Cloning of a *Thermomonospora fusca* Xylanase Gene and Its Expression in *Escherichia coli* and *Streptomyces lividans*

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*Thermomonospora fusca* chromosomal DNA was partially digested with EcoRI to obtain 4- to 14-kilobase fragments, which were used to construct a library of recombinant phage by ligation with EcoRI arms of AgtWES.AB. A recombinant phage coding for xylanase activity which contained a 14-kilobase insert was identified. The xylanase gene was localized to a 2.1-kilobase *Sall* fragment of the EcoRI insert by subcloning onto pBR322 and derivatives of pBR322 that can also replicate in *Streptomyces lividans*. The xylanase activity produced by *S. lividans* transformants was 10- to 20-fold higher than that produced by *Escherichia coli* transformants but only one-fourth the level produced by induced *T. fusca*. A 30-kilodalton peptide with activity against both Remazol brilliant blue xylan and xylan was produced in *S. lividans* transformants that carried the 2.1-kilobase *Sall* fragment of *T. fusca* DNA and was not produced by control transformants. *T. fusca* cultures were found to contain a xylanase of a similar size that was induced by growth on xylan or Solka Floc. Antiserum directed against supernatant proteins isolated from a Solka Floc-grown *T. fusca* culture inhibited the xylanase activity of *S. lividans* transformants. The cloned *T. fusca* xylanase gene was expressed at about the same level in *S. lividans* grown in minimal medium containing either glucose, cellubiose, or xylan. The xylanase bound to and hydrolyzed insoluble xylan. The cloned xylanase appeared to be the same as the major protein in xylan-induced *T. fusca* culture supernatants, which also contained at least three additional minor proteins with xylanase activity and having apparent molecular masses of 43, 23, and 20 kilodaltons.

Next to cellulose, hemicelluloses are the most abundant naturally occurring polymers, and they are present in association with lignin and cellulose in plant cell walls. A major component of hemicelluloses from monocots is xylan, a polymer which consists of a backbone of β-(1,4)-D-xylopyranoside residues. Xylans comprise only a minor fraction of dicot-hemicellulose. Xylans of different origins vary with respect to molecular weight, composition, number of side chains, and level of acetylation.

Many bacteria and fungi can utilize xylans as carbon sources; they do so by producing xylanolytic enzymes, which are either excreted into the medium or remain associated with the cell envelope. A number of activities such as endoxylanases, exoxylanases, β-xylanosidases, α-glucuronidases, α-arabinofuranosidases, and esterases have been identified in xylanolytic microorganisms (6, 10, 36). Out of all the xylanolytic proteins the endoxylanases and β-xylanosidases have been most widely studied, and xylanase genes from a wide variety of mesophilic bacteria and fungi have been cloned (4, 5, 15-17, 20, 27, 28, 31, 32, 37, 38). Studies of a xylanase gene from an extreme thermophile were presented in a preliminary report (P. A. Caughey, D. R. Love, and P. L. Bergquist, FEMS Symp. 1987, abstr. no. P4-07, p. 90), and recently a report on the cloning of a thermophilic xylanase appeared (13).

*Thermomonospora fusca* is a filamentous soil thermophile that produces cellulolytic, xylanolytic, and pectinolytic enzymes (1, 14, 33, 35; D. L. Ristroph, Ph.D. dissertation, University of Pennsylvania, 1981). The xylanases of *T. fusca* have been studied by several workers (1, 26, 29; Ristroph, Ph.D. dissertation).

In this report we describe the isolation by cloning into bacteriophage λ of a xylanase gene from *T. fusca* YX. The xylanase gene was expressed in both *Escherichia coli* and *Streptomyces lividans*. The *S. lividans* transformants excreted into the medium a 30-kilodalton (kDa) xylanase that bound to xylan and appeared to be identical to the major xylanase produced by xylan-induced cultures of *T. fusca*. In addition, we present initial studies on the regulation of xylanase production in both *T. fusca* and *S. lividans*.

