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Molecular Cloning of β-Galactosidase from *Bacillus subtilis* HP-4

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A gene coding for a β-galactosidase of *Bacillus subtilis* HP-4 was cloned in *E. coli* JM109 by inserting *HindIII* digested fragment of *B. subtilis* HP-4 chromosomal DNA into the site of pBR322 and selecting recombinant transformant showing blue color on X-gal plate. The recombinant plasmid, named pBG109, was found to contain the 1.4 Kbp *HindIII* fragment originated from *B. subtilis* HP-4 chromosomal DNA by Southern hybridization. The cloned gene was stably maintained and expressed in *E. coli* JM109 and the pBG109 encoded β-galactosidase had the same enzymatic properties as those of β-galactosidase produced by *B. subtilis* HP-4.

β-Galactosidase (EC. 3.2.1.23) catalyzes the spilitting of lactose to glucose and galactose and is important for the treatment of milk for milk-intolerlant people who cannot digest lactose. Above 50℃, most microorganisms except thermophilic bacteria cannot grow, and these temperature operations may protect against bacterial development of the treated dairy products and the enzyme inactivation due to contaminants. Therefore, thermophilic β-galactosidases have attracted increasing interest because of their industrial importance. Extensive enzymatic and genetic studies have been reported on the β-galactosidase of *Escherichia coli* (1, 4, 7, 12) but there is little information on the study for β-galactosidase of *Bacillus* except for *B. stearothermophilus*. Hirata *et al.* (6) cloned the two β-galactosidase structural genes (bga A and bga B) from *B. stearothermophilus*, a mesophilic bacterium, into *E. coli* recently. We have isolated *B. subtilis* HP-4 which a *B. subtilis* HP-4 which synthesize thermophilic β-galactosidase at 50℃ constitutively, and studied on the immobilized enzyme with regard to the possibility of an industrial application (8). In this study, the gene of β-galactosidase from *B. subtilis* HP-4 was cloned and expressed in *E. coli* and the properties of enzyme by *E. coli* transformant habouring chimeric plasmid were characterized.

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MATERIALS AND METHODS

Bacterial Strains and Plasmid

Bacillus subtilis HP-4 isolated from soil was used for cloning of β-galactosidase gene from a gene library constructed. *E. coli* JM109 (relA1, endA1, GyrA96, thi, hsdR 17, supE44 (lac-proAB), F′ (traD36, $proA^+$, $proB^+$, $lacI^q$, lacZ M15) was used as a host in the cloning experiments and the plasmid pBR322 (Amp^r, Tet^r) was used as a vector. *B. subtilis* HP-4 was grown on NB medium containing meat extract (10 g), peptone (10 g) and NaCl (5 g) per liter (pH 7.0) at 50℃ on a reciprocal shaker. *E. coli* was grown on LB medium containing tryptone (10 g), yeast extract (5 g) and NaCl (10 g) per liter (pH 7.5) at 30℃. Transformants were selected on LB agar containing 50 μg/*ml* ampicillin, 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 20 μg/*ml* X-gal.

DNA Cloning

B. subtilis HP-4 chromosomal DNA was prepared by spool method (16). Plasmid DNA was isolated by the method of Birnboim and Doly (3). The chromosomal DNA and pBR322 vector were digested with *HindIII* and the mixture was ligated in buffer containing 50 mM of Tris-HCl (pH 7.6), 10 mM of $MgCl_2$, 20 mM of dithiothreitol, and 0.1 unit of T_4 DNA ligase at 14℃ for 24 h. The ligated DNA was transformed into *E. coli* JM109 by the method of Kushner (10) and ampicillin-resistant transformants with blue color were selected on LB plate

containing X-gal.

