Identification of Two Distinct *Bacillus circulans* Xylanases by Molecular Cloning of the Genes and Expression in *Escherichia coli*†

ROBERT C. A. YANG,* C. ROGER MACKENZIE, DORIS BILOUS, AND SARAN A. NARANG

Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6

Received 7 September 1988/Accepted 29 November 1988

Two genes coding for xylanase synthesis in *Bacillus circulans* were cloned and expressed in *Escherichia coli*. After digestion of genomic DNA from *Bacillus circulans* with *EcoRI* and *PstI*, the fragments were ligated into the corresponding sites of pUC19 and transformed into *Escherichia coli*. Restriction enzyme mapping of the two inserts permitted determination of distinctly different nucleotide sequences. Cross-hybridization assays confirmed the absence of sequence homology between the two genes. In vitro transcription-translation assays indicated that the cloned genes encoded for proteins with molecular weights of 22,000 and 59,000. The gene products displayed different substrate specificities. The 22,000-dalton enzyme readily hydrolyzed aspen, larchwood, and oat spelt xylans, whereas the second was unable to extensively depolymerize oat spelt xylan and resulted in very limited reducing sugar release from any of the xylan substrates tested. Both of the xylanases had isoelectric points of approximately 9.0.

Hemicellulose constitutes 30 to 40% of the carbohydrate found in plant cell walls. Xylan is a major component of hemicelluloses, and there is considerable interest in its degradation because of possible applications in ruminal digestion, waste treatment, fuel and chemical production, and paper manufacture (1, 7, 12, 14, 16). The structure of xylan is quite complex and varies according to its origin. It is a branched β-1,4-linked α-xylan polymer with substituents that include acetyl, arabinosyl, and uronyl groups (1).

As a result of its structural complexity, complete xylan breakdown requires the action of several distinct enzyme activities (1, 9). However, endoxylanase, as the depolymerizing activity, is a key enzyme. Most xylanolytic organisms also produce cellulases, and this is undesirable in some applications (7, 20). Cloning and expression of endoxylanase in noncellulolytic organisms provides a solution to this problem.

Several *Bacillus* spp. produce xylanases, and the genes coding for these enzymes have been characterized in some instances (4, 5, 15, 22). *Bacillus circulans* has been reported to produce at least two xylanases, but the xylan-degrading system of this organism has not been studied at the gene level (3). We have initiated genetic and biochemical studies on this organism to determine the suitability of xylanolytic *B. circulans* genes for particular applications. Here we report on the cloning and expression of two *Bacillus circulans* xylanase genes in *Escherichia coli*.

**MATERIALS AND METHODS**

**Bacteria, plasmids, and growth conditions.** *B. circulans* (NRCC 9024; USDA 729) was chosen as the donor strain, and *Escherichia coli* HB101 was chosen as the recipient strain. 2YT broth, containing 10 g of tryptone (Difco Laboratories, Detroit, Mich.), 10 g of yeast extract (Difco), and 5 g of NaCl was used for growth of both organisms. *B. circulans* was grown at 30°C with shaking, and *E. coli* was grown at 37°C with shaking. Xylanase-producing clones were detected on 2YT agar plates containing 2% (wt/vol) Noble agar (Difco) and 0.15% Remazol brilliant blue-xylan (Sigma Chemical Co., St. Louis, Mo.). Filter-sterilized ampicillin was added to *E. coli* transformant cultures at 50 μg/ml. Plasmids pBPX277 (22) and pRH200 (15) carry *Bacillus polymyxa* and *Bacillus subtilis* xylanase genes, respectively. *E. coli* harboring pRH200 was kindly provided by M. Paice, Pulp and Paper Research Institute of Canada, Pointe Claire, Quebec.

**DNA manipulations.** Bacterial genomic DNA was prepared as described previously (22). Plasmid DNA was isolated by the alkaline-sodium dodecyl sulfate method of Birnboim and Doly (2), followed by CsCl-ethidium bromide ultracentrifugation. *B. circulans* and pUC19 DNA were digested separately and to completion with *EcoRI*. After phenol treatment, ethanol precipitation, and resuspension, 500 ng of unfractonated bacterial DNA fragments and 50 ng of plasmid were mixed and ligated with T4 DNA ligase overnight at 12°C. This ligated mixture was used to transform competent *E. coli* cells. The *PstI* library was constructed in a similar manner. Subcloning, physical mapping, and filter hybridization were performed as described by Maniatis et al. (11).