**MATERIALS AND METHODS**

**Bacterial strains and vectors.** The bacterial strains and vectors used in this study are listed in Table 1. *E. coli* LE392 was obtained from the *E. coli* Genetic Stock Center, Department of Biology, Yale University, and was used as a host for λgtWES.AB and its derivatives (21). λgtWES.AB DNA and λgtWES.AB EcoRI arms were purchased from either Bethesda Research Laboratories, Inc., or Amersham Corp. λgtWES.AB SaI1 arms were prepared as described below. *S. lividans* TK24 and plasmid pIJ702 were kindly provided by D. A. Hopwood, John Innes Institute, Norwich, England.

**Growth of organisms.** Unless indicated otherwise, *T. fusca* YX was grown as described previously (12). *E. coli* strains were grown in Luria Bertani (LB) medium (24). When used as a host for a phage growth, LE392 was grown in 1% tryptone–2.5% sodium chloride–10 mM MgCl₂–0.2% maltose. *E. coli* strains carrying recombinant plasmids were grown in LB medium containing ampicillin (50 μg/ml).

The media for *S. lividans* protoplast preparation and regeneration were described previously (11, 12). Unless indicated otherwise, *S. lividans* cultures were grown on tryptone soya broth as previously described (12).

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gesting fractions (11).

The DNA was digested with restriction enzymes EcoRI, HindIII (Amersham), and XhoI, and subsequently separated on a 0.7% agarose gel and stained with ethidium bromide. The DNA was visualized by ultraviolette light, and the fragments were excised and separated on a preparative gel. The DNA was purified by treatment with proteinase K and phenol-chloroform. The DNA was precipitated by the addition of ethanol and lyophilized. About 3 to 4 g of dried xylan was obtained.

Preparation of insoluble xylan. After the soluble xylan was removed as described above, the insoluble xylan was suspended in 200 ml of H2O, the pH was adjusted to 7.0 with 1 M acetic acid, and the preparation was filtered on Whatman no. 1 filter paper and extensively washed with water. The white paste was carefully removed and dried by lyophilization.

Screening of plaques for xylanase activity with Congo red. Plates to be screened for xylanase activity were overlaid with 7 ml of xylan-agarose (1% agarose, 0.05% soluble xylan, 10 mM Tris [pH 6.5]) and incubated at 35°C for 6 h. After incubation, the plates were stained with 0.1% Congo red for 15 min. The dye solution was removed, and the plates were destained with 1 M NaCl. A yellow ring resulting from xylan hydrolysis appeared around positive plaques (27, 34).

Screening of colonies with RBB-xylan. Remazol brilliant blue (RBB)-xylan was prepared by the procedure of Biely et
FIG. 2. Construction of shuttle plasmids pGG92 (A) and pGG93 (B) that are capable of replicating in *E. coli* and *S. lividans* and carry *T. fusca* DNA coding for xylanase. Symbols: ( ) pBR322 sequence, ( ) sequences originating from pIJ702, ( ) *Thermomonospora* sequences carrying the xylanase gene. Amp, Ampicillin resistance gene expressed in *E. coli*; Tsr, thiostrepton resistance gene expressed in *S. lividans*. Only the sites that were important for this study are shown. Additional sites are shown in Fig. 1 and reference 8.

Enzyme assays. Xylanase was assayed by *E. coli* by growing a 100-ml overnight culture and harvesting it by centrifugation (10 min at 10,000 × g); the pellet was suspended in 3 ml of 0.05 M potassium phosphate buffer (pH 6.5) (buffer I), and an extract was prepared in a French press at 10,000 lb/ in². The culture supernatant was concentrated to 3 ml with an Amicon UM10 membrane. Xylanase activity was determined by measuring the rate of release of reducing sugar from xylan. Cell extract or concentrated culture supernatant was mixed with buffer I to give a final volume of 0.3 ml, 0.10 ml of 1% soluble xylan solution made up in buffer I was added, and the mixture was incubated at 55°C for 30 min. After incubation, 0.75 ml of dinitrosalicylic acid reagent (35) was added, and the samples were incubated in a boiling water bath for 15 min. The samples were allowed to cool to room temperature, and then the *A*₅₀₀ was determined. One unit of xylanase is defined as the amount of enzyme that produces 1 μmol of reducing sugar per min in the above procedure.

