Molecular Basis of Isozyme Formation of β-Galactosidases in *Bacillus stearothermophilus*: Isolation of Two β-Galactosidase Genes, *bgaA* and *bgaB*

HARUHISA HIRATA,[1,2] SEIJI NEGORO,[1]* AND HIROSUKE OKADA[1]

Department of Fermentation Technology, Osaka University, Yamada-oka, Suita-shi, Osaka 565,[1] and Wakamoto Pharmaceutical Co., Ltd., Nihonbashi-muromachi 1-8, Chuo-ku, Tokyo 103,[2] Japan

Bacillus stearothermophilus IAM11001 produced three β-galactosidases, β-galactosidase I, II, and III (β-gal I, II, and III), which are detectable by polyacrylamide (nondenatured) gel electrophoresis. By connecting restriction fragments of the chromosomal DNA to plasmid vectors, followed by transformation of *Escherichia coli*, two β-galactosidase genes (*bgaA* and *bgaB*) located close to each other on the chromosome were isolated. Identification of the gene products and Southern hybridization analyses with a 2.7-kilobase-pair *Eco*RI fragment containing the *bgaA* gene as probe revealed that a single *bgaA* gene exists on the genome and that β-gal II and β-gal III consist of a common subunit (the *bgaA* gene product; molecular weight, 120,000), but differ in their assembly (β-gal II is a dimer, and β-gal III is a tetramer). The *bgaB* gene product (molecular weight, 70,000) in *Bacillus subtilis* harboring pHG5 (a hybrid plasmid consisting of pUB110 and a 2.9-kilobase-pair *Eco*RI fragment) was estimated to be the β-gal I protein from its heat stability. Southern hybridization and immunological testing indicated that the two genes have no homology.

β-Galactosidase, an important industrial enzyme for producing lactose-free milk, hydrolyzes β-1,4-D-galactosidic linkages. Extensive enzymatic and genetic studies have been made on the β-galactosidases of *Escherichia coli*, and thermophilic β-galactosidases have attracted increasing interest (5, 15, 16, 23, 33) because of their industrial importance. Despite the growing interest, however, little information is available on the genetic backgrounds of these enzymes.

Recent advances in recombinant DNA techniques have provided a tool for studying enzymes in terms of their gene structures, in addition to the conventional physicochemical study of their proteins. This paper describes the cloning of two β-galactosidase structural genes (*bgaA* and *bgaB*) of *Bacillus stearothermophilus* IAM11001, a mesothermophilic bacterium, into *E. coli*. Immunological and electrophoretical studies of the gene products revealed that two of the three β-galactosidases (β-galactosidases II and III [β-gal II and III]) produced by this bacterium are encoded on the same gene (*bgaA*), whereas the other (β-gal I) is encoded on *bgaB*.

MATERIALS AND METHODS

Strains and plasmids. *B. stearothermophilus* IAM11001, provided by the Institute of Applied Microbiology, University of Tokyo, was used as the source of heat-stable β-galactosidases. The β-galactosidase-deficient mutant, *E. coli* 294-43, which was derived from strain 294 (*endA thi hsdR*) (1) by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment, was used as the recipient for transformation. *Bacillus subtilis* MI111 (*arg-15 leuA8* $r_M^- m_M^-$) was provided by K. Sakaguchi, Mitsubishi-kasei Institute for Life Sciences. Plasmids pBR322 (4), pACYC177 (7), pNL212 (a hybrid plasmid consisting of pBR322, the *lac*UV5 promoter fragment [1], and a DNA fragment containing the *nylB* gene [structural gene of 6-aminohexanoic acid linear oligomer hydrolase]) (27), and pUB110 (18) were used as vector DNA for cloning experiments.

Media and culture conditions. *B. stearothermophilus*

IAM11001 cells were grown on LL medium containing bactotryptone (10 g), yeast extract (5 g), NaCl (5 g), and lactose (2 g) in 1 liter (pH 7.0) at 55°C on a reciprocal shaker to give 10^9 cells per ml. *E. coli* and *B. subtilis* strains were grown at 37°C on LL medium to a density of 2×10^9 cells per ml. When necessary, ampicillin (50 μg/ml), tetracycline (10 μg/ml), or kanamycin (5 μg/ml) was added to the medium.

