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Molecular Cloning and Expression of the β-Xylosidase Gene (*xylB*) of *Bacillus stearothermophilus* in *Escherichia coli*

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The second β-Xylosidase gene (*xylB*) from *Bacillus stearothermophilus* was isolated from the genomic library, cloned into pBR322, and subsequently transferred into *Escherichia coli* HB101. Six out of 10,000 transformants were selected from the selective LB medium supplemented with *p*-nitrophenyl-α-L-arabinofuranoside (pNPAf) and ampicillin (50 μg/ml) based on their ability to form a yellow ring around the colony. One of the clones was found to harbor the recombinant plasmid with 5.0 kb foreign DNA, which was identical to the α-L-arabinofuranosidase gene (*arfI*) previously cloned in this lab, while the other five had 3.5 kb of the foreign DNA. Southern blotting experiments confirmed that the 3.5 kb insert DNA was from *B. stearothermophilus* chromosomal DNA. A zymogram with 4-methylumbelliferyl-α-L-arabinofuranoside as the enzyme substrate revealed that the cloned gene product was one of the mutiple α-L-arabinofuranosidases produced by *B. stearothermophilus*. Unlike the *arfI* gene product, the product of the gene on the insert DNA (*xylB*) showed an activity not only on pNPAf but also on oNPX suggesting that the cloned gene product could be a bifunctional enzyme having both α-L-arabinofuranosidase and β-xylosidase activities.

Xylan is the second most abundant polysaccharide next to cellulose and accounts for nearly 30% of the dry weight of monocots and hardwoods (24). Unlike cellulose which is composed of β-1,4-linked glucose residues with a nonbranching structure, xylan is composed of a β-1,4-linked xylopyranoside backbone substituted with arabinosyl, acetyl, glucuronosyl, mannosyl, and glucosyl side chains (5, 9). Thus, the complete enzymatic digestion of xylan requires not only the major xylanolytic enzymes such as endo-xylanase and β-xylosidase but also the side-chain removing enzymes such as α-L-arabinofuranosidase, acetyl xylan esterase, α-glucuronidase, mannosidase, and glucosidase (2, 23).

In this lab, a bacterium possessing the xylanolytic enzyme system was isolated from soil and identified as *Bacillus stearothermophilus* (25). The genes for endo-xylanase (7), β-xylosidase (21), acetyl xylan esterase (14), and α-L-arabinofuranosidase (10) were cloned into *E. coli*, and all the cloned gene products were purified and characterized (6, 11, 13, 15, 18). Furthermore, the nucleotide sequence of the endo-xylanase (8) and β-xylosidase (20) genes were determined.

The present work reports the cloning of the second *B. stearothermophilus* β-xylosidase gene into *E. coli* and the characterization of the gene product based on its substrate specificity.

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Key words: α-L-arabinofuranosidase, *Bacillus stearothermophilus*, molecular cloning

MATERIALS AND METHODS

Bacterial Strains and Plasmid

E. coli HB101 (F⁻, *hsdS*20, *recA*13, *ara*14, *proA*2, *lacY*1, *galK*2, *rpsL*20, *xyl*5, *mtl*1, *supE*44) (3) and pBR 322 (26) were used as a host strain and a cloning vector, respectively.

The bacterium producing α-L-arabinofuranosidase was *B. stearothermophilus* isolated from soil and used as a donor strain (25).

Chemicals and Enzymes

Lysozyme, ribonuclease A, oat spelt xylan, 4-methylumbelliferyl-α-L-arabinofuranoside, nitrophenylglycosides were purchased from Sigma Chemical Co. (St. Louis, USA) and rye arabinoxylan was obtained from Megazyme (Sidney, Austrailia).

The DNA labeling and detection kit was obtained from Boeringer Mannheim (Mannheim, Germany). All the restriction enzymes and T4 DNA ligase were from either New England Biolabs (Beverly, MA.) or Promega (Madison, Wis). All other materials used were of analytical grade.

Media and Culture Condition

B. stearothermophilus was cultured in an optimal

medium (25) prepared in this lab and incubated at 45°C.

E. coli was grown in LB medium at 37°C. Ampicillin was added when needed at the final concentration of 50 μg/ml.

Chromosomal and Plasmid DNA Isolation and Transformation

Chromosomal DNA was isolated according to Doi *et al.* (22), and plasmid DNA was purified based on the rapid alkaline lysis method or PEG precipitation method.

Transformation of *E. coli* HB101 with recombinant plasmids was performed according to Simanis *et al.* (12).

Agarose Gel Electrophoresis and Elution

Gel electrophoresis was performed based on the methods of Sambrook *et al.* (17), and DNA elution from the agarose gel was performed using a Gene Clean II kit according to the manufacturer's instructions.

