Molecular Cloning and Expression of a Xylanase Gene of Alkalophilic
Aeromonas sp. no. 212 in Escherichia coli

By TOSHIK I KUDO, ATSUSHI OHKOSHI and KOKI HORIZKOSHI*
The Riken Institute, Wako-shi, Saitama-ken 351-01, Japan

(Received 3 April 1985; revised 19 May 1985)

A gene coding for a xylanase activity of alkalophilic Aeromonas sp. no. 212 (ATCC 31085) was cloned in Escherichia coli HB101 with pBR322. Plasmid pAX1 was isolated from transformants producing xylanase, and the xylanase gene was located in a 6.0 kb HindIII fragment. The pAX1-encoded xylanase activity in E. coli HB101 was about 80 times higher than that of xylanase L in alkalophilic Aeromonas sp. no. 212. About 40% of the enzyme activity was observed in the periplasmic space of E. coli HB101. The pAX1-encoded xylanase had the same enzymic properties as those of xylanase L produced by alkalophilic Aeromonas sp. no. 212, but its molecular weight was lower (135000 vs 145000, as estimated by SDS polyacrylamide gel electrophoresis).

INTRODUCTION

In our laboratory, many alkalophilic bacteria which grow well at high pH (10.0-11.0) have been isolated. These bacteria produce many kinds of extracellular enzymes which have optimum pH values for activity in the alkaline region (Horikoshi & Akiba, 1982). There have been several reports of multi-enzyme systems in alkalophilic Bacillus strains. The alkalophilic Bacillus sp. strain C-125 produced two types of xylanases: xylanase A, with a very broad pH–activity curve (pH 5–11), and xylanase N, with a pH optimum of 7-0 (Honda et al., 1985b). These two enzymes are entirely different protein molecules, encoded by different genes (Honda et al., 1985a). Another multi-enzyme system is carboxymethylcellulase (CMCase) produced by Bacillus sp. strain N-4 (Horikoshi et al., 1984). We cloned two CMCase genes from strain N-4 in Escherichia coli with pBR322 (Sushihara et al., 1984). The plasmid-encoded enzymes had very broad pH–activity curves, and were probably not a mixture of two or three enzymes, because the cloned DNA fragments were too small to encode more than one enzyme. Therefore, two of the CMCases produced by alkalophilic Bacillus strain N-4 were synthesized by different genes.

Recently it was found that an alkalophilic non-sporeforming bacterium, Aeromonas sp. no. 212 (ATCC 31085), isolated from soil produced three types of xylanases, L, M and S, with molecular weights of 145000, 37000 and 23000, respectively (A. Ohkoshi and co-workers, unpublished). This paper deals with the cloning and expression of the xylanase L gene of alkalophilic Aeromonas sp. no. 212 in E. coli with pBR322; some properties of the plasmid-borne xylanase are also discussed.

METHODS

Bacterial strains, plasmids and media. Alkalophilic Aeromonas sp. no. 212 (ATCC 31085) was grown aerobically with continuous shaking at 37 °C in a medium containing 5 g glucose, 5 g polypeptone, 5 g yeast extract, 1 g K2HPO4, 0.2 g MgSO4, 7H2O and 5 g NaHCO3 (autoclaved separately) per litre (Horikoshi & Akiba, 1982). E. coli HB101 (F- proA leuB6 thi-1 lacY1 hsdR hsdM ara-14 galK2 xyl-5 mtl-1 supE44 recA4571) and the plasmid pBR322 (Bolivar, 1978) were kindly provided by Dr R. H. Doi (University of California, Davis, USA). E. coli HB101 was grown aerobically in LB medium (Miller, 1972) at 37 °C with shaking. When required, antibiotics (Sigma) were added to the medium at a final concentration (µg ml−1) of 50 and 100 for tetracycline and ampicillin.
respectively. For plasmid amplification, M9 medium (Maniatis et al., 1982) was supplemented with chloramphenicol at a final concentration of 200 µg ml\(^{-1}\) (Bolivar et al., 1977). Xylan plates (LBX) for screening of the xylanase-producing colonies contained 0.5% xylan from larchwood in LB medium without glucose.

