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Molecular Cloning and Expression of β-Xylosidase Gene from Thermophilic Alkalophilic *Bacillus* sp. K-17 into *Escherichia coli* and *Bacillus subtilis*

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고온, 호알칼리성 *Bacillus*속 K-17 균주의 β-Xylosidase 유전자의 *Escherichia coli* 및 *Bacillus subtilis*에의 클로닝 및 발현

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The chromosomal DNA fragments of thermophilic alkalophilic *Bacillus* sp. K-17, a potent xylan-hydrolyzing bacterium, were ligated to a vector plasmid pBR322 and transformed into *Escherichia coli* HB101. The plasmid pAX278, isolated from a transformant forming yellow color on the LB agar plate containing 1 mM *p*-nitrophenyl- β-xylopyranoside, was found to enable the transformants to produce β-xylosidase. The 5.0 kilobase insert of pAX278 had single sites for *Eco*RI, *Pst*I, *Xba*I, and *Pvu*II, and 2 sites for *Bgl*II. Biotinylated pAX278 was hybridized to 0.9 kb as well as 5.0 kb fragment from *Bacillus* sp. K-17 DNA on nitrocellulose filter. pGX718 was constructed by inserting the 5.0 kb *Hind*III fragment of pGX278 at the *Hind*III site of pGR71, *E. coli* and *B. subtilis* shuttle vector. The enzymatic properties of β-xylosidase from *E. coli* HB101 carrying recombinant plasmid were the same those of β-xylosidase from *Bacillus* sp. K-17.

Xylose is a good fermentable substrate for the production of fuel and chemicals, and is produced by chemical and enzymatic degradation of xylan. Xylan can be degraded to xylose by the synergistic action of xylanase and β-xylosidase. Panbangred *et al* isolated two genes of β-xylosidase I and β-xylosidase II from *Bacillus pumilus* and compared their gene products(1). Thermophilic alkalophilic *Bacillus* sp. K-17, a potent xylan degrader, produces an extracellular β-xylosidase(2). The enzyme was purified and characterized, and its action on the xylan hydrolizate by xylanase was also investigated (2). The β-xylosidase of *Bacillus* sp. K-17 had an optimal pH for reaction at 7.0, and was stable at alkaline pH ranges. The enzyme had an optimal temperature at 45°C, and was stable at 40°C for 10 min, but was completely inactivated by incubation at 60°C for 10 min(2).

We have started to clone the gene to xylan degradation to elucidate gene structure and to develop a hyperproducing strain. Here, we deal with the cloning of β-xylosidase gene from *Bacillus* sp. K-17 and its expression in *E. coli* and *B. subtilis*.

Materials and Methods

Bacterial strains and plasmids

E. coli HB101 (F^-, *hsd*S20, *rec*A13, *ara*14, *pro*A2, *lac*Y1, *gal*K2, *rps*L20, *xy*l5, *mtl*1, *sup*E44, λ) and *B. subtilis* RM-141 (*arg*15, *leu*B8, *his*A1, r^-, m^-) were

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used as the host organisms. Thermophilic alkalophilic Bacillus sp. K-17 reported previously used as a gene source strain (2). pBR322 and pGR71 were used as cloning vector

Media and growth condition

E. coli and *B. subtilis* was grown aerobically at 37°C in LB medium containing 10g of tryptone, 5g of yeast extract and 10g of NaCl in liter of deionized water. Antibiotics were added when appropriate: ampicillin, 50 μg/ml; tetracycline, 15 μg/ml; kanamycin sulfate, 20 μg/ml. LB agar containing ampicllin and 1 mM *p*-nitrophenyl- β-D-xylopyranside (pNPX) was used as a selective medium (LBPX) for transformant producing β-xylosidase. *Bacillus* sp. K-17 was grown as described previously (2).

Recombinant DNA technique

Chromosomal DNA of *Bacillus* sp. K-17 was purified by the method of Saito (3). For plasmid isolation, the method of Birnboim and Doly was employed (4). The chromosomal DNA of *Bacillus* sp. K-17 was partially digested with *Hind*III, and 3 to 10 kilobase (kb) fragments were collected by sucrose gradient centrifugation. The fragments were ligated to the linear pBR322 cutted with *Hind*III and the ligated mixture was used to transform *E. coli* HB101.

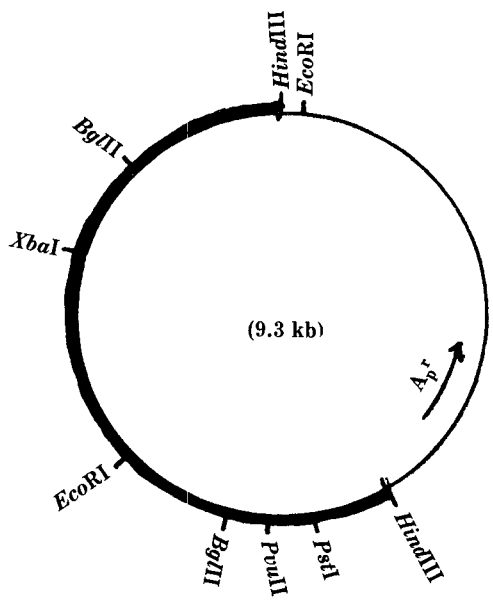

Fig. 1. Restriction map of pAX278 containing β-xylosidase gene.