Isolation of the Recombinant Colony

The transformants were spreaded on the LB-ampicillin plate coated with 200 μl of 10 mM *p*-nitrophenyl-α-L-arabinofuranoside solution (pH 6.5, 0.05 M phosphate buffer) and incubated at 37°C for 18 h. Colonies which were able to form yellowish circles around them were isolated.

Southern Blotting

DNA fragments from agarose gel electrophoresis were transferred to Hybond-N^+ membrane according to the capillary blot transfer method, and DNA hybridization was performed based on the methods of Oh *et al.* (21).

DNA labeling and detection were performed according to the dioxygenin-anti-digoxygenin ELISA method (1). The probe DNA used was the digoxygenin-labelled insert DNA of pKMG12 and digoxygenin-labelled λ DNA-*Bst*EII digests.

Enzyme Preparation

The recombinant bacteria were cultured in LB media supplemented with ampicillin at 37°C for 18 h, and *B. stearothermophilus* was grown in the optimal media at 45°C for 22 h. Bacterial cells were obtained by centrifugating the culture broth at 5000 rpm for 10 min, and the supernatant was used as the extracellular enzyme fraction. The intracellular fraction was obtained by washing the cell pellet with sodium phosphate buffer (0.05 M, pH 6.5) followed by sonication for 3 min at 28 kHz. The resultant supernatant after centrifuagation was used as an intracellular fraction.

Enzyme Assay and Protein Measurement

Various kinds of nitophenylglycosides were dissolved in 0.05 M sodium phosphate buffer (pH 6.5) at a final concentraion of 0.01 M. Enzyme solution at 0.1 ml was added to the same amount of the substrate solution and incubated at 45°C for 20 min. Reaction was terminated by adding 2 ml of 0.6 M Na_2CO_3, and the released *p*-nitrophenol was quantitated by reading the absorbance at 405 nm. One unit of enzyme was defined as the amount of enzyme required to release 1 μmol of *p*-nirophenol per min.

Protein concentration was measured according to the methods of Lowry *et al.* (16).

Zymogram

Enzyme fractions were electrophoresed on two identical 6% nondenaturing polyacrylamide gels, and one was stained with Coomassie blue to detect protein bands while the other was washed with 0.05 M sodium phosphate buffer (pH 6.5) followed by overlaying 5 mM 4-umbelliferyl-α-L-arabinofuranoside agarose gel at 45°C. Activity bands were then observed under UV.

RESULTS AND DISCUSSION

Cloning of α-Arabinofuranosidase Gene

B. stearothermophilus chromosomal DNA was partially digested with *Eco*RI, and 3 - 9 kb fragments were obtained. The fragments were then completely digested with the same restriction enzyme and ligated with the *Eco*RI-treated pBR322. The recombinant plasmids were then transformed into *E. coli* HB101. In order to isolate transformed cells containing the α-L-arabinofuranosidase gene, the recombinant cells were plated on the LB agar plate supplemented with *p*-nitrophenol-α-L-arabinofuranoside, and out of 10,000 transformants 6 colonies with a yellowish ring around them were obtained (Fig. 1).

Plasmids of the transformants were purified, and insert DNAs were isolated. Out of 6 transformants, one had 5

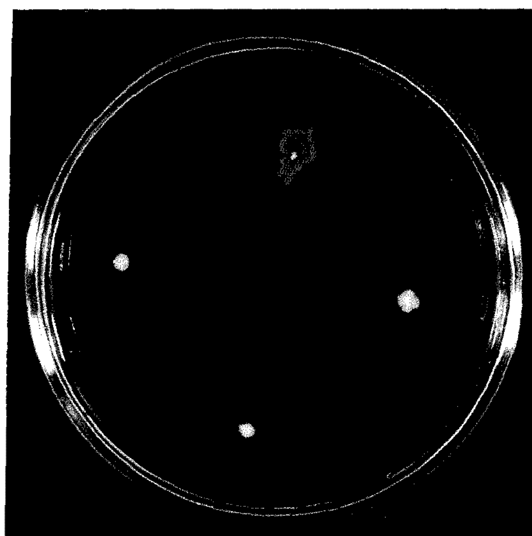

Fig. 1. The expression of the α-L-arabinofuranosidase activity of the *B. stearothermophilus* gene in *E. coli* HB101.
1, *Bacillus stearothermophilus*; 2, *E. coli* HB101/pKMG11; 3, *E. coli* HB101/pKMG12; 4, *E. coli* HB101/pBR322. *E. coli* was incubated on LB-ampicillin plate containing pNPAf at 37°C for 18 h followed by the incubation at 45°C for 18 h for *B. stearothermophilus*.

kb-long insert DNA, while the other five contained 3.5 kb of foreign DNA. The former was found to be just the same recombinant plasmid as pKMG11 (10) which had been previously obtained in this lab (data not shown). The latter 5 recombinant plasmids were supposed to contain another α-arabinofuranoside gene and were named pKMG12.

