Molecular Cloning and Expression of a Xylanase Gene from *Bacillus polymyxa* in *Escherichia coli*†

ROBERT C. A. YANG, C. ROGER MacKENZIE, DORIS BILOUS, VERNER L. SELIGY,
AND SARAN A. NARANG*

*Division of Biological Sciences, National Research Council of Canada, 100 Sussex Drive, Ottawa, Ontario,
Canada K1A 0R6*

Genomic fragments of *Bacillus polymyxa* derived from separate and complete digestion by *Eco*RI, *Hin*dIII, and *Bam*HI were ligated into the corresponding sites of pBR322, and the resulting chimeric plasmids were transformed into *Escherichia coli*. Of 6,000 transformants screened, 1 (pBPX-277) produced a clear halo on Remazol brilliant blue xylan plates. The insert in the pBPX-277 recombinant, identified as an 8.0-kilobase *Bam*HI fragment of *B. polymyxa*, was subsequently subjected to extensive mapping and a series of subclonings into pUC19. A 2.9-kilobase *Bam*HI-*Eco*RI subfragment was found to code for xylanase activity. Xylanase activity expressed by *E. coli* harboring the cloned gene was located primarily in the periplasm and corresponded to one of two distinct xylanases produced by *B. polymyxa*. Xylanase expression by the cloned gene occurred in the absence of xylan and was reduced by glucose and xylose. Southern blot hybridization with the cloned fragment as a probe against complete genomic digests of the bacilli *B. polymyxa*, *B. circulans*, and *B. subtilis* revealed that the cloned xylanase gene was unique to *B. polymyxa*. The xylanase expressed by the cloned gene had a molecular weight of approximately 48,000 and an isoelectric point of 4.9.

Xylan, a β-1,4-glycoside-linked polymer of D-xylose, is a major component of forest and agricultural materials such as hardwood, grain straw, corn cobs, and grasses (27). Xylan can be enzymatically hydrolyzed to xylose (2) and converted into economically valuable products such as xylulose (26), xylitol, and ethanol (11, 23). Although xylan is generally insoluble in nature, a number of microorganisms which produce extracellular xylanolytic enzymes can solubilize xylan rather readily. Among these enzymes, D-xylanase is a key enzyme in that it initiates the degradation of xylan into xylooligosaccharides which are substrates for other enzymes. Xylanases are generally quite small proteins, with molecular weights ranging from 15,000 to 30,000, although higher-molecular-weight xylanases have occasionally been isolated (2).

Recent studies on the molecular characterization of bacterial xylanases (4, 7, 16, 18, 22, 24) suggest that this enzyme class will be a useful model for fundamental studies on protein secretion, expression, and structure as well as applied research. The long-term objective of our interest in xylanases is to elucidate the mechanism of enzyme-substrate interaction and to engineer the enzyme molecule for more desirable properties, such as heat stability. To achieve this goal, a thorough understanding of gene structure and function is required. This report details the cloning and expression of a *Bacillus polymyxa* xylanase gene in *Escherichia coli* and analysis of some of its biological characteristics.

MATERIALS AND METHODS

Bacteria and culture media. *B. polymyxa* (NRC 2822; also called NRRL 8505) and *Bacillus circulans* (NRC 9024; USDA 729) were obtained from the U.S. Department of Agriculture. *Bacillus subtilis* (NRC 3052; ATCC 6051) was obtained from the American Type Culture Collection. *B. polymyxa* NRC 2822 was chosen as the donor strain. *E. coli*