Electrophoresis. Electrophoresis of DNA (plasmid or chromosomal DNA) digested with a restriction endonuclease was carried out as described previously (26). Polyacrylamide gel (7.5%) electrophoresis of proteins was carried out at pH 9.0 by the method of Gabriel (14). For estimation of molecular weight of native enzymes, electrophoresis was performed with gels of different concentrations (5.0, 6.1, 7.2 and 8.6%) according to the method of Hedrick and Smith (21). The following proteins were used as molecular weight standards: bovine serum albumin (68,000), yeast alcohol dehydrogenase (140,000), rabbit muscle lactate dehydrogenase (142,000), catalase (240,000), and *E. coli* β-galactosidase (466,000). The molecular weight of the subunits of the proteins was determined by electrophoresis on polyacrylamide gel (10%) containing 0.1% sodium dodecyl sulfate (SDS) by the procedure of Laemmli (24). Gel electrofocusing was carried out on polyacrylamide gel (4%) containing 2% Ampholine (pH range, 4 to 6; LKB-Produkter AB, Bromma, Sweden) by the procedure of Wrigley (34).

Enzyme assay. β-Galactosidase activity was measured at 55°C with *o*-nitrophenyl-β-D-galactopyranoside as substrate by the method of Craven et al. (10). One unit of the enzyme activity was defined as the amount of enzyme hydrolyzing 1 μmol of the substrate in 1 min. To detect β-galactosidase activity on polyacrylamide gel, the gel was incubated with 0.25 mg of 6-bromo-2-naphthyl-β-D-galactopyranoside per ml and then with 1 mg of diazo-blue B per ml according to the procedure of Erickson and Steers (12).

Preparation of crude enzyme solution. *B. stearothermophilus* cells grown on 150 ml of LL medium to a density of 10^9 cells per ml were harvested by centrifugation, washed with buffer A (100 mM sodium phosphate buffer [pH 7.0] contain-

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* Corresponding author.

ing 10 mM KCl, 1 mM MgSO$_4$, and 50 mM β-mercaptoethanol), and suspended in 3 ml of the same buffer. The cell suspension was sonicated for 4.5 min at 20 kHz. Supernatant obtained by centrifugation at 20,000 × g for 10 min was used as the crude enzyme solution. To prepare crude enzymes of E. coli and B. subtilis, cells grown on LL medium to a density of 2 × 10^9 cells per ml were harvested, washed with buffer A, and sonicated as described above.

Purification of β-galactosidases. The crude enzyme solutions (12 mg/ml, 17 U/ml, 305 ml) were applied on a DEAE-Sephadex A-50 column (22 by 8 cm) equilibrated with buffer B (0.02 M sodium phosphate buffer [pH 7.0] containing 10% glycerol) and were eluted stepwise with 6-liter portions of buffer B containing 0.1, 0.25, or 0.7 M NaCl. β-Galactosidases eluted at 0.25 M NaCl were pooled (0.26 mg/ml, 3 liters), precipitated by the addition of ammonium sulfate (50% saturation), and centrifuged (10,000 × g, 10 min). Afterwards, the precipitates were dissolved in 5 ml of buffer B and dialyzed against the same buffer, and the concentrated sample (9.0 mg/ml, 7 ml) was applied to a Toyopearl HW55 column (φ2.6 by 95 cm) and eluted with the same buffer. The active fractions were pooled and concentrated, and then the sample (0.85 mg/ml, 12 ml) was subjected to 7.5% polyacrylamide slab gel electrophoresis (15 by 15 by 0.6 cm) as described previously (14). After the run, the gel was incubated in 2.2 mM o-nitrophenyl-β-D-galactopyranoside at 25°C for 5 min. Bands of β-gal II and III were cut out, extracted, and dialyzed against buffer B.