Production and Localization of β-Galactosidase in *B. subtilis* HP-4 and Recombinant *E. coli*

For the preparation of crude enzymes of *B. subtilis* HP-4 and recombinant *E. coli*, cells grown on 50 m*l* of LB medium to a density 10^9 cells per m*l* were harvested by centrifugation, washed with 100 mM sodium phosphate buffer (pH 7.0) and suspended in small amount of the same buffer. The cell suspension was sonicated for 3 min at 20 KHz on ice and the supernatant obtained by centrifugation at 20,000×g for 10 min was used as a crude enzyme. To investigate a localization of β-galactosidase in recombinant, osmotic shock was done as described by Neu and Heppel (15). β-galactosidase induction of *B. subtilis* HP-4 and *E. coli* recombinant was tested as described by Miller (13). *B. subtilis* HP-4 cultured in NB medium was inoculated into NB medium containing 0.5% lactose or 10 mM IPTG as inducer. *E. coli* recombinant also cultured in minimal medium (K_2HPO_4 of 10.5 g, KH_2PO_4 of 4.5 g, $(NH_4)_2SO_4$ of 1.0 g and sodium citrate·$2H_2O$ of 0.5 g per liter) supplimented with B_1, $MgSO_4$ and glycerol (0.5%) at 37℃ overnight and diluted with the same medium containing above inducers. Organisms were subjected to partial disruption by addition of one drop of toluene with a pasteur pipette and the intracellular β-galactosidases were assayed with o-nitrophenol-β-D-galactopyranoside (ONPG).

Nick Translation and Hybridization

A 1.4 Kbp *Hin*dIII fragment of pBG109 was labeled by nick translation to a specific activity of 2×10^7 cpm/μg with [α-^{32}P]dCTP. The labeled DNA was used for hybridization at 42℃ with blotted DNA fragments on a nitrocellulose filter by the procedure of Southern (17).

Restriction Mapping and DNA Electrophoresis

Restriction mapping of insert DNA was performed by the method of Ausubel (2). Size of restricted DNA fragments were analyzed by 0.8% agarose gel electrophoresis using 89 mM Tris-borate buffer (pH 8.3).

β-Galactosidase Assay

β-Galactosidase activities were determined by the modified method of Miller (13). One unit of the enzyme activity was defined as the amount of enzyme hydrolysing 1 μmole of ONPG per min at 50℃.

RESULTS

Cloning of β-Galactosidase Gene of *B.subtilis* HP-4 in *E. coli*

A gene library of *B. subtilis* HP-4 DNA was constructed in *E. coli* JM109 and a positive clone, *E. coli*/pBG 109, showing blue color on LB/X-gal plate was selected. Recombinant plasmid DNA, named pBG109, was isolated and reintroduced into the original *E. coli* JM109 to verify the presence of β-galactosidase gene on the plasmid. It was confirmed that the chimeric plasmid contained the gene coding β-galactosidase from the enzyme assay.

Characterization of the Recombinant Plasmid

The recombinant plasmid, pBG109, was digested with *Hin*dIII and a 1.4 Kbp insert was confirmed by agarose gel electrophoresis. For mapping the insert DNA, plasmid DNA was digested with *Eco*RI, *Eco*RV, *Pst*I, *Pvu*II, *Bam*HI, and *Cla*I. It was confirmed that the insert had the only recognition site for *Pst*I and the others were not found from the electrophoretic analysis (Fig. 1). Southern hybridization demonstrated that the 1.4 Kbp insert of pBG109 was originated from *B. subtilis* HP-4 (Fig. 2). The stability of recombinant plasmid by replica plating test was genetically quite stable during the course of 30 successive subcultures. Spontaneous loss of the recombinant was not observed.

Production and Properties of the β-Galactosidase in Recombinant

Time course of growth and β-galactosidase production by the *B. subtilis* HP-4 and *E. coli* recombinant were determined (Fig. 3). Both strains showed high β-galactosidase activity after the late logarithmatic phase of growth and about 90% of the total enzyme activity was detected in the cellular fraction (Tabel 1). No significant difference was observed between the β-galactosidase of *B. subtilis* and that of *E. coli* recombinant in the temperature optimum of 50℃ and pH optimum of 6.5 (Fig. 4 and Fig. 5).