Transformants containing recombinant plasmid were enriched by the method of Bolivar*et al* (5).

Southern blot analysis

Transfer of DNA fragments from agarose gel to nitrocellulose paper was performed as described by Southern (6) and biotinylation of DNA for hybridization probes by nick translation was performed as described previously (7).

Transformation

Transformation of *E. coli* and *B. subtilis* were done by the method of Cohen (8) and Imanaka (9), respectively.

β-Xylosidase assay and localization

The activity of β-xylosidase was measured as described previously (2). Fractionation was performed by the method of Cornelis *et al* (10).

Results

Cloning of *Bacillus* sp. K-17 β-xylosidase gene into *E. coli*

The chromosomal DNA of *Bacillus* sp. K-17 was digested with *Hind*III and resulting DNA fragments were ligated in pBR322 digested with *Hind*III. The ligated DNA was used to transform the competent cells of *E. coli* HB101. The β-xylosidase producing transformant was detected directly on the plates because the yellow color of *p*-nitrophenol was formed around the colony producing β-xylosidase on an LB agar plate containing 1 mM PNPX. Among the 14,000 Ap^r, Tc^s transformants, one strain forming a yellow color was selected. The recombinatant plasmid pAX278 isolated from this strain was used to retrasform into *E. coli* HB101. All the resulted transformants formed yellow color on the LBPX plate. The pAX278 was digested with restriction endonucleases and the resulting fragments were analyzed by agarose gel electrophoresis.

The physical map of pAX278 showed that pAX278 contained the 5.0 kb chromosomal DNA of *Bacillus* sp. K-17 at the *Hind*III site of pBR322. The 5.0 kb insert of pAX278 had single site for *Eco*RI, *Pst*I, *Xba*I, *Pvu*II, and 2 sites for *Bgl*II (Fig. 1). No cutting site for *Bam*HI, *Sal*I, *Cla*I, *Kpn*I, and *Xho*I was observed.

To confirm that the cloned 5.0 kb fragment was

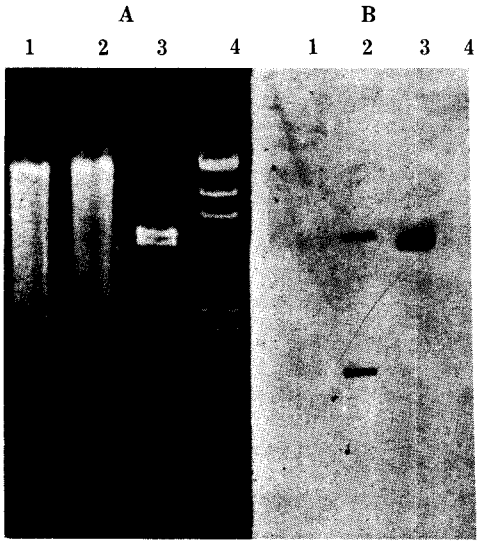

Fig. 2. Homology between the cloned fragment of pAX278 and the chromosomal DNA of *Bacillus* sp. K-17.

A) 1: *Hind*III digest of *E. coli* HB101 chromosomal DNA
2: *Hind*III digest of *Bacillus* sp. chromosomal DNA
3: *Hind*III digest of pAX278
4: λ DNA *Hind*III digest
B) Hybridization analysis of Southern transfer from A

derived from *Bacillus* sp. K-17, we performed Southern blot hybridization using the biotinylated pAX278 as a probe and *Hind*III digested chromosomal DNA of *Bacillus* sp. K-17 as traget. We found that the biotin-labeled pAX278 hybridized to 0.9 as well as 5.0 kb *Hind*III fragment from chromosomal DNA of *Bacillus* sp. K-17 on the nitrocellulose filter (Fig. 2).

Introduction of the cloned β-xylosidase gene into *B. subtilis*

To introduce the cloned β-xylosidase gene into *B. subtilis*, the cloned *Hind*III fragment of pAX278 was inserted into the *Hind*III site of pGR71, shuttle vector between *E. coli* and *B. subtilis*. The 5.0 kb *Hind*III. fragment of pAX278 was electroeluted and ligated in pGR71 digested with *Hind*III. *E. coli* HB101 was transformed with ligation mixture and plated onto the LBPX media.Recombinant plasmid (pGX718) was isolated from yellow color forming kanamycin resistant *E. coli* transformant, and transformed into *B. subtilis* RM141. All kanamycin resistant *B. subtilis* transformants produced yellow color on the LBPX media.

Table 1. Distribution and activity of β-xylosidase in *E. coli* HB101 and *B. subtilis* RM-141 carrying plasmids.