Isolation of DNA samples and transformation. Chromosomal DNA of B. stearothermophilus IAM11001 was prepared by the method of Saito and Miura (30). Plasmid DNAs (pBR322, pACYC177, and pNL212) were prepared from E. coli strains harboring one of them by the procedure of Birnboim and Doly (3), followed by purification by cesium chloride-ethidium bromide density gradient centrifugation. Plasmid pUB110 was obtained from B. subtilis MI111 by the same procedure. E. coli strains were transformed by the method of Cohen et al. (9), and the B. subtilis strain was transformed by "protoplast transformation" (8).

Enzymes. Restriction endonuclease digestions were performed as described previously (11). Ligation was carried out at 4°C in the reaction mixture containing 67 mM Tris-hydrochloride (pH 7.5), 10 mM MgCl$_2$, 1 mM ATP, 10 mM dithiothreitol, 0.2 to 1 μg of DNA, and 1 U of T4 DNA ligase. Restriction endonucleases, T4 DNA ligase, DNA polymerase I, and alkaline phosphatase were obtained from Takara Shuzo Co., Kyoto, Japan. Nuclease BAL 31 was obtained from New England Biolabs, Inc., Beverly, Mass.

Nick translation and DNA-DNA hybridization. A 2.7-kilobase-pair (kbp) EcoRI fragment of pHG10 and a 2.95-kbp PstI fragment of pHG2 (0.7 μg) were labeled by nick translation to a specific activity of 2 × 10^7 cpm/μg with [α-^{32}P]dATP (800 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) (29). The ^{32}P-labeled DNA was used for hybridization at 65°C with blotted DNA fragments on a nitrocellulose filter by the procedure of Southern (31). Colony hybridization was performed by the method of Grunstein and Hogness (17).

Immunological assay. Antiserum against β-gal II of B. stearothermophilus IAM11001 was raised in rabbits by hypodermic injection of the purified enzyme (1.5 mg) emulsified in Freund adjuvant (complete type). Rabbits were bled after three injections. The double immunodiffusion test was carried out by the procedure of Ouchterlony (28). The colony immunoassay was performed as described by Kemp and Cowman (22).

RESULTS

β-Galactosidases produced in B. stearothermophilus IAM11001. B. stearothermophilus IAM11001 produced thermostable β-galactosidases at the highest level (1.4 U/mg of cellular protein) among five strains tested (B. stearothermophilus IAM11001, IAM11002, IAM11003, IAM11004, and IFO12550). To identify the β-galactosidases in strain IAM11001, a cell extract was fractionated by polyacrylamide slab (nondenatured) gel electrophoresis, followed by histochemical staining for the enzyme activity. Three bands were found, of which the corresponding enzymes were designated β-gal I, II, and III in order of decreasing electrophoretic mobility (Fig. 1A). We purified β-gal II, which gave the densest band in Fig. 1A, from the cell extracts by the method described above (yield, 8.2%). Polyacrylamide disc gel electrophoresis of the purified enzyme gave a single band with a relative mobility of 0.32 (Fig. 1B). The mobilities of the bands detected by the protein staining and by the activity staining were identical. The purified enzyme (420 U/mg of protein) was used for the preparation of anti-β-gal II serum.