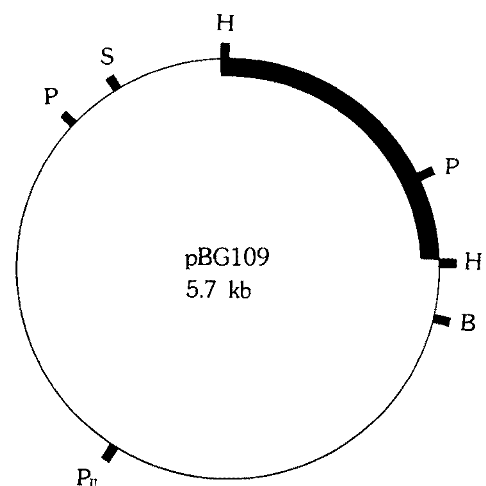

Fig. 1. Restriction map of recombiant plasmid pBG109. The thick line indicates the insert containing β-galactosidase gene. Restriction sties: H; *Hin*dIII, B; *Bam*HI, P; *Pst*I, P_{II}; *Pvu*II

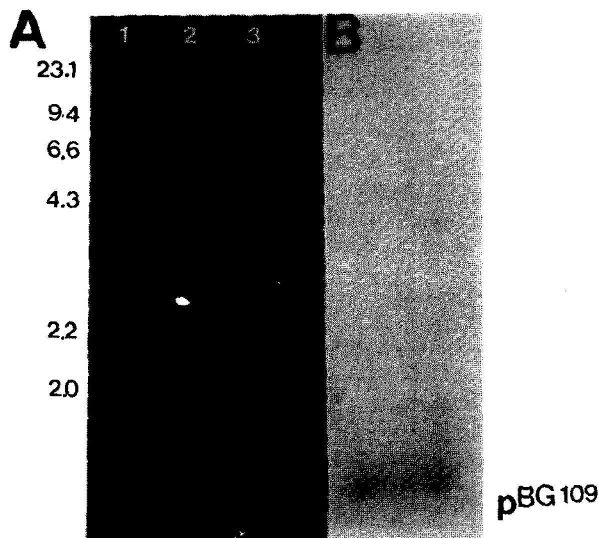

Fig. 2. Southern hybridization of the *B. subtilis* HP-4 chromosomal DNA digested with *Hind*III and pBG 109 containing β-galactosidase gene probe.
Lanes, A: 1; λ DNA as size marker, 2; *B. subtilis* HP-4 chromosomal DNA digested with *Hind*III, 3; pBG109 DNA digested with *Hind*III. B: Hybridization pattern of *B. subtilis* HP-4 chromosomal DNA digested with *Hind*III.

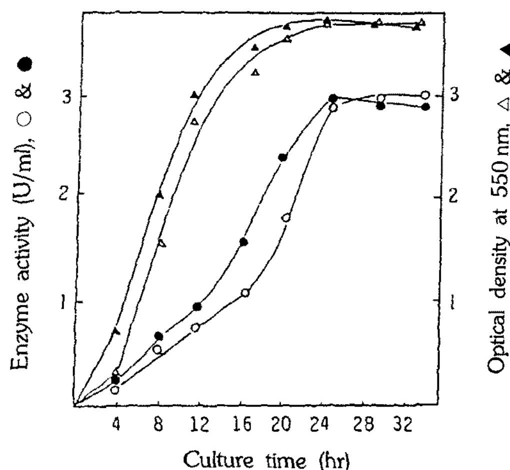

Fig. 3. Growth and β-galactosidase production by pBG 109/*E. coli* and *B. subtilis* HP-4.
▲–▲, △–△; Growth of pBG109/*E. coli* and *B. subtilis* HP-4, respectively.
●–●, ○–○; Intracellular enzyme activity of pBG109/*E. coli* and *B. subtilis* HP-4, respectively.