HB101 (F⁻ *hsdS20* [r_B⁻ m_B⁻] *supE44 ara-14 galK2 lacY1 proA2 apsL20* Str^r *xyl-5 mtl-1* λ⁻ *recA13*) (8) was used as the recipient strain. *E. coli* harboring pRH200 was kindly provided by M. Paice; pRH200 carries a *B. subtilis* xylanase gene (18). 2YTP broth, consisting of 16 g of Bacto-tryptone (Difco Laboratories), 10 g of yeast extract (Difco), 5 g of NaCl, 5 g of KH₂PO₄, and 5 g of xylan per liter, was used for growth of the bacilli as well as *E. coli*. BM medium suitable for the growth of bacilli was also used; it contained (per liter) 6 g of Na₂HPO₄, 3 g of KH₂PO₄, 0.5 g of NaCl, 1 ml of 1 M MgSO₄, 1 ml of 1 M CaCl₂, 1 g of yeast extract (Difco), 2.5 g of urea, and a suitable carbon source. For enzyme studies, *B. polymyxa* was grown at 30°C for 24 h with shaking, and *E. coli* was grown at 37°C for 24 h with shaking. For agar plates 2.0% (wt/vol) Noble agar (Difco) was included. Filter-sterilized ampicillin was added to *E. coli* transformant cultures at 100 mg/liter.

DNA preparations. Bacterial genomic DNA was prepared as follows. A 75-ml amount of an overnight culture grown at 30°C was spun at 5,000 rpm at 4°C for 15 min. The cell pellet was washed once by suspension in 2 ml of 10× TE buffer (100 mM Tris hydrochloride [pH 7.6], 10 mM EDTA), followed by centrifugation for 1 min in two 1.5-ml microfuge tubes. The cell pellet (300 μl) in each tube was thoroughly mixed with lysozyme (10 mg/ml), RNase A (1 mg/ml), and TEG buffer (25 mM Tris hydrochloride [pH 8.0], 10 mM EDTA, and 50 mM glucose) in a total volume of 1 ml. Sodium dodecyl sulfate (SDS; 0.5%) was then added, and the tube contents were gently mixed by inverting the tubes several times by hand. After incubation overnight at 37°C, the mixture in each tube was extracted thrice with 500 μl of phenol-CHCl₃ (1:1). DNA was sedimented from the aqueous phase following addition of NaCl (0.5 M) and isopropanol (40%). The flocculent DNA thus formed was spooled with a polypropylene pipette tip and suspended in 300 μl of TE (10 mM Tris hydrochloride [pH 7.6], 1 mM EDTA). Usually 800 to 1,000 μg of genomic DNA is obtained by this procedure. The DNA sample was sufficiently pure and intact for the

* Corresponding author.
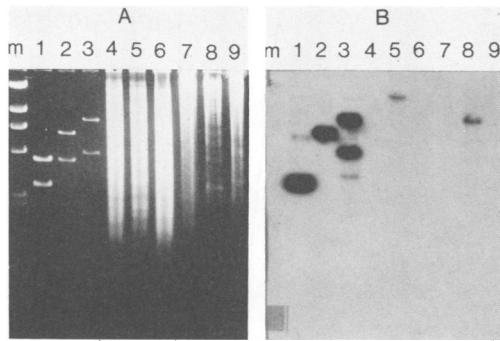† National Research Council of Canada paper no. 28495.

cloning work. Large preparations of plasmid DNA were isolated by the alkali-SDS method of Birnboim and Doly (1), followed by CsCl-ethidium bromide ultracentrifugation (15). Small plasmid preparations were isolated from a lawn culture of a recombinant clone grown on a quarter of an 85-mm plate as described previously (5, 6).

Construction of genomic libraries. *B. polymyxa* genomic DNA and pBR322 DNA were digested separately to completion with *Bam*HI. After phenol treatment, ethanol precipitation, and resuspension, 3 μg of bacterial DNA and 1 μg of plasmid were mixed and ligated with T4 DNA ligase overnight at 12°C. This ligated mixture was used to transform competent *E. coli* HB101 cells (15). The *Hind*III and *Eco*RI libraries were constructed in a similar manner.

Southern blot hybridization. After electrophoresis, DNA fragments were transferred by blotting from the agarose gel to a Hybond-N nylon filter (Amersham) as described by Southern (25). The filter was baked and hybridized with the appropriate ^{32}P-labeled DNA probe. DNA was labeled by nick translation (20) with *E. coli* DNA polymerase I, pancreatic DNase I, and [α-^{32}P]dATP (3,000 Ci/mmol; Amersham).