Restriction Mapping

Insert DNA was isolated from pKMG12 and analyzed by using various restriction enzymes to construct a restriction map. As a result, pKMG12 insert DNA was found to have unique *Ava*I, *Bam*HI and *Hin*dIII site. Based on these results, a restriction map of the insert DNA was made as shown in Fig. 2.

Subcloning was further attempted and 2 kb-long fragment (the *Hin*dIII-*Eco*RI fragment) of the original insert showed enzyme activity though at a lower level. Therefore, it was supposed that the region responsible for the gene regulation might have been lost in the subcloned DNA fragment.

Southern Blotting

In order to confirm that the cloned DNA fragment was from *B. stearothermophilus* genomic DNA, southern hybridization was performed by using the probe DNA obtained from the dig-labeled insert DNA of pKMG12 and *B. stearothermophilus* chromosomal DNA treated with *Eco*RI or *Hin*dIII (Fig. 3). The probe DNA was hybridized with *Eco*RI-treated chromosomal DNA at the same region as the band formed between the probe and *Eco*RI-treated pKMG12. Furthermore, the probe DNA also formed two bands with *Hin*dIII-treated genomic DNA at 2.7 and 1.26 kb. Therefore, it was safely concluded that the insert DNA was from *B. stearothermophilus* chromosomal DNA.

Localization of the Cloned Gene Product

Extracellular, periplasmic, and intracellular fractions were prepared according to the osmotic shock method (19) in order to localize the gene product of the insert DNA. As shown in Table 1, more than 95% of the enzyme produced was found to reside intracellularly.

Substrate Specificity

Various substrates were used to compare two α-L-arabinofuranosidases produced from the recombinant HB 101/pKMG12 and the HB101/pKMG11. The cloned gene product was found to act less well on pNPAf than *arfI*, revealing that the *arfI* gene product was a primary α-L-arabinofuranosidase from *B. stearothermophilus*. But when β-xylosidase activity was tested with pNPX, the cloned gene product showed 10 fold higher activity than that of *arfI*. Interestingly, the cloned gene worked well on oNPX similar to the β-xylosidase from *Butyrivibrio fibrisolvens* (27). Then, we named the cloned gene as *xyl*B and the gene product as β-xylosidase B, in order to distinguish the *xyl*A gene and its product of this *B. stearothermophilus* strain (20). It is therefore assumed

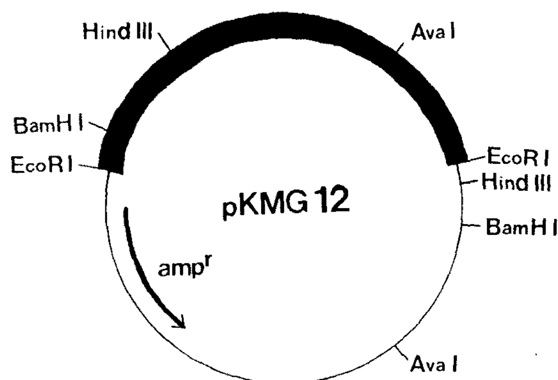

Fig. 2. Restriction map of pKMG12.
The thick line represents cloned insert DNA. The thin line represents pBR322.

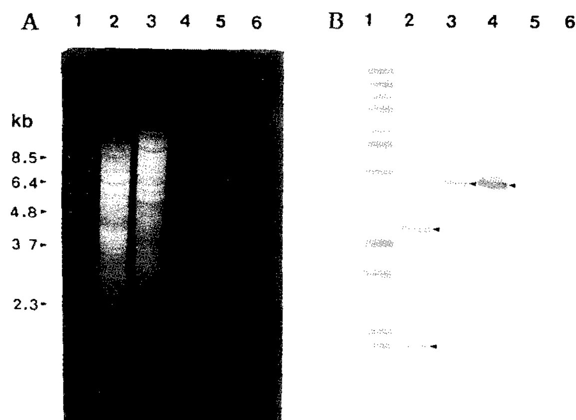

Fig. 3. Southern hybridization of *B. stearothermophilus* genomic DNA with random-primed DNAs synthesized from the insert DNA of pKMG12.
A, 1% agarose gel electrophoretic pattern. B, Southern blot pattern. Lane 1, λ DNA digested with *Bst*EII; lane 2, Chromosomal DNA digested with *Hin*dIII; lane 3, Chromosomal DNA digested with *Eco*RI; lane 4, pKMG12 DNA digested with *Eco*RI; lane 5, pKMG11 DNA digested with *Eco*RI; lane 6, pBR322 DNA digested with *Eco*RI. Arrows indicate the bands which are specifically hybridized with the DNA insert of pKMG12.

