Kor. J. Appl. Microbiol. Bioeng.
Vol. 17, No. 3, 178-182 (1989)

Molecular Cloning and Expression of a Xylanase Gene from Thermophilic Alkalophilic *Bacillus* sp. K-17 in *Escherichia coli*

Sung, Nack-Kie[1*], Hyo-Kon Chun[1], Ki-Hwan Shim[1], In-Soo Kang[1] and Teruhiko Akiba[2]

Department of Food Science and Technology, Gyeong Sang National University, Chinju 660-701, Korea
Microbial Ecology Lab., RIKEN, Wako, Saitama 352-01, Japan

高溫, 好알칼리성 *Bacillus* sp. K-17 Xylanase 遺傳子의 *Escherichia coli* 에의 클로닝 및 發現

成洛癸[1*]・全孝坤[1]・沈奇煥[1]・姜仁秀[1]・秋葉晄彥[2]

[1]慶尚大学校 食品工学科 [2]日本理化学研究所 微生物生態学研究室

A gene coding for a xylanase of thermophilic alkalophilic *Bacillus* sp. K-17 was cloned in *Escherichia coli* C600 with pBR322. Plasmid pAX113 was isolated from a transformant producing xylanase, and the xylanase gene was located in a 4.3 Kb *Hind*III fragment. Biotinylated pAX113 hybridized to a 4.3 Kb *Hind*III fragment from chromosomal DNA of thermophilic alkalophilic *Bacillus* sp. K-17. The xylanase activity was observed in the extracellular curture fluid of *E. coli* carrying pAX113. The pAX113-encoded xylanase had the same enzymatic properties as those of xylanase I produced by thermophilic alkalophilic *Bacillus* sp. K-17.

Xylan is one of the main components in plant biomass and accounts for nearly 30% of total sugar in some plants(1). Enzymatic degradation of xylan to xylose is thus important for the use of agricultural wastes. Xylan can be degraded to xylose by the sequential reaction of xylanase and β-xylosidase(2). Recently, the structural genes for the xylanase of several bacterial strains were cloned with plasmid vectors in *Escherichia coli*(3-7).

In our laboratory, many thermophilic alkalophilic bacteria which produced xylan degrading enzyme were isolated. One of them, thermophilic alkalophilic *Bacillus* sp. K-17 produced two types of xylanases: xylanase I, with a pH optimum of 7.0, and xylanase II, with a very broad pH-activity curve (pH 5-11)(8). We have started to clone the genes for xylan degradation of thermophilic alkalophilic *Bacillus* sp. K-17 to obtain hyperproducer of xylanase and to analize their genetic information for the multicomponents of the xylanases.

This paper deals with the cloning and expression of the xylanase I gene of thermophilic alkalophilic *Bacillus* sp. K-17 in *E. coli* C600 with pBR322; some properties of the plasmid-borne xylanase are also discussed.

Materials and Methods

Bacterial strains, plasmid and media

Thermophilic alkalophilic *Bacillus* sp. K-17 was grown aerobically with continuous shaking at 48 °C in a medium containing 5g xylose, 5g polypeptone, 5g yeast extract, 1g K_2HPO_4, 0.2g $MgSO_4 \cdot 7H_2O$ and 10g Na_2CO_3 (autoclaved separately) per litre of distilled water. *E. coli* C600 (F *thi*-1 *thr*-1 *leu*B6 *lac*Y1 *ton*A21 *sup*E44) and the plasmid pBR322

Key words: Alkalophilic *Bacillus* K-17, xylanase I gene, cloning
* Corresponding author

were used as the host strain and the vector plasmid for this experiment, respectively. *E. coli* strain was grown aerobically in LB medium (10g tryptone, 5g yeast extract and 10g NaCl per litre of distilled water) at 37 °C with shaking. When required, antibiotics (Sigma) were added to the medium at a final concentration (μg/m*l*) of 20 and 100 for tetracycline and ampicillin, respectively. For plasmid amplification, M9 medium was supplemented with chloroamphenicol at a final concentration of 170 μg/m*l*. Xylan plates(LBX) for screening of the xylanase-producing colonies contained 0.5% xylan from larchwood in LB medium.