Subcloning of primary clone. Plasmid DNA was first digested with the appropriate enzyme(s), and the resulting fragments were separated by electrophoresis on a 1.2% low-melting-point agarose gel. The subfragment to be recloned was isolated by melting the excised gel at 70°C, followed by phenol-chloroform extraction and ethanol precipitation as described previously (5). Ligation of the purified subfragment with an appropriate vector and transformation of *E. coli* HB101 were then carried out as described earlier (1).

Xylanase assays. Assays contained 1% oat spelt xylan and were carried out as described by MacKenzie et al. (13). One unit was defined as the amount of enzyme releasing 1 μmol of reducing sugar per min.

Components of xylanase preparations were separated by analytical isoelectric focusing. Xylanase bands were detected by the technique described by MacKenzie and Williams (14). Focused gels were placed in contact with agar gels containing 0.1% oat spelt xylan. Xylan gels were then stained with Congo red. Clear zones were observed in areas exposed to xylanase activity.

Localization of xylanase activity. Periplasmic fractions (from 100-ml cultures) were obtained by the Huang and Forsberg modification (10) of the osmotic shock technique described by Neu and Heppel (17). Following osmotic shock, the cells were suspended in 50 mM phosphate, pH 6, supplemented with 0.8 M NaCl and sonicated for nine 20-s bursts with 1-min intervals on ice. After centrifugation at 35,000 × g for 30 min, the supernatant was retained as the cytoplasmic fraction.

Xylanase purification procedures. Xylanase preparations from *E. coli* and *B. polymyxa* were fractionated by high-pressure liquid chromatography (HPLC). Samples were applied to a TSK DEAE-3SW column (7.5 by 150 mm) and eluted at a flow rate of 1 ml/min with a 0 to 1 M linear NaCl gradient in 10 mM phosphate buffer, pH 6. Fractions containing xylanase were concentrated by ultrafiltration, applied to a TSK G2000SW gel permeation column (7.5 by 600 mm), and eluted with 50 mM phosphate buffer, pH 6. Protein in xylanase fractions obtained by HPLC were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10 to 15% polyacrylamide gradient gels in a PhastSystem (Pharmacia) and by native PAGE with 8 to 25% gradient gels in a Phastsystem.

FIG. 1. Restriction map of recombinant plasmid pBPX-277. The 8-kb *Bam*HI fragment of *B. polymyxa* DNA (linear map) was inserted by ligation at the *Bam*HI site of the pBR322 vector. A total of 14 restriction enzymes were used for the construction of this physical map. DNA size is estimated in kilobases. The numbers inside the circle also represent kilobases.

RESULTS

Cloning of a xylanase gene from *B. polymyxa* into *E. coli*. One among 6,000 transformants examined was found to produce a clear halo on 0.5% oat spelt xylan plates and 0.15% Remazol brilliant blue-xylan plates. The chimeric plasmid was named pBPX-277. Isolated pBPX-277 DNA was digested with various restriction enzymes, and the resulting fragments were analyzed by electrophoresis on agarose gels. The *B. polymyxa* DNA insert was estimated to be an 8-kilobase (kb) *Bam*HI fragment. A detailed physical map was constructed (Fig. 1).

Functional mapping of the xylanase gene domain in pBPX-277. The 8-kb *B. polymyxa* DNA insert was scanned by subcloning to localize the xylanase gene domain. The strategy for subcloning and the results of xylanase assays are summarized in Fig. 2. The vector was pUC19, except for subclones pBPX-532R, pBPX-532H, pBPX-532Sc, pBPX-532A, and the original clone, pBPX-277, which contained pBR322 as the vector. During this mapping, we also ob-

FIG. 2. Mapping of the functional domain of the *B. polymyxa* xylanase gene cloned in pBPX-277. The 8-kb *B. polymyxa* DNA insert was subjected to subcloning at various restriction sites (A, *Ava*I; Bm, *Bam*HI; R, *Eco*RI; H3, *Hind*III; Sc, *Sac*I; Sp, *Sph*I). Deleted areas are indicated with dotted lines. The new clones containing the subfragments (solid lines) were examined for extracellular xylanase production. The location and extent of the xylanase gene domain are marked by the shaded box.