Cloning of the structural gene of β-gal II (bgaA) in E. coli. EcoRI fragments of the chromosomal DNA of B. stearothermophilus IAM11001 was ligated with plasmid pNL212, which had been cleaved with EcoRI and treated with alkaline phosphatase. Since the single EcoRI site on pNL212 is located just downstream of the lac promoter region, a gene located in the inserted fragment in the correct orientation is likely to be expressed. After transformation of E. coli 294-43 with the ligated DNA, β-galactosidase-producing clones were screened by colony immunoassay by using anti-β-gal II serum and ^{125}I-labeled protein A. Of 1,600 ampicillin-resistant colonies examined, 7 clones produced β-gal II antigenic protein with no β-galactosidase activity. EcoRI digestion of the plasmid DNA contained in the seven clones revealed that they harbored a 9.0-kbp plasmid (named pHG10; Fig. 2) consisting of pNL212 and an inserted 2.7-kbp fragment. Figure 3 shows the clear precipitin lines formed between the cell extract of E. coli harboring pHG10 and anti-β-gal II serum, whereas no precipitin lines were formed between the cell extracts of E. coli (strains 294 and 294-43) and anti-β-gal
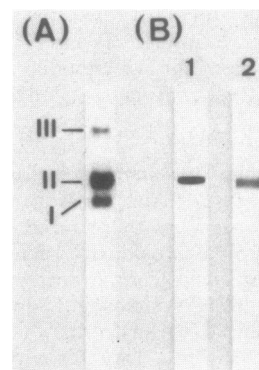

FIG. 1. Polyacrylamide gel electrophoresis of β-galactosidase in the cell extract of B. stearothermophilus (A) and purified β-gal II (B). (A) The cell extract of B. stearothermophilus IAM11001 (containing 0.09 U of β-galactosidase activity and 36 μg of protein) was subjected to electrophoresis on a polyacrylamide disc (pH 9.0, nondenatured) gel, and then β-galactosidase bands were detected by activity staining with 6-bromo-2-naphthyl-β-D-galactopyranoside as substrate. (B) The purified β-gal II (4.2 U, 10 μg of protein) was subjected to electrophoresis. Lane 1, Protein staining; lane 2, activity staining.
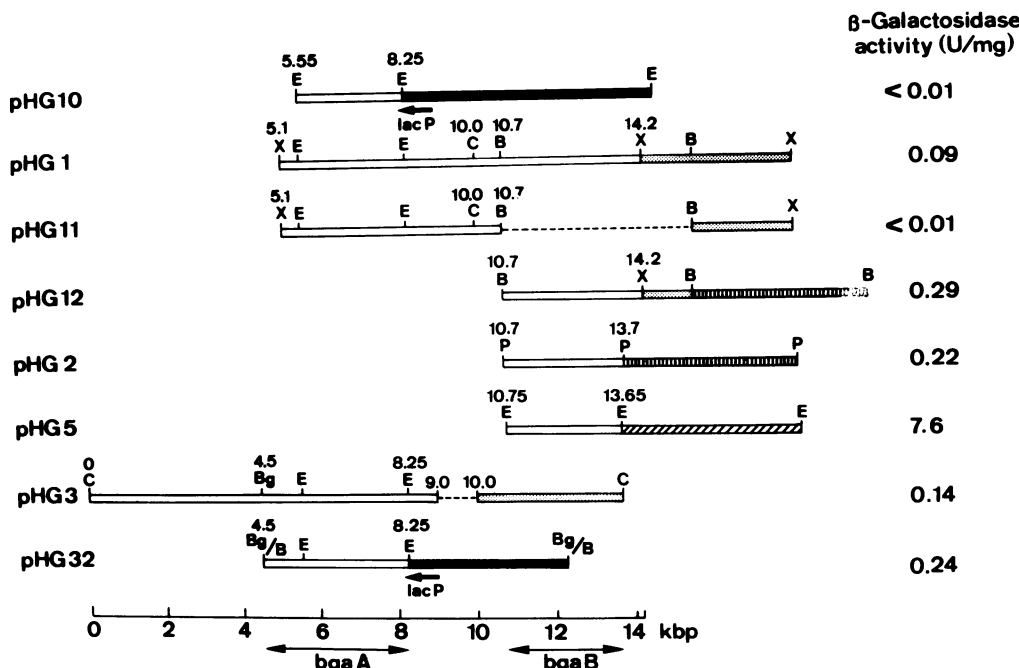
β-Galactosidase
activity (U/mg)