Isolation of DNA

Bacterial chromosomal DNA was isolated by the method of Saito and Miura(9), Plasmid DNAs were prepared according to Bolivar *et al.*(10). The small-scale extraction of plasmids was done by the method of Birnboim and Doly(11).

Construction of recombinant plasmids

DNAs were digested with *Hind*III at 37 °C for 1hr (plasmid DNA) or for 14 hr (chromosomal DNA). After the digestion, 1 μg of plasmid and 3 μg of chromosomal DNA were mixed and ligated with T4 DNA ligase for 12 hr at 14 °C. This ligated DNA mixture was used to transform *E. coli* C600 (11).

Nick-translation and hybridization

Plasmid pAX113 was labelled by nick translation using *E. coli* DNA polymerase I in the presense of biotinylated dATP (BRL) as described by Takahashi *et al.*(12). The genomic hybridization analysis was done as described by Southern (13).

Xylanase assay

Enzyme in 50 μ*l* of 0.05M phosphate buffer (pH 6.5) was mixed with 0.5 m*l* of xylan in the same buffer. The mixture was incubated for 10 min at 60°, and then reducing sugar was determined by the method of Sumner and Somers (14). One unit of enzyme activity is defined as the amount of enzyme which liberates 1 mg of reducing sugar expressed as xylose per minute under above conditions.

Analysis of xylanase distribution

Fractionation of extracellular, periplasmic and intracellular xylanase was done by the method of Kato *et al.*(15).

Xylanase purification

All purification steps were carried out at 4 °C. The buffer used throughout the purification was 0.05M phosphate buffer. The culture broth was centrifuged at 8,000 rpm for 15 min to remove cells. To the supernatant, ammonium sulfate was added to 30% saturation. After standing for one day, the resulting precipitate was collected by centrifugation at 10,000 rpm, dissolved in the buffer and dialyzed for two days against the same buffer. The resulting insoluble materials were removed by centrifugation. The dialyzed enzyme solution was charged onto a DEAE-Sephadex A-50 column (2.2 × 80 cm) equilibrated with the buffer. The column was washed with the same buffer until 1 litre of elute had been passed. The remaining fraction was eluted with a linear gradient of 0 to 0.5M NaCl in the buffer. The pooled active fraction was applied to a CM-Sephadex C-50 column (2.2 × 60 cm) equilibrated with the buffer. The column was washed with a linear gradient of 0 to 0.3M NaCl in the buffer. The active fraction of CM-Sephadex step was purified by gel filtration on Sephadex G-100 column (2.0 × 80 cm).

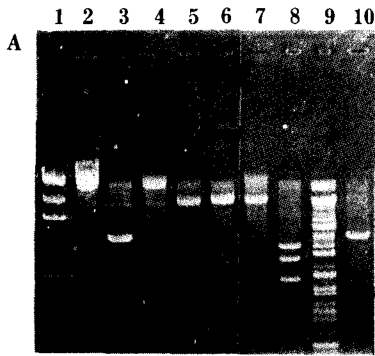
Gel electrophoresis

DNA molecules were separated on a 1% agarose gel in the running buffer, 90 mM Tris, 90 mM-boric acid, 1 mM-Na_2EDTA (pH 8.0) and DNA bands were visualized by staining with ethidium bromide.

Results

Cloning of the xylanase gene from *Bacillus* sp. K-17 into *E. coli*

E. coli C600 was transformed with the ligated mixture described in Materials and Methods with an efficiency of 10^5 ampicillin resistant transformants per μg total DNA. About 10% of the isolated colonies were tetracycline sensitive, indicating the possible insertion of foreign DNA fragments. Xylanase activity was detected on the plates by the appearance of a clear zone around a xylanase-producing colony on LBX plate supplemented with ampicillin. One among 8,000 ampicillin resistant and tetracycline sensitive transformants was found