**Isolation of DNA.** Alkalophilic Aeromonas sp. no. 212 was grown aerobically to the early stationary phase at 37°C in the medium described above and chromosomal DNA was purified by the method of Saito & Miura (1963). Plasmid DNAs were prepared according to Bolivar et al. (1977). The microscale extraction of plasmids was done by the method of Holmes & Quigley (1981).

**Construction of recombinant plasmids.** DNAs were digested with HindIII at 37°C for 1 h (plasmid DNA) or for 5 h (chromosomal DNA). After the digestion, 1 µg of plasmid and 3 µg of chromosomal DNA were mixed and ligated with T4 ligase overnight at room temperature. This ligated DNA mixture was used to transform *E. coli* HBl101 as described by Lederberg & Cohen (1974).

**Nick-translation and hybridization.** Plasmid pAX1 was labelled by nick-translation using *E. coli* DNA polymerase I in the presence of \[^{32}\text{P}\]dATP (New England Nuclear) as described by Rigby et al. (1977). The genomic hybridization analysis was done essentially as described by Southern (1975).

**Xylanase purification.** After 20 h cultivation at 37°C with shaking in the presence of ampicillin (50 µg ml\(^{-1}\)), cells were harvested by centrifugation, washed in 50 mM-sodium phosphate buffer (pH 6.0) and resuspended in 60 ml of the same buffer. Crude enzyme extract was prepared by sonic treatment. Cell debris was removed by centrifugation at 10000 g for 30 min at 4°C.

The supernatant fluid (50 ml) was applied to a DEAE-cellulose column (30 x 2.5 cm) previously equilibrated with 0.05 M-sodium phosphate buffer (pH 6.0). The enzyme was eluted with a linear gradient of 0-0.5 M-NaCl in the same buffer at a flow rate of 30 ml h\(^{-1}\). The fractions containing xylanase activity were dialysed against 10 mM-sodium phosphate buffer (pH 7.0). The dialysed xylanase fraction was loaded on a hydroxyapatite column (1 x 30 cm) equilibrated with 10 mM-sodium phosphate buffer (pH 7.0) and the enzyme was eluted with 50 mM-sodium phosphate buffer (pH 7.0). This fraction was concentrated with polyethylene glycol (PEG) 20000 followed by a Sephacryl S-200 gel filtration with 50 mM-sodium phosphate buffer (pH 7.0). The dialysed xylanase fraction was loaded on a hydroxyapatite column (1944). One unit of enzyme activity is defined as the amount of the enzyme which liberates 1 µmol reducing sugar (calculated as xylitol) min\(^{-1}\) under the above conditions.

**Gel electrophoresis.** DNA molecules were separated on a 1% agarose gel in the running buffer, 90 mM-Tris, 90 mM-boric acid, 1 mM-EDTA (pH 8.0), and DNA bands were visualized by staining with ethidium bromide. The SDS-polyacrylamide gels for protein separation were prepared by the method of Laemmli (1970).

**Analysis of xylanase distribution.** Fractionation of extracellular, periplasmic and intracellular xylanase was done by the method of Kato et al. (1983).

**Preparation of antisera.** The purified pAX1-encoded xylanase (about 1 mg protein) was mixed with 1 ml complete Freund’s adjuvant, and the mixture was injected into mature white rabbits. Booster injections of the samples were given in the same manner after 4 and 6 weeks, and the sera were collected 1 week after the last booster injection.

