Hyperexpression of a *Bacillus circulans* Xylanase Gene in *Escherichia coli* and Characterization of the Gene Product†

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A 4.0-kilobase (kb) fragment of *Bacillus circulans* genomic DNA inserted into pUC19 and encoding endoxylanase activity was subjected to a series of subclonings. A 1.0-kb *Hind*III-*Hinc*II subfragment was found to code for xylanase activity. Maximum expression levels were observed with a subclone that contained an additional 0.3-kb sequence upstream from the coding region. Enhancer sequences in the upstream region are thought to be responsible for these high expression levels. Southern hybridization analyses revealed that the cloned gene hybridized with genomic DNA from *Bacillus subtilis* and *Bacillus polymyxa*. Xylanase activity expressed by *Escherichia coli* harboring the cloned gene was located primarily in the intracellular fraction. Levels of up to 7 U/ml or 35 mg/liter were obtained. The protein product was purified by ion exchange and gel permeation chromatography. The xylanase had a molecular weight of 20,500 and an isoelectric point of 9.0.

The structure of xylan, a major component of plant cell walls, is quite complex in that the β-1,4-linked D-xylose backbone is highly substituted with acetyl, arabinosyl, and uronyl groups (2). The enzymatic degradation of xylan to monomeric material requires several distinct activities, but endoxylanase, as the depolymerizing enzyme, is of particular significance. Most biochemical studies have focused on fungal xylanases such as those produced by *Aspergillus niger* (6–8, 16, 23), *Trichoderma harzianum* (26–28), and *Schizophyllum commune* (5, 13, 19). It has been shown that xylanolytic organisms produce multiple forms of xylanase with different physical and kinetic properties (6, 8, 23). It is not known how these enzymes act in concert and to what extent the multiple forms are the result of differential mRNA processing or posttranslational modification.

A 20- to 22-kilodalton (kDa) xylanase has now been identified in xylanase preparations from both fungal and bacterial sources (9, 13, 18, 20, 27, 29, 31). Sequence data for such xylanases from *Bacillus circulans*, *Bacillus subtilis*, *Bacillus pumilus*, *S. commune*, and *T. harzianum* have shown extensive sequence homology (Yaguchi et al., Abstr. Meet. Am. Chem. Soc. 1988, V195, part 1, p. 127). This enzyme has now been identified in several *Bacillus* spp. It is the smallest of at least two distinct xylanases produced by these organisms (11, 29, 30). *B. pumilus* (9, 20), *B. subtilis* (18), and *B. circulans* (29, 31) genes encoding the 20- to 22-kDa enzyme have been cloned and expressed in *Escherichia coli*. On the basis of sequence data and DNA hybridization studies, a high degree of homology has been shown to exist among the 20- to 22-kDa xylanases (9, 18, 31). Expression levels, like those in the *Bacillus* spp. donor strains, have been quite low. This report details the construction of a clone in which the levels of expression of the *B. circulans* xylanase gene are much higher than those previously achieved with cloned xylanase genes or with the *Bacillus* spp. donors.

MATERIALS AND METHODS

Organisms and growth conditions. *E. coli* HB101 harboring recombinant plasmids was grown in 2YT broth as described

previously (29). Plasmids pBCX549-R1, pBCX549-R3, and pBCX549-P5 carry *B. circulans* xylanase genes and were constructed as described previously (29). Plasmid pBPX277 contains a *Bacillus polymyxa* xylanase gene (30), and pRH200, kindly provided by M. Paice, harbors a *B. subtilis* xylanase gene (18).

Subcloning of pBCX549-R3. Large preparations of plasmid DNA were isolated by the alkali-sodium dodecyl sulfate method of Birnboim and Doly (3). Small preparations were isolated as described by Guo et al. (10). Plasmid DNA was completely digested with restriction enzymes, and the resulting fragments were separated by electrophoresis in 1.2% low-melting-point agarose gels containing TAE buffer (40 mM Tris hydrochloride [pH 7.8], 20 mM sodium acetate, 2 mM EDTA). Purified subfragments were isolated from the gels (30), ligated with an appropriate vector, and transformed into *E. coli* HB101 (30).

Hybridizations. Southern hybridizations were carried out as described previously (30). *Eco*RI and *Bam*HI digests of genomic DNA from *B. circulans*, *B. polymxya*, and *B. subtilis* were electrophoresed in a 0.8% high-melting-point agarose gel containing TAE buffer and were hybridized with a ^{32}P-labeled pBCX549-R3 probe.