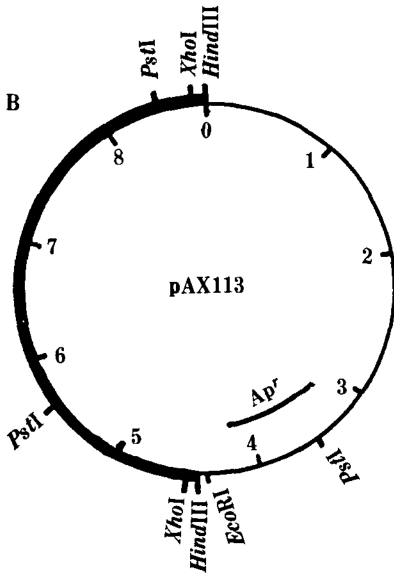


Fig. 1. Agarose gel electrophoretic pattern of pAX113 digested with restriction enzyme (A) and restriction map of pAX113 (B).

A: **lane 1**: λDNA *Hind*III digest, lane 2: intact pAX113, lane 3: pAX113 *Hind*III digest, lane 4: pAX113 *Bgl*II digest, lane 5: pAX113 *Bam*HI digest, lane 6: pAX113 *Eco*RI digest, lane 7: pAX113 *Sal*I digest, lane 8: pAX113 *Pst*I digest, lane 9: λ DNA *Hind* III + *Eco*RI digest, lane 10: pAX113 *Xho*I digest

to produce a clear halo on LBX plate. This colony harboured the chimeric plasmid, which consisted of pBR 322 and a 4.3 Kb fragment inserted in the *Hind* III site. This hybrid plasmid (pAX113) was isolated and reintroduced into *E.. coli* C600 to verify the presence of the xylanase gene on the plasmid. All the transformants obtained showed clear zone around colonies on LBX plate supplemented with ampicillin.

Partial characterization of plasmid pAX113

The 4.3 Kb *Hind*III insert of pAX113 had two

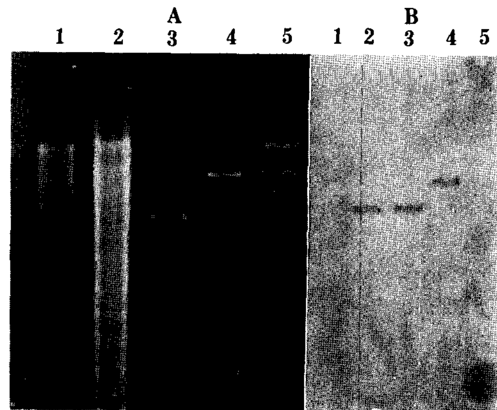


Fig. 2. Southern hybridization of the *Hind*III digested *Bacillus* sp. K-17 chromosomal DNA and the *Hind*III digest of pAX113.

biotinylated pAX113 was used as a probe

A, lane 1: *Hind*III digest of *E. coli* C600 chromosomal DNA

lane 2: *Hind*III digest of *Bacillus* sp. K-17 chromosomal DNA

lane 3: *Hind*III digest of pAX113

lane 4: *Eco*RI digest of pAX113

lane 5: λDNA digested with *Hind*III

B, Hybridization pattern of A

Table 1. Distribution of xylanase activity (U)

Strains	Extracellular	Periplasmic	Intracellular
E. coli C600 (pBR322)	0.00	0.00	0.00
E. coli C600 (pAX113)	0.92	0.28	0.08
Bacillus sp. K-17	2.35	–	0.22

cleavage sites for *Pst*I and *Xho*I. No *Bam*HI, *Bgl*II, *Eco*RI or *Sal*I sites were found (Fig. 1). In order to confirm the origin of the 4.3 Kb *Hind*III fragment in pAX113, a genomic hybridization experiment was done (Fig. 2). Biotinylated pAX113 hybridized to *Hind*III digest and *Eco*RI digest of unbiotinylated pAX113 and also to a 4.3 Kb *Hind*III fragment from chromosomal DNA of *Bacillus* sp. K-17. No sequences complementary to pAX113 were detected in *E. coli* DNA fragments.