**RESULTS**

**Cloning of the xylanase gene from alkalophilic Aeromonas sp. no. 212 into E. coli HBl101**

*E. coli* HBl101 was transformed with the ligated DNA mixture described in Methods with an efficiency of 8 x 10⁴ ampicillin-resistant (Ap\(^{r}\)) transformants per µg total DNA. About 10% of the isolated colonies were tetracycline sensitive (Tc\(^{s}\)), indicating the possible insertion of foreign DNA fragments. Xylanase activity was detected directly on the plates by the appearance of a clear zone around a xylanase-producing colony on an LBX plate supplemented with ampicillin. Out of 3000 Ap\(^{r}\)Tc\(^{s}\) transformants, four colonies were isolated. These colonies harboured the same plasmid, which consisted of pBR322 and a 6.0 kb fragment inserted in the HindIII site. This hybrid plasmid, pAX1, was introduced into *E. coli* HBl101 to verify the presence of the xylanase gene on the plasmid. All the transformants obtained showed clear zones around colonies on LBX plates supplemented with ampicillin.
Xylanase gene from alkalophilic Aeromonas sp.

Fig. 1. Restriction map of pAX1. The thin line represents pBR322 and the heavy line, the 6.0 kb chromosomal DNA insert from alkalophilic Aeromonas sp. no. 212.

Fig. 2. Homology between plasmid pAX1 and chromosomal DNAs. (a) Agarose (1%) gel electrophoresis of DNA digests. Lane 1, E. coli DNA digested with HindIII (5 µg); lane 2, alkalophilic Aeromonas sp. no. 212 DNA digested with PstI (5 µg); lane 3, alkalophilic Aeromonas sp. no. 212 DNA digested with HindIII (5 µg); lane 4, pAX1 digested with PstI (0.2 µg); lane 5, pAX1 digested with HindIII (0.2 µg); lane 6, pAX1 digested with EcoRI (0.2 µg); lane 7, phage λ DNA digested with HindIII (0.2 µg). (b) Hybridization analysis of the Southern transfer of the DNAs from the gel in (a), using 32P-labelled pAX1 as a probe. Lanes 1–7 are as described in (a).

Partial characterization of plasmid pAX1

Plasmid pAX1, containing the 6.0 kb HindIII fragment from alkalophilic Aeromonas sp. no. 212, had cleavage sites for BamHI, PstI, SstI, BglII, XhoI, SalI and HindIII. No KpnI, XbaI, BstEII or BclI sites were found (Fig. 1). In order to confirm the origin of the 6.0 kb HindIII fragment in pAX1, a genomic hybridization experiment was done (Fig. 2). Radioactively labelled pAX1 hybridized to the insert of unlabelled pAX1 (lanes 4, 5 and 6 in Fig. 2) and also to a 6.0 kb HindIII fragment and the 3.0 kb PstI fragment within the 6.0 kb HindIII fragment from DNA of alkalophilic Aeromonas sp. no. 212 (lanes 2 and 3 in Fig. 2). No sequences complementary to pAX1 were detected in E. coli DNA fragments (lane 1 in Fig. 2).
Table 1. Distribution of enzymes in E. coli HB101 carrying pAX1

Activities were measured after growth in LB medium for 20 h at 37 °C. The activities of alkaline phosphatase and β-galactosidase were measured by the methods of Kreuzer et al. (1975) and Miller (1972), respectively. The activities of xylanase and β-galactosidase are expressed as units per ml broth; alkaline phosphatase activity is expressed as milliunits per ml broth. The values in parentheses are percentages of the total activity of the enzyme.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Extracellular</th>
<th>Periplasmic</th>
<th>Cytoplasmic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylanase</td>
<td>0 (0)</td>
<td>0.63 (39)</td>
<td>1.0 (61)</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>0.3 (2)</td>
<td>10.7 (60)</td>
<td>6.7 (38)</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4.7 (100)</td>
</tr>
</tbody>
</table>

Distribution of xylanase activity in E. coli HB101 carrying pAX1

After growth of alkalophilic Aeromonas sp. no. 212 for 48 h at 37 °C, the activities of xylanases L, M and S in the culture medium were, respectively, 0.025, 1.6 and 0.60 units per mg protein of the total culture. E. coli HB101 showed no xylanase activity, but when E. coli HB101 carrying pAX1 was grown aerobically in LB broth for 20 h the xylanase activity detected in sonically treated cells was 2.0 units per mg protein of the crude extract. It is striking that the xylanase activity expressed in E. coli was about 80 times higher than that of xylanase L produced by alkalophilic Aeromonas sp. no. 212. The extracellular, periplasmic and intracellular xylanase activities of E. coli carrying pAX1 were assayed (Table 1). About 40% of the xylanase activity was detected in the periplasmic space, and the remainder in the intracellular fraction; no extracellular xylanase was detected.