pHG10   < 0.01

pHG 1   0.09

pHG11   < 0.01

pHG12   0.29

pHG 2   0.22

pHG 5   7.6

pHG 3   0.14

pHG32   0.24

0   2   4   6   8   10   12   14   kbp

bgaA   bgaB

FIG. 2. Plasmids encoding β-galactosidase genes. Plasmid pHG10 (producing β-gal II antigenic protein) was composed of vector pNL212 region and the 2.7-kbp EcoRI fragment of B. stearothermophilus IAM11001 chromosomal DNA. Plasmids pHG1 and pHG3 were selected by colony hybridization with the ^{32}P-labeled 2.7-kbp fragment of pHG10 from the hybrid plasmids (pACYC177 plus the ClaI fragment [pHG3] or the XhoI fragment [pHG1] of the chromosomal DNA) (see the text for details). Plasmid pHG32 was constructed by ligating the 3.8-kbp BglII-EcoRI fragment of pHG3 (the 4.5- to 8.25-kbp region on the map) with the 4.0-kbp BamHI-EcoRI fragment of pNL212. After BamHI digestion of pHG1, followed by ligation and transformation of E. coli, pHG11 was obtained. Plasmid pHG12 was constructed by connecting the 4.8-kbp BamHI fragment of pHG1 (coding the 10.7- to 14.2-kbp map region and the 1.3-kbp BamHI-XhoI fragment of pACYC177) and pBR322 digested with BamHI. Plasmid pHG2 was a hybrid constructed by joining the 2.95-kbp PstI fragment of pHG1 to PstI-cleaved pBR322. After the 2.95-kbp PstI fragment (5 µg) had been digested with 0.2 U of BAL 31 at 30°C for 6 min in 25 µl of the reaction mixture described by Miyanohara et al. (25), the DNA fragment (blunt end) was ligated with 8 nucleotides of EcoRI linker (GGAATTCC), followed by EcoRI digestion. By inserting the digested DNA at the EcoRI site of pUB110, followed by transformation of B. subtilis MI111, plasmid pHG5 was obtained. Symbols: ▭, chromosomal DNA of B. stearothermophilus IAM11001; ▰, pNL212; ▨, pACYC177; ▥, pBR322; ▧, pUB110. Broken lines represent deleted regions. B, Bg, C, E, P, and X indicate restriction sites for BamHI, BglII, ClaI, EcoRI, PstI, and XhoI, respectively. Some ClaI sites located in the 10.7- to 14.2-kbp region and in vector DNAs are omitted. β-Galactosidase activities in the cell extracts of E. coli harboring one of the plasmids other than pHG5 and the activity in the cell extract of B. subtilis(pHG5) were shown as the activity per 1 mg of cellular protein.
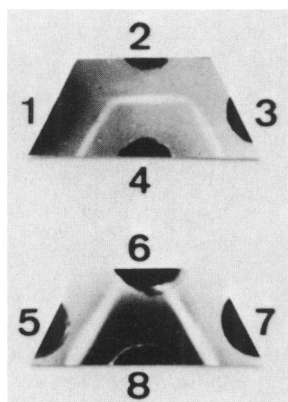
FIG. 3. Double immunodiffusion test between β-galactosidases and anti-β-gal II serum. Wells 4 and 8, Rabbit antiserum against the purified β-gal II; wells 1, 5, and 7, β-gal II (2.8 U, 6.8 µg of protein); well 2, partially purified β-gal III (1.5 U, 6.3 µg of protein); well 3, cell extract of E. coli(pHG10) (3.1 mg of protein); well 6, heat-treated (70°C, 15 min) cell extract of B. subtilis(pHG5) (6.5 U, 150 µg of protein) (see legend to Fig. 6).

II serum (data not shown). Further observations indicated that the antigenic protein is a truncated peptide of β-gal II protein. (i) The molecular weight of the subunit of the antigenic protein is 95,000, which is less than that of the β-gal II protein (molecular weight, 120,000) (Fig. 4A). (ii) The immunotitration of β-galactosidase activity of purified β-gal II protein with the antiserum was inhibited by the addition of the cell extract of E. coli(pHG10) (data not shown).