Production of xylanase by *E. coli* C600 carrying pAX113

E. coli C600 carrying pAX113 was grown aero-

bically in LB broth for 20 hr at 37 °C. The extracellular, periplasmic and intracellular xylanase activities were assayed. A significant amount of the enzyme activity was found in the extracellular fraction. As shown in Table 1, essentially no enzyme activities were detected in the culture broth of *E. coli* C600. The extracellular xylanase activity was not due to the cell rupture because β-galactosidase, typical cellular enzyme, was not found in the extracellular fraction. The production of xylanase in *E. coli* was constitutive and no effect of supplement of xylan was observed.

Comparison of the xylanase from *Bacillus* sp. K-17 and the xylanase encoded by pAX113

Adsorption on ion exchanger: The enzyme solution obtained from *E. coli* carrying pAX113 was applied on the DEAE-Sephadex A-50 column and the column was eluted as described in Materials and Methods. The xylanase was not adsorbed on this column. The active fraction of the elute was applied on CM-Sephadex C-50 column. The enzyme was adsorbed on this column and eluted with 0.05M phosphate buffer containing NaCl. Xylanase I from *Bacillus* sp. K-17 was not adsorbed on DEAE Sephadex but adsorbed on CM-Sephadex, whereas xylanase II was adsorbed on DEAE-Sephadex.

Molecular weight: After ion-exchange chromatography, the enzyme solution was further purified by gel filtration on Sephadex G-100. The molecular weight of the purified xylanase was estimated as 23,000 by SDS-polyacrylamide gel electrophoresis. The molecular weights of xylanase I and xylanase II was 23,000 and 47,000, respectively(8).

Effect of pH: The pH was adjusted with MacIlvain buffer (pH 3-8) or 0.05M-glycine/NaOH buffer (pH 9-13). No significant difference was observed between the xylanase of *E. coli* C600 carrying pAX113 and the xylanase I of *Bacillus* sp. K-17 (pH optimum 6.5).

Effect of temperature: Xylanase I, II and pAX 113-encoded xylanase were most active 60, 65 and 60 °C, respectively.

Discussion

In previous study(8), we showed that thermophilic alkalophilic *Bacillus* sp. K-17 produced two types of xylanases: xylanase I with a pH optimum of 6.5 and a molecular weight of approximately 23,000, and xylanase II with a pH optimum of 7.0 and a molecular weight of approximately 47,000 (8). In this experiment we cloned a xylanase gene from *Bacillus* sp. K-17 into *E. coli* C600 using pBR322 as vector plasmid and obtained a clone showing a clear zone on LBX plate. *E. coli* C600 carrying pAX113, containing a 4.3 Kb *Hind*III fragment of *Bacillus* sp. K-17 DNA, produced a xylanase with enzymatic properties that were similar to the xylanase I from *Bacillus* sp. K-17. Genomic hybridization showed that no homology was observed between the 4.3 Kb *Hind*III fragment of pAX113 and genomic DNA fragment except the 4.3 kb *Hind*III fragment of *Bacillus* sp. K-17 DNA. Thus it seems that the xylanases activities in *Bacillus* sp. K-17 are encoded by different genes that possess no homology.

The xylanases of *Bacillus* sp. K-17 are released into the extracellular culture fluid. The major of the xylanase activity from *E. coli* C600 carrying pAX 113 was also detected in the extracellular culture fluid. It has been demonstrated that the xylanase synthesized by *B. subtilis* harbouring the cloned gene from *B. pumilus* was secreted into the medium, but xylanase synthesized in *E. coli* bearing the same gene was retained in the cytoplasm(1). In contrast, a xylanase gene from an alkalophilic *Bacillus* coded for an enzyme which was mostly (82%) secreted into the extracellular medium by *E. coli* (5). Other diret excretion of the cloned gene products through the outer menbrane of *E. coli* was also reported: a cloned penicillinase of an alkalophilic *Bacillus* sp.(15) and a cloned protease of a *Serratia marcescens*(16). It seemed that the excretion of the cloned penicillinase from alkalophilic *Bacillus* in *E. coli* was due to the insertional activation of *kil* gene, which was originally found in pMB9(17). The excretion of the cloned xylanase from alkalophilic *Bacillus* and the cloned protease from *Serratia maecescens* in *E. coli* was due to the characteristic sequences within the enzyme genes. The excretion of the cloned xylanase from our strain in *E. coli* may be caused by the latter mechanism since the xylanase gene was cloned with pBR 322 instead of pMB9.