Xylanase assays. Xylanase assays contained 1% oat-spelt xylan and were carried out as described by MacKenzie et al. (15). One unit was defined as the amount of enzyme releasing 1 μmol of reducing sugar per min.

Localization and purification of xylanase. Periplasmic and intracellular fractions were obtained by osmotic shock and sonication procedures as described previously (30). Protein concentrations were estimated by the method described by Bradford (4), with pooled immunoglobulin as the standard. Intracellular xylanase preparations were fractionated by high-pressure liquid chromatography. Samples were applied to a TSK CM-3SW column (7.5 by 150 mm) and eluted at a flow rate of 1 ml/min with a 0 to 1 M NaCl gradient in 10 mM phosphate buffer (pH 6). Fractions containing xylanase were freeze-dried, redissolved, applied to a TSK G2000SW gel permeation column (7.5 by 600 mm), and eluted with 50 mM phosphate buffer (pH 6). Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10 to 15% polyacrylamide gradient gels in a PhastSystem (Phar-

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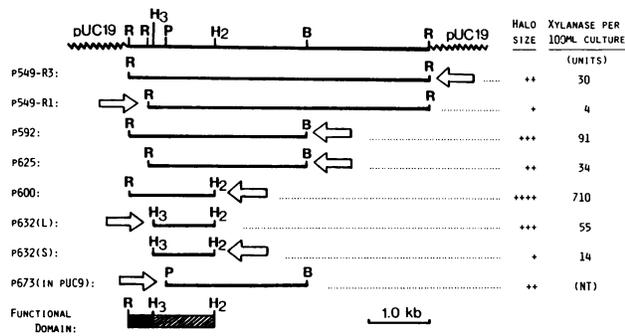† National Research Council of Canada publication 30113.

FIG. 1. Mapping of the functional domain of *B. circulans* xylanase gene cloned in pBCX549-R3. The 4.3-kb *B. circulans* DNA insert was subjected to subcloning at various restriction sites (B, *Bam*HI; H₂, *Hinc*II; H₃, *Hin*dIII; P, *Pst*I; R, *Eco*RI). Deleted areas are indicated with dotted lines. Xylanase expression levels obtained with the various subclones are given on the right. Clearing zones extending 1, 2, 3, and 4 mm from colonies are designated +, ++, +++, and ++++, respectively. The location and extent of the xylanase gene functional domain are indicated by the shaded box. The solid box indicates the location of enhancer sequence, and the arrow indicates the direction and location of the LacZ' gene promoter. All subclones carried pUC19 as the vector with the exceptions of pBCX632(L) and pBCX632(S), which carried pTZ18 and pBCX673 which carried pUC9. NT, Not tested.

macia). Protein bands were visualized by Coomassie brilliant blue staining.

RESULTS

Mapping of the xylanase gene functional domain in pBCX549-R3. The functional domain of the xylanase gene was located by subcloning of the 4.3-kilobase (kb) *B. circulans* DNA insert. The subcloning strategy and the results of the xylanase assays are summarized in Fig. 1. A 1.0-kb *Hin*dIII-*Hinc*II subfragment was found to code for xylanase activity. After 24 h of growth, virtually all of the activity produced by the various subclones was located in the cytoplasmic fraction. However, the amount of activity produced by the different clones varied greatly. Maximum levels were observed with pBCX600, a subclone in which the 0.3-kb upstream sequence was retained and the noncoding downstream sequence was deleted.

Expression levels. The influence of the upstream sequence on expression level is demonstrated by the differences in xylanase production by the two primary clones (pBCX549-R1 and pBCX549-R3) and by the effect of elimination of the sequence in some of the subclones. The difference in expression level between pBCX600 and pBCX632(S) is particularly striking. It was observed that xylanase overproduction by the pBCX600 clone was accompanied by a change in cellular morphology. Many of the cells were up to 10 times longer than normal *E. coli* cells. Deletion of the sequence downstream from the coding region resulted in a significant increase in expression level. The different xylanase levels produced by pBCX632(L) and pBCX632(S) suggest that the orientation of the gene (31) with respect to the β-galactosidase promoter is of some importance.