Both enzyme showed to be stable at temperature below 55℃ and retained 70% of their original activity even on heating at 60℃ for 30 min. The optimum temperature also was shown to be lower than those of *B. stearothermophilus* (4) or *B. acidocaldarius* (9). Fig. 6 showed that *B. subtilis* HP-4 and *E. coli*/pBG109 synthesized β-galactosidase constitutively regardless of the presence

Table 1. Location of the β-galactosidase produced by pBG109/*E. coli* and *B. subtilis* HP-4

Strain	% of activity of β-galactosidase		
	Extracellular	Periplasmic	Cellular
pBG109/*E. coli*	5	5	90
B. subtilis HP-4	5		95

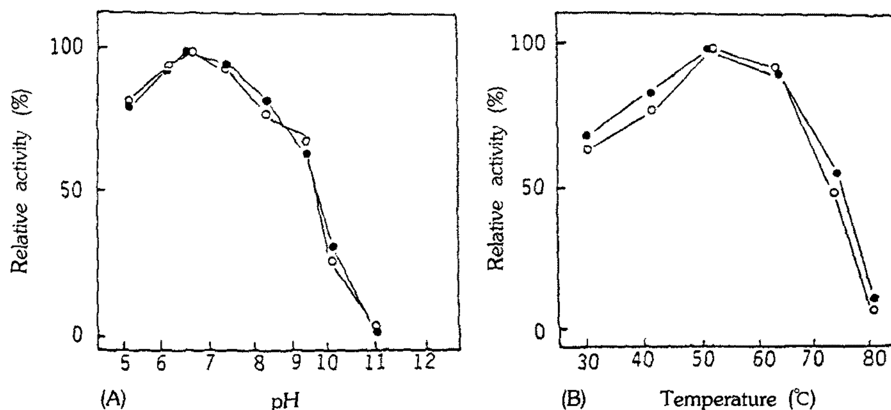

Fig. 4. Effects of pH (A) and temperature (B) on the β-galactosidese activity of pBG109/*E. coli* (●) and *B. subtilis* HP-4 (○).
Crude enzyme and ONPG were incubated at different reaction pH (5.0~12.0) and temperature (30~80℃) and then examined with ONPG as substrate.

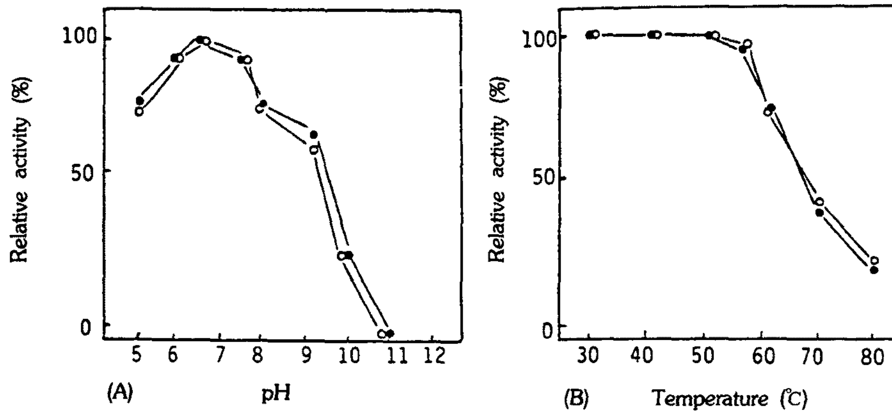

Fig. 5. Stability of pH (A) and temperature (B) on the β-galactosidase activity of pBG109/*E. coli* (●) and *B. subtilis* HP-4 (○).

Crude enzymes were held at different pH (5.0~12.0) and temperature (30~80℃) for 30 min, then cooled and assayed with ONPG as substrate.

pH preparation: McIlvaine buffer (0.1 M, pH 3.0~6.0), Sodium phosphate buffer (0.1 M, pH 7.0~8.0), Glycine-NaOH buffer (0.2 M, pH 9.0~12.0).