FIG. 3. Southern hybridization analysis with the entire pBPX-534R plasmid used as a probe against complete digests (3 μg per digest) of genomic DNA from *B. polymyxa*, *B. circulans*, and *B. subtilis*. (A) Electrophoretic banding patterns of DNA fragments on a 0.8% high-melting-point agarose gel. (B) Autoradiogram of Southern blot hybridization of Hybond-N filter (Amersham). DNA digests: lane m, lambda DNA (0.5 μg), HindIII (molecular markers); lane 1, pBCX-549R1 (0.125 μg), EcoRI; lane 2, pRH200 (0.125 μg), EcoRI; lane 3, pBPX-277 (0.125 μg), BamHI; lanes 4, 5, and 6, EcoRI digests of *B. circulans*, *B. polymyxa*, and *B. subtilis* DNA, respectively; lanes 7, 8, and 9, BamHI digests of *B. circulans*, *B. polymyxa*, and *B. subtilis* DNA, respectively. Each genomic digest contained 3 μg of DNA. Plasmid pBCX-549R1 is composed of a 4-kb EcoRI fragment from *B. circulans* and the 2.7-kb pUC19 vector (R. Yang, unpublished data). pRH200 consists of a 4-kb EcoRI DNA fragment from *B. subtilis* and the 6-kb pBR325 vector (18). The light bands shown in lanes 1 and 3 are probably either cross-lane contaminants or partial cleavage products.

served that the three positive subclones (pBPX-532Sc, pBPX-537BS, and pBPX-534R) exhibited higher extracellular xylanase activity than the original clone, pBPX-277. Judging from the width of the clear zone around each colony on Remazol brilliant blue-xylan plates, the levels were approximately 10-fold higher.

Chromosomal copy analysis of xylanase gene. BamHI and EcoRI digests of chromosomal DNA from *B. polymyxa*, *B. circulans*, and *B. subtilis* were separated by 0.8% high-melting-point agarose gel electrophoresis and hybridized with ^{32}P-labeled entire pBPX-534R DNA, which contained the 2.9-kb BamHI-EcoRI subfragment derived from the original pBPX-277 clone (Fig. 2). The probe hybridized only to *B. polymyxa* genomic DNA: a 10-kb EcoRI fragment (Fig. 3, lane 5) and an 8-kb BamHI fragment (lane 8), as well as the 8-kb insert of pBPX-277 (lane 3). The cloned *B. polymyxa* xylanase gene was not homologous at all to either the *B. circulans* xylanase gene cloned in pBCX-549R1 (lane 1) (details to be published elsewhere) or the *B. subtilis* xylanase gene cloned in pRH200 (lane 2).

Regulation of xylanase in *B. polymyxa* and pBPX-532-Sc. Xylanase production by *B. polymyxa* occurred only in medium containing xylan (Table 1). Larchwood xylan was a somewhat better inducer of activity than oat spelt xylan. The reason for the lower activity levels at higher xylan concentrations in YT is unknown. Xylanase production by pBPX-532-Sc occurred in the absence of xylan but was reduced by glucose and xylose (Table 1). Supplementation of YT with xylan did not result in elevated levels of xylanase. No xylanase production was observed in BM supplemented with xylan, possibly because of poor growth. Under all conditions which resulted in xylanase expression by pBPX-532-Sc, approximately 90% of the total activity was localized in the periplasm.