The effects of the upstream sequence and the β-galactosidase promoter orientation were further investigated by construction of a subclone (pBCX839-3) from pBCX600 in which the orientation of the *Hinc*II-*Hin*dIII fragment was reversed (data not shown). This was accompanied by a 50% reduction in expression level. Clones pBCX839-3 and
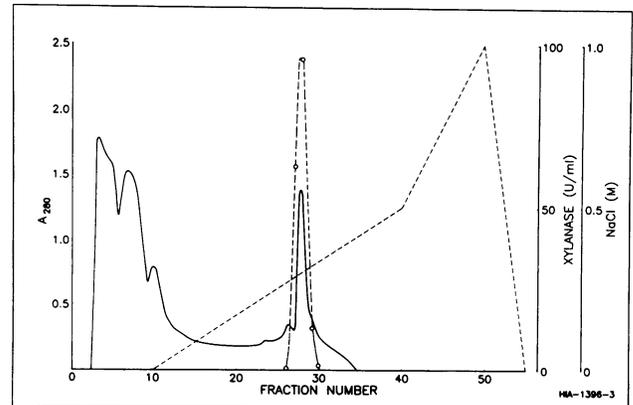

FIG. 2. TSK CM chromatography of intracellular xylanase produced by *E. coli* harboring pBCX549-R3. Symbols: ———, A_{280}; - - -, NaCl; ○, xylanase.

pBCX632(L) are identical with respect to β-galactosidase promoter and xylanase gene orientation, yet the expression level for pBCX839-3 was seven times that of pBCX632(L). This indicates that the *Eco*RI-*Hin*dIII fragment also enhances expression level when positioned downstream of the xylanase gene.

Subclone pBCX673, which was constructed by insertion of the 2.2-kb *Pst*I-*Bam*HI fragment of pBCX549-R3 into the corresponding sites of pUC9, was halo positive (Fig. 1), while a recombinant constructed in the same manner with pUC19 as the vector was halo negative. The nucleotide sequence near the *Pst*I site of pBCX673 indicated that the first 10 codons of the β-galactosidase gene contained in pUC9 had been inserted immediately upstream of the mature xylanase gene and in the same reading frame: 5'. . . ATG ACC ATG ATT ACG CCA AGC TTG GCT GCA GCT AGC. . . 3' (*Pst*I site underlined). In effect, the 10-residue N-terminal peptide encoded by the β-galactosidase gene replaced the 28-residue prepeptide of the wild-type xylanase.

Levels of xylanase production by *E. coli* harboring pBCX600 in 2YT medium and 2YT supplemented with larchwood or oat-spelt xylan were similar. Addition of isopropyl-β-D-thiogalactopyranoside was also without effect. Addition of glucose or xylose to the medium resulted in much-reduced enzyme levels. For this reason, the organism was grown in unsupplemented 2YT for xylanase production.

Southern hybridization analyses. Brief autoradiography showed that *Eco*RI digests of *B. circulans* and *B. subtilis* genomic DNA contained a common 4.0-kb band that hybridized with pBCX549-R3 (data not shown). The existence of a common 22-kDa xylanase gene in these organisms was further confirmed by hybridization between pBCX549-R3 and *Bam*HI digests of genomic DNA. Digests of *B. circulans* and *B. subtilis* DNA contained 9- and 15-kb bands, respectively, that hybridized strongly with pBCX549-R3.

Xylanase purification. After 24 h of growth, more than 95% of the activity was located in the intracellular fraction. Fractionation of the intracellular material on a TSK CM column separated the xylanase activity, which adsorbed to the column, from most of the other UV-absorbing activity (Fig. 2). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the xylanase fraction revealed the presence of small amounts of impurities. The xylanase fraction obtained by ion exchange chromatography was further purified by permeation chromatography on a TSK G2000 column. The activity was associated with the larger of two protein peaks
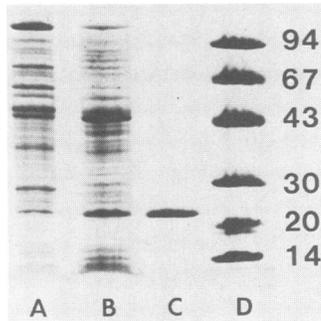
FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of intracellular fraction of *E. coli* harboring pUC19 (A), intracellular fraction of *E. coli* harboring pBCX549-R3 (B), purified xylanase (C), and molecular weight markers (D). Numbers on the right are molecular weights (10^3) of the markers. Approximately 20 μg of intracellular protein and 2 μg of purified xylanase were applied to the gel.

and eluted late in the run (data not shown). The enhanced expression of the enzyme in *E. coli* harboring pBCX600 is evident from the size of the xylanase peak in the TSK CM column chromatogram (Fig. 2) and the intensity of the xylanase band in the intracellular fraction of cells harboring this plasmid (Fig. 3).