**In vitro transcription-translation.** The protein products of the cloned *B. circulans* inserts were identified by using the in vitro transcription-translation system of Zubay (23) (Amerham Corp., Arlington Heights, Ill.).

**Xylanase assays.** *E. coli* transformant cultures were harvested by centrifugation. Periplasmic and intracellular fractions were obtained as described previously (22). Xylanase assays were carried out as described by MacKenzie et al. (8). Oat spelt and larchwood xylan were obtained from Sigma, and aspen xylan was prepared by the method of Jones et al. (6). One unit was defined as the amount of enzyme releasing 1 μmol of reducing sugar equivalent per min. Xylanase preparations were subjected to analytical isoelectric focusing, and the location of xylanase bands in these gels was determined by Congo red staining as described previously (10). The products of xylanase action were analyzed by high-performance liquid-chromatography, using an HPX-42A column (Bio-Rad Laboratories, Rich-

---

† Corresponding author.

† National Research Council of Canada paper no. 29887.
mmond, Calif.) maintained at 85°C. Samples were eluted with water at a flow rate of 0.4 ml/min.

RESULTS

Physical mapping of the cloned xylanase genes. Of 1,224 transformants derived from the EcoRI library, 2 were found to produce clear halos on Remazol brilliant blue-xylan plates. The chimeric plasmids containing the xylanase genes were named pBCX549-R1 and pBCX549-R3. Of 2,120 transformants derived from the PstI library, 1 was halo positive; the corresponding plasmid was designated pBCX549-P5. The DNA inserts of the plasmids were subjected to restriction enzyme mapping, and detailed physical maps were constructed (Fig. 1 and 2). The pBCX549-R1 and pBCX549-R3 inserts were identical except for opposite insert polarity and the fact that the pBCX549-R1 insert (4.0 kilobases [kb]) was 0.3 kb smaller than that of pBCX549-R3. The chimeric plasmid pBCX549-P5 harbored a 7.2-kb PstI insert which was obviously derived from incomplete PstI digestion of B. circulans DNA, since there are five PstI sites on the insert.

Hybridization analyses. Southern hybridization results revealed that the xylanase gene carried by the pBCX549-P5 insert was not homologous to that carried by pBCX549-R1 or pBCX549-R3, which indicated that these plasmids harbored at least two distinct xylanase genes (Fig. 3). The hybridizations also showed that the pBCX549-P5 insert was not homologous to the B. subtilis (15) or B. polymyxa (22) xylanase genes that had been cloned and expressed in E. coli as pRH200 and pBCX277, respectively.

In vitro transcription-translation. The protein products of pBCX549-R1, pBCX549-R3, and pBCX549-P5 were analyzed by in vitro transcription-translation (Fig. 4). The 30-kilodalton β-lactamase encoded by the pUC19 ampicillin resistance gene was clearly visible in each instance. The B. circulans DNA inserts in pBCX549-R1 and pBCX549-R3 coded for a 22-kilodalton protein, whereas the 7.2-kb insert in pBCX549-P5 apparently coded for a protein with a molecular weight of 59,000. The nature of the 42,000-dalton product obtained with all three recombinant plasmids is unknown.

Characteristics of cloned xylanases. Xylanase production in E. coli by pBCX549-R1 and pBCX549-R3 occurred in the absence of xylan but was reduced by glucose and xylose. Under optimum conditions, xylanase levels of 0.04 U per ml of culture were obtained. Gene orientation did not significantly affect expression levels. Xylanase expression by pBCX549-R5 was not affected by the presence of xylan, glucose, or xylose and reached levels of 0.03 U/ml of culture. Under all conditions resulting in xylanase expression by pBCX549-R1, pBCX549-R3, and pBCX549-P5, more than 90% of the activity was located in the cytoplasm after 24 h of growth.

Quantitation of expression levels of the two xylanase activities in E. coli was influenced by the choice of substrate (Table 1). Whereas the enzyme expressed by pBCX549-R1 and pBCX549-R3 functioned effectively on all three xylans, that expressed by pBCX549-P5 displayed similar activity on aspen and larchwood xylan but much lower activity on oat spelt xylan. It was also noted during more prolonged assays that there was very limited digestion of all three xylans by the pBCX549-P5 enzyme, since less than 1% of the substrate was converted to reducing sugar on a xylose-equivalent basis. The larchwood and aspen xylans were observed to be more water soluble than the oat spelt xylan. This is a property that has been linked to a higher uronic acid content.
FIG. 2. Restriction map of recombinant plasmid pBCX549-P5. The 7.2-kb PstI fragment of \textit{B. circulans} DNA (linear map) was inserted by ligation at the \textit{PstI} site of the pUC19 vector. Abbreviations for restriction enzyme sites are as for Fig. 1.