Properties of *B. polymyxa* xylanases and activity expressed

TABLE 1. Effect of carbohydrate source on xylanase activity of *B. polymyxa* and *E. coli* (pBPX-532-Sc)

Medium and addition	Xylanase (U/100 ml of culture)	
	B. polymyxa supernatant fraction	*E. coli*(pBPX-532-Sc), periplasmic fraction
2YTP	0	3.5
0.2% glucose	0	0.9
0.5% glucose	0	0
0.2% xylose	0	0.9
0.5% xylose	0	0
0.2% larchwood xylan	140	3.4
0.5% larchwood xylan	24	3.7
0.2% oat spelt xylan	65	3.3
0.5% oat spelt xylan	49	2.4
BM		
0.2% larchwood xylan	170	0
0.5% larchwood xylan	180	0
0.2% oat spelt xylan	26	0
0.5% oat spelt xylan	56	0

by *E. coli* harboring pBPX-532-Sc. Zymogram analysis of the extracellular xylanase from *B. polymyxa* indicated that it produced two major xylanases with isoelectric points of approximately 4.9 and 9.0 and that *E. coli* containing pBPX-532-Sc produced the pI 4.9 component (Fig. 4). Two zones of hydrolysis were evident in each preparation, with the major xylanase band expressed by the cloned gene being slightly more acidic than the equivalent *B. polymyxa* enzyme.

The elution profiles of xylanase activity from TSK-DEAE columns were in good agreement with the activity stain results. Fractionation of the extracellular protein from *B. polymyxa* yielded an unadsorbed and adsorbed xylanase peak (Fig. 5A), while fractionation of the periplasmic fraction from *E. coli* harboring pBPX-532-Sc yielded only an adsorbed xylanase fraction (Fig. 5B). The plasmid-encoded xylanase bound slightly more strongly to the ion-exchange matrix than did the equivalent *B. polymyxa* xylanase.

Gel permeation chromatography of the adsorbed *B. polymyxa* xylanase gave a single xylanase peak (Fig. 6). The molecular weight of this fraction was estimated to be 48,000. The periplasmic fraction from *E. coli* harboring pBPX-532-Sc also gave a single xylanase peak. However, this component eluted slightly earlier and had an estimated molecular weight of 51,000 (data not shown).
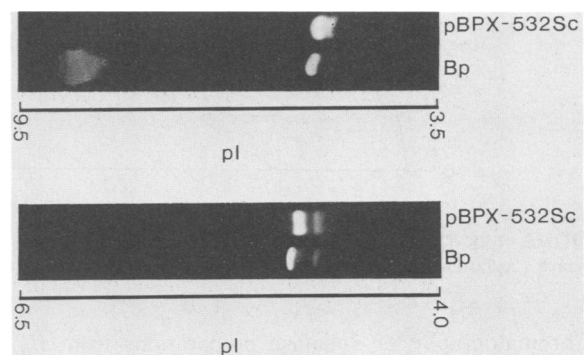