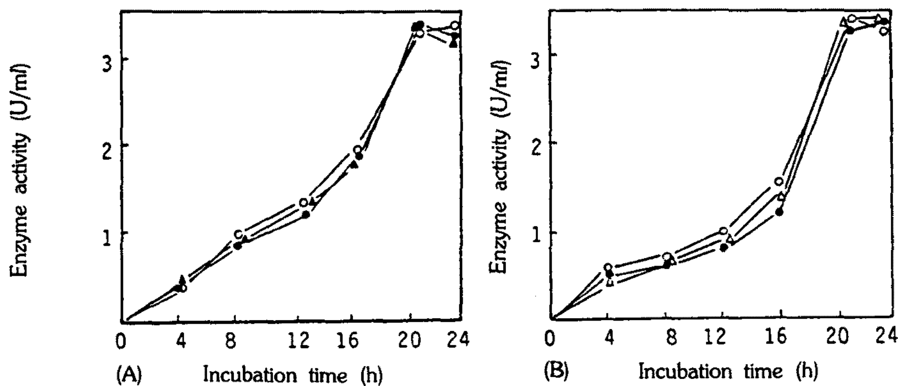

Fig. 6. Effects of lactose and IPTG on the induction of β-galactosidase synthesis from pBG109/*E. coli* (A) and *B. subtilis* HP-4 (B).

●; lactose, 0.5%, ○; IPTG, 10 mM, △; glycerol, 0.5%

of some known inducer of the enzyme synthesis in the *E. coli* system such as lactose or IPTG.

DISCUSSION

This study revealed that a 1.4 Kbp insert fragment including β-galactosidase structural gene of the mesothermophilic bacterium, *B. subtilis* HP-4, was cloned and expressed in *E. coli* JM109 strain. Hirata *et al.* (6) reported that two inserts containing β-galactosidase genes (*bga* A gene on 2.7 Kbp and *bga* B gene on 2.9 Kbp) from *B. stearothermophilus* IAM11001 strain were cloned into *E. coli*. Herman and Mckay (5) also reported that β-galactosidase positive clone containing a 4.15 Kbp insert from chromosomal DNA of lactic *Streptococcus thermophilus* 19258 was isolated in *E. coli* using pBR322 as a cloning vector and a 5.67 Kbp insert from *S. thermophilus* A054 was cloned into a lactic streptococcal specific cloning vector (pTG222). *B. subtilis* HP-4 produced one β-galactosidase protein, of which molecular weight was to be estimated approximately 105 KDa by detection in non-denaturing polyacrylamide gel electrophoresis, followed by histochemical staining for detection of enzyme activity and partial purification of the enzyme (data not shown). Both β-galactosidases produced by *B. subtilis* HP-4 and recombinant *E. coli*/pBG109 also showed same molecular size of enzyme protein and very similiar enzymatic properties. *B. subtilis* HP-4 and *E.*

coli recombinant synthesize β-galactosidase constitutively. This stands for marked constrast to the normally inducible synthesis of β-galactosidase by other members of the genus *Bacillus* as *B. megaterium* (11), *B. subtilis* (1) and *B. acidocaldarius* (9). However, Goodman (4) reported that thermophilic *B. stearothermophilus*, which was isolated from hot spring and had optimum temperature of 65℃ for the enzymes, synthesized β-galactosidase constitively under absence of inducers, but the constitutivity of the enzyme was apparently not the result of a temperature-sensitive repressor. From these results, it was suggested that gene structure and expression of β-galactosidase in *B. subtilis* HP-4 may be different system compared with those of *E. coli* or other *Bacillus* species. Therefore, the recombinant *E. coli*/pBG109 may well serve as model system for the study of β-galactosidase induction in *Bacillus*.

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