Table 1. Cellular distribution of *arfI* and *xyl*B gene products in the recombinant *E. coli* HB101/pKMG11 and HB101/pKMG12 cells, respectively.

	Enzyme activity (unit)	Relative activity (%)
Extracellular	0.11	2.68
Periplasm	0.06	1.41
Intracellular	4.08	95.91

Enzyme activity was measured at 45°C for 20 min using pNPAf as the substrate. One unit of enzyme was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per min.

Table 2. Substrate specificity of *arfI* and *xylB* gene products against nitrophenyl-glycosides.

Substrates	Specific activity (U/mg)		
	pKMG11	pKMG12	pBR322
p-nitrophenyl-α-L-arabinofuranoside	17.9	0.22	ND*
p-nitrophenyl-β-D-xylopyranoside	0.023	0.25	0.016
o-nitrophenyl-β-D-xylopyranoside	0.16	1.90	0.013
p-nitrophenyl-α-L-arabinopyranoside	0.0068	0.0066	0.0063
p-nitrophenyl-α-D-galactopyranoside	ND	ND	ND
o-nitrophenyl-α-D-galactopyranoside	ND	ND	ND
o-nitrophenyl-β-D-galactopyranoside	0.015	0.015	0.013
p-nitrophenyl-α-L-rhamnopyranoside	ND	ND	ND
p-nitrophenyl-α-L-fucopyranoside	ND	ND	ND
p-nitrophenyl-β-L-fucopyranoside	ND	ND	ND
p-nitrophenyl-β-D-fucopyranoside	ND	ND	ND
o-nitrophenyl-β-D-fucopyranoside	ND	ND	ND
p-nitrophenyl-α-D-glucopyranoside	0.0033	0.0030	0.0034
p-nitrophenyl-β-D-glucopyranoside	ND	ND	ND
o-nitrophenyl-β-D-glucopyranoside	ND	ND	ND
p-nitrophenyl-α-D-mannopyranoside	ND	ND	ND
p-nitrophenyl-β-D-mannopyranoside	ND	ND	ND

Specific activity was expressed as mol of *p*-nitrophenol released per min per mg of protein. *ND: Not Detected.

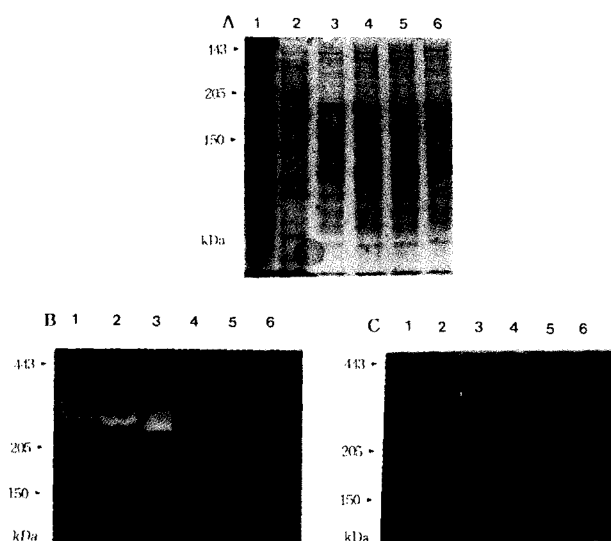

Fig. 4. Zymogram of A-L-arabinofuranosidase and B-xylosidase from *B. stearothermophilus* and recombinant *E. coli* strains.
(A) Protein bands. (B) α-L-arabinofuranosidase activity band. (C) β-xylosidase activity band. Lane 1, Intracellular proteins of *B. stearothermophilus*; lane 2, Extracellular proteins of *B. stearothermophilus*; lane 3, *E. coli* HB101/pKMG11; lane 4, *E. coli* HB101/pKMG12; lane 5, *E. coli* HB101/pBR322; lane 6, *E. coli* HB101/pMG01 (21).

that the *xyl*B gene product could be a bifunctional enzyme having both β-xylosidase and α-L-arabinofuranosidase activities (Table 2).

Zymogram

A substrate such as 4-methylumbelliferyl-α-L-arabinofuranoside was used to compare *xyl*B gene product with that of *B. stearothermophilus* by using a zymogram technique. As shown in Fig. 4, the original bacterium produced more than one α-L-arabinofuranosidase, and one of these bands corresponded to the bifuctional enzyme from the *xyl*B gene product. Furthermore, the result showed that the pKMG12 gene product was an extracelluar enzyme, while it was found intracellularly in the recombinant cell HB101/pKMG12.

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