Comparison of the xylanases from alkalophilic Aeromonas sp. no. 212 and the xylanase encoded by pAX1

Molecular weight. The synthesis of xylanase in E. coli carrying pAX1 was constitutive and no effect of supplementation with xylan was observed. The xylanase thus produced was purified by ion-exchange chromatography followed by gel filtration. The molecular weight of the purified xylanase was estimated as 135 000 by SDS-polyacrylamide gel electrophoresis (not shown). The molecular weights of xylanases L, M and S from alkalophilic Aeromonas sp. no. 212 were 145 000, 37 000 and 23 000, respectively (A. Ohkoshi and co-workers, unpublished).

Effect of pH. The pH was adjusted with MacIlvain buffer (pH 3-8) or 0.05 M-glycine/NaOH buffer (pH 9-13). Other conditions were the same as those of the standard assay. No significant difference was observed between the xylanase of E. coli carrying pAX1 and xylanase L of alkalophilic Aeromonas sp. no. 212 (pH optimum 7.0-8.0).

Effect of temperature. Xylanases L, M and S and pAX1-encoded xylanase were most active at 50 °C, 50 °C, 60 °C and 50 °C, respectively.

Hydrolysis of xylan. About 100 mU of each enzyme was mixed with 2 ml 0.5% xylan. Samples of the reaction mixture were analysed by high-performance liquid chromatography. The end-products were oligosaccharides such as xylobiose, xylotriose, xylotetraose and higher oligosaccharides. Xylose was not detected as a major end-product.

Immunological studies. The results of an Ouchterlony double-diffusion test using the four purified enzymes are shown in Fig. 3. Xylanase L gave a line of precipitation which fused with that for xylanase purified from E. coli HB101 carrying pAX1.

DISCUSSION

Some xylanases produced by micro-organisms are multienzyme systems (Esteban et al., 1982). This may be due to (i) processing or modification by proteases or other enzyme systems during production process, or (ii) multiple genes. The results of the immunodiffusion test indicated that E. coli HB101 carrying pAX1 produced a xylanase which immunologically cross-reacted with
xylanase L from alkalophilic *Aeromonas* sp. no. 212. The pAX1-encoded xylanase had the same properties as those of xylanase L, except that its molecular weight was smaller than that of xylanase L. This difference might be due to modification of the protein molecules, e.g. by glycosylation, that did not occur in *E. coli*. No homology was observed between the 6 kb HindIII DNA fragment of pAX1 and genomic DNA fragments except the 6 kb HindIII fragment of alkalophilic *Aeromonas* sp. no. 212. A similar lack of hybridization to DNA other than that associated with the pAX1 clone was observed after *PstI* digestion. Thus it seems (though it is not definitely proved) that the xylanase activities in alkalophilic *Aeromonas* sp. no. 212 are encoded by several genes that possess no homology (as detected in our experimental conditions).

The structural genes for the xylanases of *Bacillus* spp. have been cloned in *E. coli* by Bernier et al. (1983), Panbangred et al. (1983) and Honda et al. (1985a). The xylanase activities produced by these transformants were about 20–25% of those of the donor bacteria except in the study of Honda et al. (1985a) (50%). Although we have not shown directly that the expression of the xylanase gene in *E. coli* was higher than that in *Aeromonas* sp. no. 212, it is noteworthy that the activity of the pAX1-encoded xylanase in *E. coli* was about 80 times higher than that of xylanase L produced by alkalophilic *Aeromonas* sp. no. 212. This high enzyme activity may be explained by several possibilities, such as the derepression of the xylanase L gene, plasmid copy number, or expression from the tetracycline promoter in the plasmid vector.

We thank Zeria Pharm. Co. Ltd for preparation of the antiserum.

**REFERENCES**