TABLE 2. Expression of a *T. fusca* xylanase gene in *E. coli* and *S. lividans*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Xylanase (U/ml)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell extracts</td>
<td>Culture supernatant</td>
<td></td>
</tr>
<tr>
<td>HB101(pTX101)</td>
<td>0.52</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>HB101(pBR322)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>HB101(pGG92)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>HB101(pGG93)</td>
<td>0.22</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>TK24(pGG82)</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td></td>
</tr>
<tr>
<td>TK24(pGG92)</td>
<td>1.0 ± 0.5</td>
<td>7.0 ± 2.0</td>
<td></td>
</tr>
</tbody>
</table>

Each plate was overlaid with 5 ml of 1% agarose containing 10 mM Tris (pH 6.5) and 0.07% RBB xylan and incubated at 55°C for over 4 h. If necessary, the plates were destained with a solution of 50 mM sodium acetate (pH 5.4) in 95% ethanol (1:2, vol/vol); positive plaques were surrounded by a clear ring.

al. (7). Each plate was overlaid with 5 ml of 1% agarose containing 10 mM Tris (pH 6.5) and 0.07% RBB xylan and incubated at 55°C for over 4 h. If necessary, the plates were destained with a solution of 50 mM sodium acetate (pH 5.4) in 95% ethanol (1:2, vol/vol); positive plaques were surrounded by a clear ring.
T. fusca and S. lividans cultures were assayed for xylanase activity after removing the mycelia either by centrifugation or by filtration through glass wool. Carboxymethyl cellulase activity was determined by replacing xylan with carboxymethylcellulose in the above assay (35).

**RESULTS**

**Xylanase gene cloning.** The transformation efficiency of the recombinant λgt phage T. fusca genome bank was 8 × 10^6 PFU/µg of DNA. Of 3,500 plaques screened for xylanase activity by both the Congo red and the RBB-xylan procedures, 2 showed xylanase activity. Based on restriction maps, the DNA of both phage contained the same ~14-kb EcoRI insert. This recombinant phage was designated λxyl-1 (Fig. 1).

**Subcloning of the xylanase gene.** Since there were no EcoRI sites in the insert DNA, λxyl-1 DNA was subcloned by using λgtWES.AB SalI arms as described in Materials and Methods. About 1,500 plaques were screened by the Congo red procedure, and 11% were xylanase positive. Seven positive plaques were purified, and their xylanase activities and restriction maps were determined. One phage had an insert DNA containing 3.4- and 2.1-kb SalI arms, whereas all of the other phage contained both of these fragments along with at least one additional fragment. The xylanase-positive phage with the smallest insert was called λxyl-2, and its restriction map is shown in Fig. 1.

λxyl-2 DNA was digested with SalI, and the 3.4- and 2.1-kb fragments were separated by gel electrophoresis and subcloned into pBR322. Transformants were screened for
xylanase activity by the Congo red procedure. All of the colonies from the 3.4-kb fragment ligation were negative, whereas 35% of the transformants from the 2.1-kb ligation were positive. Restriction maps of several transformants showed that they all contained only the 2.1-kb insert. This plasmid was designated pTX101, and its restriction map is also shown in Fig. 1.

Construction of E. coli-S. lividans shuttle plasmids containing the xylanase gene. The multicopy plasmid pIJ702 (18) commonly used for cloning in S. lividans contains five SalI restriction sites and therefore is not convenient for cloning SalI fragments. Instead, a derivative of pIJ702, pGG82, was constructed and used for expressing the T. fusca xylanase gene in S. lividans. In plasmid pGG82 the smaller BglII-SphI fragment of pIJ702 is replaced by a BamHI-SphI fragment of E. coli plasmid DNA. The structure of pGG82 appears in Fig. 2. The EcoRI-SphI fragment of pGG82 is from pBR322, and the BamHI-BglII fragment is derived from pUR222 (30). Plasmid pGG82, unlike plasmid pIJ702, no longer produces melanin (Mel− phenotype) and contains single EcoRI and HindIII sites. The BglII site of pIJ702 is inactivated in pGG82, due to its fusion to the BamHI site of pUR222.