FIG. 4. Zymogram of extracellular xylanases produced by *E. coli* containing pBPX-532-Sc and *B. polymyxa* (Bp). Prior to activity staining, enzyme preparations were electrofocused on gels with pH ranges of 3.5 to 9.5 (top) and 4.0 to 6.5 (bottom).
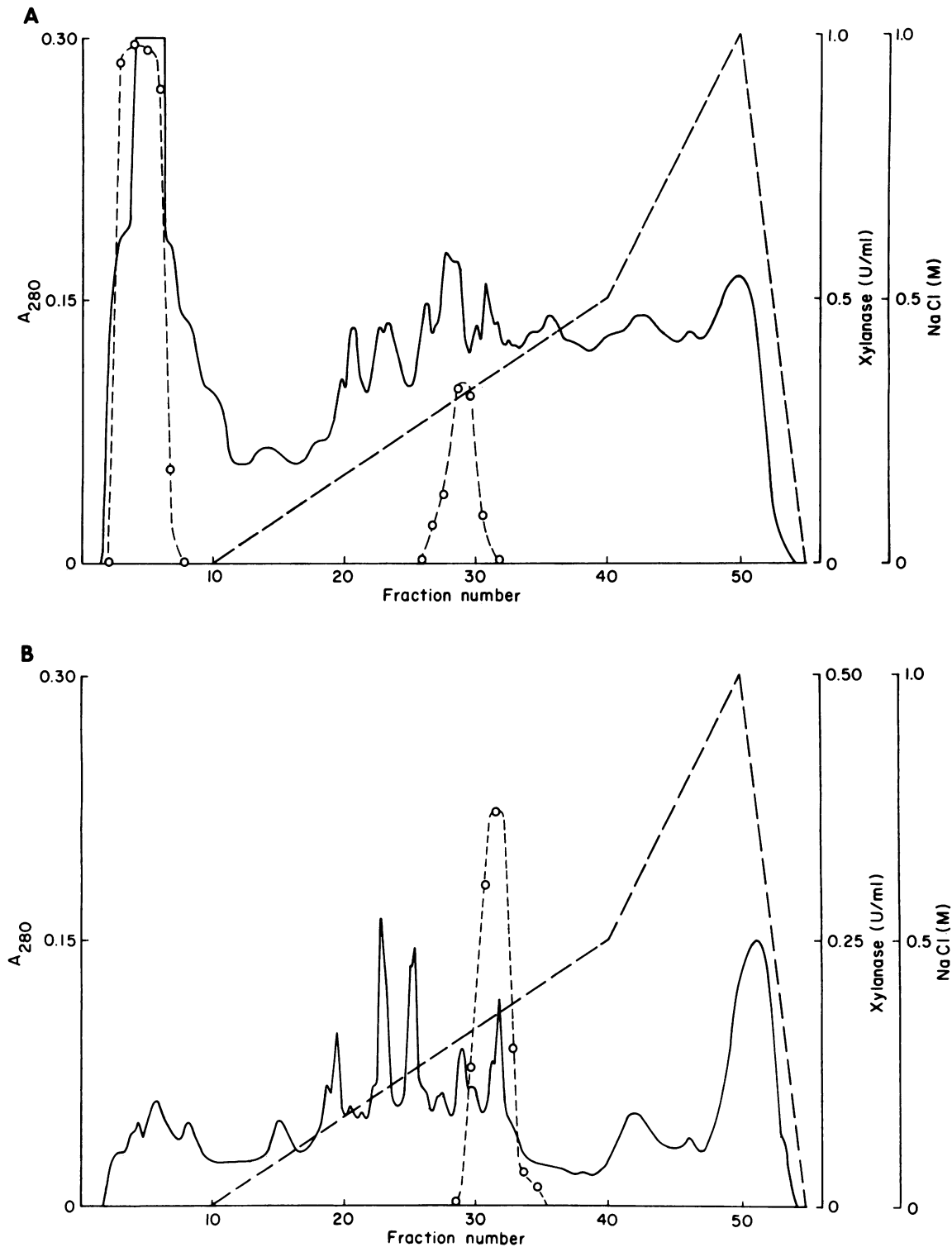
FIG. 5. TSK-DEAE chromatography of periplasmic xylanase produced by *E. coli* containing pBPX-532-Sc (A) and of extracellular *B. polymyxa* xylanase (B). Symbols: ——, A_{280}; ---, NaCl; O, xylanase.

Chromatography of xylanase preparations from *E. coli* harboring pBPX-532-Sc and from *B. polymyxa* did not yield fractions with single bands following SDS-PAGE. Since activity strains are difficult following this treatment, xylanase molecular weights were not estimated by this method.