Strains and plasmids	β-Xylosidase activity (mU)		
	Extracellular	Periplasmic	Intracellular
E. coli HB101			
pBR322	0	0	0
pAX278	280	20	1
pGR71	0	0	0
pGX718	251	15	1
B. subtilis RM141			
pGR71	4	–	–
pGX718	870	–	–

Strains were aerobically grown in LB media for 24 hr at 37°C.
–: not determined

β-Xylosidase localization and production

E. coli carrying pAX278 was cultured aerobically in LB broth for 24 hr at 37°C. β-Xylosidase activities in the extracellular, periplasmic and cytoplasmic fractions were assayed. As shown in Table 1, about 90% of the total β-xylosidase activity was found in the medium. *E. coli* HB101 without plasmid or with pBR322 did not produce β-xylosidase activity. *B. subtilis* RM141 containing the plasmid pGX718 produce 3 times as much extracellular β-xylosidase as *E. coli* carrying the same plasmid and about 1.5 times as *Bacillus* sp. K-17.

Some properties of β-xylosidase produced by *E. coli* HB101 carrying pAX278

Stability of the pAX278-encoded enzyme was investigated in buffer solution of various pH values. The mixtures were incubated at 40°C for 10 min. The enzyme was stable from pH 6 to 10. The optimum pH value for enzyme activity was 7.0. The enzyme was heated at various temperature for 30 min, and the residual activity was measured at pH 7.0. The enzyme was stable up to 40°C. The optimal temperature for enzyme activity was 45°C. The enzymatic properties of pAX278 encoded β-xylosidase were the same as those of β-xylosidase produced by *Bacillus* sp. K-17.

Discussion

The thermophilic alkalophilic *Bacillus* sp. K-17 has the ability to degrade xylan. The strain has an optimal pH for growth at 10.3, which makes it interesting for possible use in fermentation processes. The β-xylosidase of this strain is different from that of other *Bacillus* species in excretion. With objective of developing microorganisms which could degrade more efficiently than naturally occurring organisms, we started to clone the genes responsible for xylan degradation. For this reason we have constructed a *Bacillus* sp. K-17 gene bank in *E. coli,* and the colonies were screened for their capacity to produce β-xylosidase. *E. coli* HB101 carrying the plasmid pAX278 could produce β-xylosidase and secrete the enzyme into medium. Protein excretion through the outer membrane of *E. coli* cells was recently reported with a cloned penicillinase of an alkalophilic *Bacillus* strain (11) and a cloned protease of a *Serratia marcescens* (12). The excretion of a cloned penicillinase of an alkalophilic *Bacillus* was elucidated to be caused by the insertional activation of *kil* gene (13). The specific excretion of a cloned protease of *Serratia* seems to be directed by characteristic sequence within the protease gene (12). The mechanism for the excretion of cloned β-xylosidase will be elucidated after carrying out further research. Hybridization experiment showed that the biotinylated pAX278 hybridized to 0.9 kb as well as 5.0 kb fragment of *Bacillus* sp. K-17 DNA. The possible gene product of this 0.9 kb fragment possessing homology with 5.0 kb fragment coding for the β-xylosidase may be one of the followings: 1) another β-xylosidase excluded in the purification step. 2) inactive xylanase II; since xylose was produced from xylan by xylanase II but not by xylanase I, the xylanase II might undergo the same molecular evolutions as the β-xylosidase. 3) β-glucosidase; this enzyme carry out very similar function with β-xylosidase. 4) The gene product of unknown gene existed in the 5.0 kb fragment since the size of the fragment cloned in pAX278 was large enough to contain more than one gene. To elucidate this point, further research will be required.

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요 약

고온, 호알칼리성 *Bacillus*속 K-17 균주에서 β-xylosidase 유전자를 pBR 322를 벡터로 이용하여 클로닝시켰다. *p*-Nitrophenyl-β-xylopyranoside를 함유하는 LB 한천배지에서 노란색을 형성하는 대장균 형질전환주에서 재조합 플라스미드 pAX 278을 분리하였으며, 본 pAX 278은 pBR 322와 고온, 호알칼리성 *Bacillus* K-17 균주 염색체 DNA의 5.0 kb *Hind*III 절편으로 구성되어 있었다. Biotin으로 표식된 pAX 278을 probe로 하여 상동성 시험을 하여 본 결과, pAX 278에 존재하는 5.0 kb *Hind*III 절편은 *Bacillus* K-17 균주의 염색체 DNA *Hind*III 절편 중에서 5.0 kb 뿐만 아니라 0.9 kb 절편과도 상동성이 있었다. pAX 278의 5.0 kb 절편을 pGR 71에 연결시켜 *B. subtilis*에서도 발현시켰다. pAX 278을 가지는 *E. coli* 균주가 생성하는 β-xylosidase는 균체외에 존재하였으며 그 효소학적 성질은 *Bacillus*속 K-17의 β-xylosidase와 동일하였다.

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