Further restriction analysis of pTX101 showed that it had no sites for SstI and BglII, one site each for EcoRI, SphI, HindIII, and BamHI, and three sites for SacAI. The construction of shuttle plasmid pGG92 from pTX101 and pGG82 is depicted in Fig. 2A. The three E. coli HB101 transformants tested had the correct restriction pattern. This plasmid is stable in E. coli HB101. The construction of shuttle plasmid pGG93 from pGG82 and pTX101 is shown in Fig. 2B. The 12.8-kb plasmid DNA was isolated from six E. coli transformants, and all six had the orientation shown in Fig. 2.

Expression of shuttle plasmids in E. coli. Unlike plasmid pTX101, which had strong xylanase activity in E. coli HB101 (Table 2), shuttle plasmid pGG92 failed to confer a xylanase-positive phenotype on HB101 (Fig. 3). The tetracycline resistance promoter is deleted during the construction of pGG92 while leaving the T. fusca DNA sequence intact, and when this promoter was present as in pGG93, xylanase activity was present in the HB101 transformants.

Transformation of S. lividans and expression of the T. fusca xylanase gene. Plasmid DNA was isolated from E. coli transformed with pGG92 and pGG93 and was used to transform S. lividans protoplasts. pGG92 consistently gave more transformants than pGG93 when equal amounts of DNA were used. Furthermore, all of the pGG92 transformants were xylanase-positive, whereas only a few of the pGG93 transformants were xylanase positive, possibly because most pGG93 transformants were unstable due to recombination between the duplicated regions of pBR322 present in pGG93. The xylanase activities of transformed and control strains are given in Table 2.

When supernatant proteins from S. lividans transformants were separated on an SDS gel and stained for xylanase activity, a 30-kDa protein was the major active band. This band was not present in control cultures (Fig. 4).

The xylanase in S. lividans transformants is similar to an induced xylanase in T. fusca. Table 3 shows the specific induction of extracellular xylanase activity in T. fusca cultures grown for 2 days on xylan. Xylan-grown cultures had low levels of cellulase and high levels of xylanase activity, whereas cellulose (Solka floe)-grown cultures had high levels of both activities. The level of xylanase was higher than the level of cellulase in glucose-grown cultures. The proteins present in the supernatants of cultures grown on the different carbon sources were separated by gel electrophoresis (Fig. 5). The major protein present in the supernatant of xylan-grown cells migrated in the same position as the cloned xylanase (Fig. 4). Four bands of xylanase activity were present in stained gels (43, 30, 23, and 20 kDa) run on the T. fusca sample. The larger two (43 and 30 kDa) are probably distinct gene products, whereas the smaller forms may be derived by proteolysis of the larger enzymes, especially as the lower-molecular weight bands are similar in size to the proteolysis products present in S. lividans transformants.

Xylanase activity of S. lividans transformants was inhibited by an antiserum prepared against proteins isolated

Table 4. Xylanase production in S. lividans gene in glucose and xylan.