Xylanase characteristics. The active fraction obtained by the purification procedure was shown to contain a single band with a molecular weight of approximately 22,000 (Fig. 3). On the basis of sequence data (31), the actual molecular weight of the protein is known to be 20,500. The protein eluted from the gel permeation column at a position much later than would be expected for a molecule of this size. The enzyme had an adsorption maximum at 280 nm and a molar extinction coefficient of 53,000. It apparently bound Coomassie blue poorly, since the Bradford protein assay gave a value that was 1/10 the actual amount, as determined from the weight of lyophilized pure enzyme.

DISCUSSION

It was previously reported (31) that the coding sequence of the pBCX600 insert was virtually identical to that described earlier (18) for the *B. subtilis* gene which codes for 22-kDa xylanase. The expression level data reported in the present article point to the significance of the 0.3-kb upstream fragment, since its deletion resulted in a drastic drop in intracellular xylanase levels. The nucleotide sequence of this fragment has been determined and has been shown to contain a modified promoter sequence and two pairs of tandem repeats resembling some animal virus enhancer elements (1, 31, 32). The *Eco*RI-*Hin*dIII fragment described in this report behaves like eucaryotic transcriptional enhancer elements in that expression levels are influenced by the location of the fragment in positions both upstream and downstream of the xylanase gene. The reason for the increased expression that was observed upon deletion of the sequence downstream from the coding region is unknown. It could be the result of increased copy number of the smaller plasmid and enhanced translation of the xylanase gene in the smaller plasmid. There is a (N) . . . -Ala-Ala-Ser- . . . (C) juncture sequence between the prepeptide and mature peptide (31). This sequence is also found in the pBCX673 subclone, in the genes encoding several extracellular *B. subtilis* proteins (14), and in other xylanases (9, 18). The

fragment also contains a Shine-Dalgarno sequence (24) or putative ribosome-binding site. The 3'-flanking sequence of the insert contains a pair of inverted repeats which have been proposed as transcription termination signals (21, 31).

The upstream sequence contained in the clone described here is considerably longer than that reported for other clones of homologous genes from other bacilli. A ribosome-binding site and possible promoter sequences were identified in a 60-base-pair (bp) sequence upstream of the initiation codon in a clone of the *B. pumilus* gene (9). A clone of the *B. subtilis* gene contained a 115-bp sequence upstream from the initiation codon (18). Possible promoter sequences were also identified in this sequence. The upstream sequence in the *B. circulans* clone described here contains 391 bp. A putative promoter sequence is located at 50 to 57 bp upstream of the initiation codon. The two pairs of tandem repeats that are thought to act as enhancer elements (31) are located at 256 to 269 bp and 286 to 302 bp upstream and are not present in the *B. pumilus* or *B. subtilis* clones. Very high expression levels in *E. coli* have been reported for two xylanase genes from two strict anaerobes, *Bacteroides succinogenes* (25) and *Clostridium acetobutylicum* (33). The *C. acetobutylicum* enzyme has a molecular weight of approximately 28,000 (33). The *Bacteroides succinogenes* enzyme has not been characterized. Regulation of gene expression was not studied in either instance.

Commercial application of cloned endoglycanase activities such as use of cellulases and xylanases in the processing of lignocellulosic activities is hampered by low enzyme yields. However, there have now been several reports of high expression levels of these enzymes in *E. coli*. O'Neill et al. (17) obtained overexpression of a *Cellulomonas fimi* cellulase gene in *E. coli* by placing the gene under the control of the leftward promoter of the bacteriophage lambda. Enhanced gene expression was obtained by thermal inactivation of the heat-sensitive lambda cI857 repressor. The same strategy has been used to obtain high levels of *Clostridium thermocellum* cellulase expression in *E. coli* (22). *C. thermocellum* cellulase overproduction in *E. coli* has also been reported by Joliff et al. (12). As with the cloned *B. circulans* xylanase gene described here, overexpression was fortuitous. Since the *C. thermocellum* gene was not sequenced, no attempt was made to explain the molecular basis of overproduction.

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