Native PAGE followed by activity staining of the adsorbed xylanase fractions obtained by TSK-DEAE chromatography of extracellular *B. polymyxa* xylanase and periplasmic *E. coli* xylanase revealed the presence of three active components (Fig. 7). Each preparation contained a component that
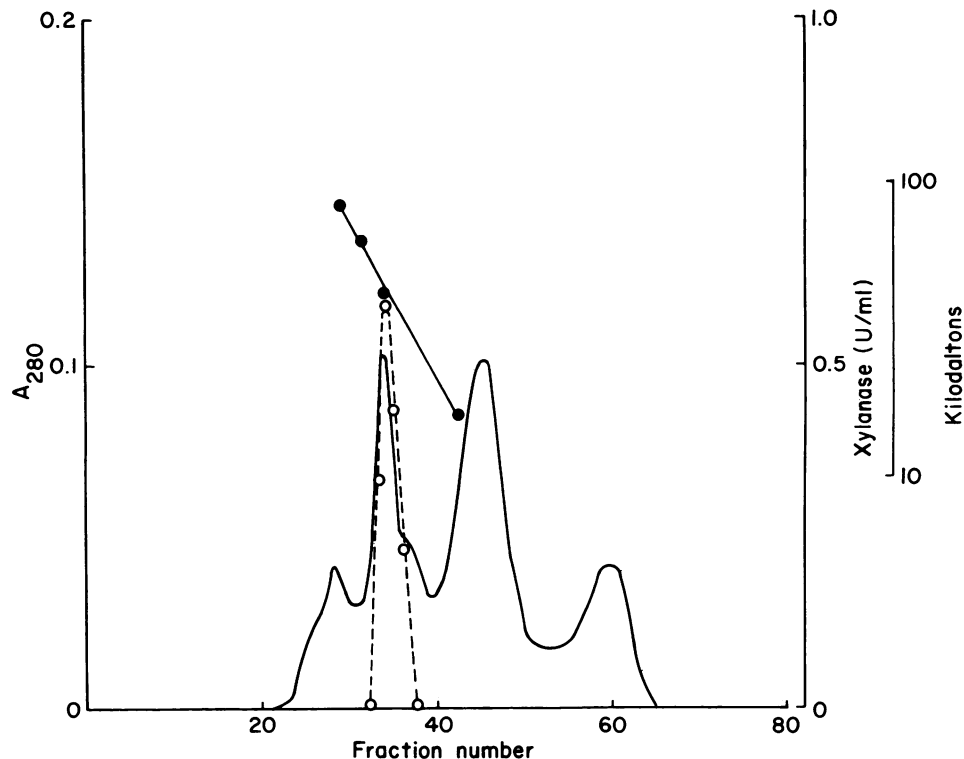
FIG. 6. HPLC gel permeation chromatography of adsorbed xylanase fraction obtained by TSK-DEAE chromatography of extracellular *B. polymyxa* Xylanase. Symbols: ——, A_{280}; O, xylanase; ●, molecular mass markers.

was common to both and one that was not. The slower mobility of one component of the *E. coli* preparation suggested that it was a higher molecular weight than the *B. polymyxa* bands. Although accurate molecular weight measurements cannot be made by this method, the mobilities of the active bands relative to those of molecular weight markers suggest a xylanase molecular weight in the 50,000 to 70,000 range.

DISCUSSION

In this study we have shown that *B. polymyxa* NRC 2282 produces two xylanases. One is a very basic protein with a pI of 9, while the second has a pI of 4.9 and a molecular weight of approximately 48,000. Furthermore, we show that the two enzymes are probably coded by two sets of distinctive sequences. The xylanase product from the primary clone, pBPX-277, containing an 8-kb *Bam*HI fragment of *B. polymyxa* DNA, produced a xylanase with electrophoretic properties that were similar to the low-pI xylanase from *B. polymyxa*. Through a series of subclonings for locating the xylanase coding region, the insert DNA was reduced to 2.9 kb (as in pBPX-534R) while retaining the ability to code for a xylanase with properties that were similar to the equivalent *B. polymyxa* xylanase. Moreover, elimination of nonfunctional sequence of xylanase gene resulted in a level of expression about ten times that of the pBPX-277 gene (unpublished result).