(21). Whereas the pBCX549-R1 and pBCX549-R3 enzymes hydrolyzed xylans to small oligosaccharides, the pBCX549-P5 enzyme did not. High-performance liquid chromatography analyses indicated that the main products obtained with the pBCX549-R1 and pBCX549-R3 enzymes were xylobiose and xylotriose, with trace amounts of xylose, xylotetraose, and higher oligosaccharides (data not shown). None of these sugars was detected in the pBCX549-P5 digest. On the basis of reducing sugar assays and high-performance liquid chromatography analyses, it was concluded that the two activities had different substrate specificities and that this was manifested in the degree to which they could depolymerize xylans. This difference in specificity was also evident when activity stains were performed on xylanase preparations that...
The encoding patterns indicated on radioactive label. Molecular weights (in kilodaltons) of products are indicated on the right.

had been fractionated by analytical isoelectric focusing. Although both activities produced clearing zones on aspen and larchwood xylans (Fig. 5), only the pBCX549-R1 and pBCX549-R3 gene products gave hydrolysis zones on oat spelt xylan gels (data not shown). The pBCX549-R1 and pBCX549-R3 gene products gave identical hydrolysis zones on all three xylans. The pBCX549-P5 product gave identical hydrolysis zones with aspen and larchwood xylan but did not result in any clearing of oat spelt xylan. The pl of both enzymes was approximately 9.0.

**DISCUSSION**

Xylanolytic *Bacillus* spp. characteristically produce an acidic and a basic xylanase. Esteban et al. (3) reported that

**TABLE 1. Effect of xylan source on the quantitation of xylanase expressed by pBCX549-R1 and pBCX549-P5**

<table>
<thead>
<tr>
<th>Xylan source</th>
<th>Xylanase activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pBCX549-R1</td>
</tr>
<tr>
<td>Aspen</td>
<td>0.071</td>
</tr>
<tr>
<td>Larchwood</td>
<td>0.086</td>
</tr>
<tr>
<td>Oat spelt</td>
<td>0.046</td>
</tr>
</tbody>
</table>

*B. circulans* produces two xylanases: a high-molecular-weight enzyme with a pl of 4.5 and a low-molecular-weight enzyme with a pl of 9.0. It has been shown that *B. polymyxa* produces a basic xylanase with a pl of 9 and an acidic xylanase with a pl of 4.9 (22). The latter, which has a molecular weight of 48,000, has been cloned and expressed in *E. coli* (22). Cloning in *E. coli* of a xylanase gene from *B. polymyxa* has also been reported by Sandhu and Kennedy (18), but the gene and gene product were not characterized. It appears that these bacilli carry a common gene that codes for a 22,000-molecular-weight xylanase. Genes that code for such proteins have now been identified in *B. circulans, B. polymyxa*, and *B. subtilis* (this work; 4, 15). A 22,000-dalton xylanase has also been identified in culture filtrates from the fungus *Trichoderma harzianum* (19). All of these enzymes have pl values of approximately 9.0. There is now sufficient molecular detail, including some preliminary X-ray data (13, 17), known about these 22,000-dalton xylanases to allow for fundamental studies on the structure and catalytic mechanisms of these enzymes.

The results presented here establish the existence of a previously undetected *B. circulans* xylanase. The cloning experiments show that *B. circulans* has two distinct genes that code for basic xylanases that are indistinguishable on the basis of isoelectric point (pl = 9.0). This charge similarity may explain why this activity was not detected in a previous study (3). The fact that this xylanase releases only small amounts of reducing sugar from native xylans would also make it difficult to detect during chromatographic analysis and may explain why it was discovered only by gene cloning.

The observation that *B. circulans* produces xylanases with distinctly different substrate specificities is interesting and potentially important. Elucidation of these specificity differ-
ences will require the availability of substrates that are better defined from a chemical standpoint. Since xylan heterogeneity is a function of side chain composition, it seems probable that xylan backbone substituents are involved in substrate recognition by xylanases. This may be an important consideration in the selection of xylanases for specific applications. One possible explanation for the inability of pBCX549-P5 xylanase to extensively hydrolyze xylans is that it recognizes only unsubstituted areas on the xylan backbone. Such an activity could result in depolymerization that was sufficient to eliminate Congo red binding but not extensive enough to give substantial release of reducing sugar.

LITERATURE CITED