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