Southern blot hybridization revealed that no related sequence was located within the genomes of either *B. circulans* or *B. subtilis* when the cloned xylanase gene of *B. polymyxa* was used as the probe. Among the three *Bacillus* strains tested, the 48,000-molecular-weight xylanase was unique to *B. polymyxa*. On the other hand, when a plasmid (pBCX-549R1) containing a 4.0-kb *Eco*RI fragment coding for a 22,000-molecular-weight xylanase from *B. circulans* (R. Yang, unpublished data) was used as the probe, it hybridized not only strongly to an *Eco*RI fragment of identical size from *B. subtilis* but also modestly to a 12-kb *Bam*HI fragment from *B. polymyxa* under medium-stringent hybridization conditions. This indicated that the 22,000-molecular-weight xylanase gene of *B. circulans* is a common gene which is quite well conserved in *B. subtilis* but only partly conserved in *B. polymyxa*. Assuming that there is no other gene coding for xylanase, it is likely that the common sequence codes for the basic *B. polymyxa* xylanase, which had a pI similar to that of the *B. circulans* and *B. subtilis* enzymes. An alternative explanation is that the common sequence is a conserved unexpressed sequence.
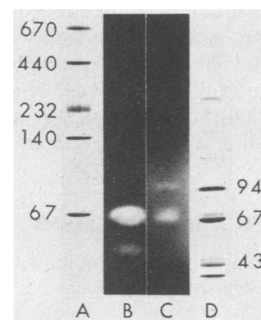

FIG. 7. Zymogram of xylanase fraction which adsorbed to the TSK-DEAE column during chromatography of extracellular *B. polymyxa* xylanase (B) and periplasmic xylanase from *E. coli* harboring pBPX-532-Sc (C). Prior to activity staining, enzyme preparations were subjected to native PAGE. Molecular weight markers (in thousands) are shown in lanes A and D.

Like *B. polymyxa*, several other *Bacillus* spp. also have multiple forms of xylanase activity (3, 9, 18). There are a number of possible explanations for these multiple forms of xylanase: (i) proteolytic modification of a parental enzyme, (ii) the existence of separate xylanase genes with distinct DNA sequences, or (iii) differential readout from mRNA. In *B. polymyxa*, it appears that two xylanase genes exist, one coding for an alkaline xylanase and one for a slightly acidic xylanase. In addition, at least two forms of acidic xylanase are produced by both *B. polymyxa* and *E. coli* harboring the acidic xylanase gene. Since the *E. coli* product seemed to have a higher molecular weight, one of these forms may be a prexylanase.

The xylanases of *B. polymyxa* are released into the extracellular culture fluid. However, less than 10% of the cloned xylanase activity was localized in the extracellular fluid at the stationary growth phase of *E. coli* harboring the pBPX-532-Sc recombinant plasmid. The majority of the xylanase activity was detected in the periplasmic space. It has been demonstrated that the xylanase synthesized by *B. subtilis* harboring the cloned gene from *Bacillus pumilus* was secreted into the medium like that of the *B. pumilus* IPO host, but xylanase synthesized in *E. coli* bearing the same gene was retained in the cytoplasm (19). In contrast, a xylanase gene from an alkalophilic bacillus coded for an enzyme which was mostly (82%) secreted into the extracellular milieu by *E. coli* (9). In the former instance, in spite of the intracellular location of the xylanase synthesized in *E. coli*, the signal peptide was processed in the same manner as in the *B. pumilus* host, and the resulting mature enzyme derived from either *E. coli* or *B. pumilus* culture fluid was verified as identical in size and in amino acid sequence (19). Prexylanase synthesized by *E. coli* bearing a cloned *B. subtilis* xylanase gene was processed similarly by a membrane protease, and the mature xylanase was secreted into the periplasm (18). Secretion of extracellular proteins by *E. coli* seems to involve mechanisms other than simple removal of the signal peptide by a membrane-bound protease. Of interest was the recent finding that expression of the *kil* gene, which was originally found in pMB9, rendered the outer *E. coli* membrane permeable to most cloned foreign gene products (12). One of the forms of xylanase produced by *E. coli* harboring pBPX-532-Sc was electrophoretically distinct from the acidic xylanases produced by *B. polymyxa*, suggesting that the enzyme was processed differently in these organisms.

Cloning in *E. coli* of a xylanolytic enzyme gene from *B. polymyxa* was also reported by Sandhu and Kennedy (22). However, since insufficient molecular details (restriction mapping, subcloning, and protein analysis) were given in their paper, it is impossible to determine whether their gene is the same as the one reported here. Nucleotide sequence analysis of the 48,000-molecular-weight xylanase gene and cloning of the second xylanase gene of *B. polymyxa* are under way.

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