<table>
<thead>
<tr>
<th>Plasmid contained</th>
<th>Carbon source</th>
<th>Xylanase in culture filtrate (U/ml) on day:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>pGG82</td>
<td>Glucose</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Xylan</td>
<td>0.0</td>
</tr>
<tr>
<td>pGG92</td>
<td>Glucose</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Xylan</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Transformed colonies were grown in tryptone soya broth containing thiostrepton. Two-day cultures (mycelial volume, about 10%) were centrifuged and taken up in modified Hagerdal medium (22) lacking a carbon source. Then 1 ml of this culture was transferred to 50 ml of the indicated medium containing 10 μg of thiostrepton per ml and 0.25 g (0.5%) of either glucose or xylan. The cultures were shaken at 30°C, and at the indicated times samples were removed, centrifuged, and assayed for xylanase activity.
FIG. 6. Comparison of cloned xylanase and T. fusca proteins by activity staining (A) and Western blotting (B). Supernatants from T. fusca and S. lividans transformants were diluted with loading dye and left in a boiling water bath for 5 min. The boiled samples were subjected to electrophoresis on an SDS-12.5% polyacrylamide gel to separate the peptides. After SDS was removed as described in the text, the gel was incubated with xylan agar replica for 30 min in a 55°C oven. The replica was stained with Congo red. Proteins in the acrylamide gel were electroblotted onto nitrocellulose (0.45-μm pore size) and probed with antiserum directed against Solka Floc-grown culture supernatant proteins. Lanes: 1. T. fusca culture grown in glucose; 2. T. fusca culture grown in xylan; 3. T. fusca culture grown in Solka floc; 4. S. lividans(pGG82) transformant culture; 5. S. lividans(pGG92) transformant culture. Std, protein molecular mass standards of 43, 25.7, 18, and 14 kDa.

from the culture supernatant of cellulose-grown T. fusca. The activity was not inhibited by control serum or serum prepared against purified cellulases from T. fusca (data not shown). Immunoblotting of S. lividans transformants revealed a 30-kDa protein; control cultures lacked this band (Fig. 6).

Regulation of xylanase production of S. lividans transformants and its binding to xylan. S. lividans transformants containing the control plasmid (pGG82) did not produce host xylanase in medium lacking xylan (Table 4), whereas the plasmid-encoded T. fusca xylanase was produced in minimal medium containing glucose, celllobiose, or xylan and in tryptone soya broth. The activity of pGG92 cultures grown on xylan was about two- to threefold the activity of control cultures and contained both induced host activity and T. fusca xylanase.

The lack of xylanase activity at early time points in the supernatants of cultures grown on xylan could indicate that the activity binds to undegraded insoluble xylan. In fact, xylanase activity quickly disappeared from the solution after the addition of insoluble xylan, and the bound enzyme released soluble reducing sugars at 55°C in 50 mM KP (pH 6.5) (Fig. 7).

DISCUSSION

The cloned T. fusca xylanase gene was expressed at a low level in E. coli and at a higher level in S. lividans, which also efficiently excretes the activity into the culture medium. The results of expression in S. lividans indicate that the xylanase gene uses its own promoter. However, efficient expression in E. coli seems to require additional promoter activity that may be provided by the adjoining T. fusca or λ DNA on the λxyl-I and λxyl-2 phages or the tet promoter in pTX101 or pGG93. The xylanase produced by the T. fusca gene present in plasmid pGG92 clearly differed from S. lividans xylanase activity in its regulation, electrophoretic mobility, and reaction with T. fusca antiserum.

S. lividans xylanase activity is induced by xylan; an S. lividans xylanase gene cloned into S. lividans on plasmid pIJ1702 was reported to be inducible (27). However, the T. fusca xylanase gene described here was not induced by xylan in S. lividans, although it was induced in T. fusca.

A number of xylanase genes from different organisms have been expressed in E. coli; however, this report appears to be the first example of heterologous xylanase expression in S. lividans. These studies demonstrate the usefulness of the S. lividans system for expressing T. fusca genes (11, 12).

Streptomyces transformants that carry the T. fusca xylanase gene produce a 30-kDa protein that displays xylanase activity and appears in all respects to be the same as the major protein in xylan-induced T. fusca supernatants. The xylanase activity of Streptomyces transforms can be specifically and quantitatively inhibited by an antiserum raised against induced T. fusca extracellular protein. The availability of large amounts of xylanase protein in S. lividans culture supernatants free from other T. fusca proteins and the xylan-xylanase binding demonstrated in this
this study should facilitate purification and structure-function studies of this protein.

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LITERATURE CITED