Nucleotide sequence analysis of the xylanase I gene and cloning of the xylanase II gene will be required for the clear explanation on the excretion of

Bacillus sp. K-17 xylanase in E. coli.

요 약

고온, 호알칼리성 *Bacillus* K-17 균주에서 한가지 xylanase 유전자를 pBR 322를 벡터로 이용하여 클로닝시켰다. Xylan을 함유하는 LB 한천배지에서 분해환을 형성하는 대장균 형질전환주에서 재조합 플라스미드 pAX 113을 분리하였으며, 본 pAX 113은 pBR 322와 고온, 호알칼리성 *Bacillus* K-17 균주 염색체 DNA의 4.3 Kb *Hind* III 절편으로 구성되어 있었다. Biotin으로 표식된 pAX 113을 probe로 하여 상동성시험을 하여 본 결과, pAX 113에 존재하는 4.3 Kb *Hind* III 절편은 고온, 호알칼리성 *Bacillus* K-17 균주 유래임을 확인하였다. pAX 113을 가지는 *E. coli* 균주가 생성하는 xylanase는 균체 외에 존재하였으며 그 효소학적 성질은 고온, 호알칼리성 *Bacillus* K-17 균주의 xylanase I과 II중에서 xylanase I과 동일하였다.

Acknowledgement

This work was supported by grants-in-aids for genetic engineering research of 1985 from the Minister of Education.

References

1. Panbangred, W., E. Fukusaki, E.C. Epifanio, A, Shinmyo and H. Okada: *Appl. Microbiol. Biotechnol.*, **22**, 259 (1985).
2. Sung, N.K., I.S. Kang, H.K. Chun, T. Akiba and K. Horikoshi: *Kor. J. Appl. Microbiol. Bioeng.*, **15**, 267 (1987).
3. Bernier, R.J., H. Driguez and M. Desrochers: *Gene,* **26**, 59 (1983).
4. Panbangred, W., T. Kondo, S. Negoro, A. Shinmyo and H. Okada: *Mol. Gen. Genet.*, **192**, 335 (1983).
5. Honda, H., T. Kudo and K. Horikoshi: *J. Bacteriol.*, **161**, 784 (1985).
6. Kudo, T., A. Ohkoshi and K. Horikoshi: *J. Gen. Microbiol.* **131**, 2825 (1985).
7. Yang, R., C. Mackenzie, D. Bilous and V. Seligy: *Appl. Environ. Microbiol.*, **54**, 1023 (1988).
8. Kang, I.S., N.K. Sung, H.K. Chun, T. Akiba and K. Horikoshi: *Kor. J. Appl. Microbiol. Bioeng.*, **14**, 447 (1986).
9. Saito, H. and K.I. Miura: *Biochem. Biophys. Acta,* **72**, 619 (1963).
10. Bolivar, F., R.L. Rodriguez, P.J. Greene, M.C. Beltlach, H.L. Heynecker and H.W. Boyer: *Gene,* **2**, 95 (1977).
11. Birnboim, H.C. and J. Doly: *Nucleic Acids Res.*, **7**, 151 (1979).
12. Takahashi, T., S. Yanoma, I. Akimoto, T. Shigematsu and K. Okada: *Medical Technol.*, **13**, 999 (1985).
13. Southern, E.M.: *J. Mol. Biol.*, **98**, 503 (1975).
14. Sumner, J.R. and G.F. Somers: Laboratory Experiments in Biological Chemistry, Academic Press, New York, 34 (1944).
15. Kato, C., T. Kudo, K. Watanabe and K. Horikoshi: *Eur. J. Appl. Microbiol. Biotech.*, **18**, 339 (1983).
16. Yanagida, N., T. Uozumi and T. Beppu: *J. Bacteriol.*, **168**, 937 (1986).
17. Kato, C., T. Kobayashi, T. Kudo and K. Horikoshi: *FEMS Microbiol. Lett.*, **36**, 31 (1986).

(Received March 4, 1989)