Since pHG10 must lack the DNA region encoding either the amino or carboxyl terminal of the protein, we tried to clone a DNA fragment which covers the whole structural gene. Southern hybridization experiments had revealed that the 10-kbp ClaI and 9.2-kbp XhoI fragments of the chromosomal DNA hybridized to the 2.7-kbp EcoRI fragment of pHG10, and these chromosomal DNA segments were therefore fractionated by agarose gel (1%) electrophoresis and were recovered from the gel. After ligation of the fractionated DNA with pACYC177 that had been digested with ClaI (for the ClaI fragment) or XhoI (for the XhoI fragment), the ligated DNA was used to transform E. coli 294-43, and transformants were selected by using ampicillin resistance as a selection marker. Colonies harboring a hybrid plasmid coding the bgaA gene were screened by the colony hybrid-
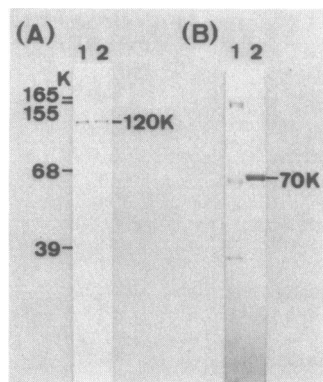
FIG. 4. SDS-polyacrylamide gel electrophoresis of β-galactosidases. (A) Purified β-gal II (0.068 μg of protein) (lane 1) and β-gal III (0.094 μg of protein) (lane 2) were electrophoresed on an SDS-polyacrylamide slab gel. After the run, proteins contained in the gel were electrophoretically transferred to a nitrocellulose filter by the method of Towbin et al. (32). The filter was incubated in 2.5% anti-β-gal II serum for 16 h and washed with 50 mM Tris-hydrochloride buffer (pH 7.5) containing 0.9% NaCl, and then the antigenic protein bands were detected by enzyme immunoassay with goat anti-rabbit immunoglobulin G horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, Calif.). (B) Purified β-galactosidase (0.7 μg of protein) of *B. subtilis* (pHG5, containing the *bgaB* gene) obtained from the heat-treated cell extracts (see legend to Fig. 6), followed by fractionation through polyacrylamide (nondenatured) gel electrophoresis, was analyzed by SDS-polyacrylamide gel electrophoresis (lane 2). Marker proteins for molecular weight determination were RNA polymerase (165,000, 155,000, 39,000), bovine serum albumin (68,000), and trypsin inhibitor (21,500) (lane 1). The protein bands were detected by staining with Coomassie brilliant blue.

ization technique with the 2.7-kbp *Eco*RI fragment of pHG10 as the probe. Plasmid pHG3, constructed from pACYC177 and the 10 kbp *Cla*I fragment, was 1.0 kbp smaller than expected (Fig. 2). As *Cla*I digestion of the plasmid gave a single 12.8-kbp fragment, a 1.0-kbp DNA fragment covering one of the *Cla*I sites may have been deleted. Further restriction endonuclease analysis indicated that the deleted region corresponds to the 9.0- to 10.0-kbp region on the map (expressed by the distance from one of the *Cla*I sites; see Fig. 2). β-Galactosidase activity was detected in *E. coli* harboring pHG3 and pHG32, the latter of which was constructed from the 3.8-kbp *Bgl*II-*Eco*RI fragment of pHG3 (the 4.5- to 8.25-kbp region on the map) and the 4.0-kbp *Eco*RI-*Bam*HI fragment of pNL212. From this result the structural gene was estimated to be in the 3.8-kbp fragment.

Relationship between the β-gal II and β-gal III proteins. Although β-gal II and β-gal III differ in their mobilities on polyacrylamide disc gel electrophoresis, we concluded from the following observations that they are products of the same *bgaA* gene. (i) β-gal III protein cross-reacts with anti-β-gal II serum on the Ouchterlony plate (Fig. 3). (ii) β-Galactosidase activity of purified β-gal III was immunotitratable with anti-β-gal II serum but not with nonimmune serum, and the titration was inhibited by the cell extract of *E. coli*(pHG10) (data not shown). (iii) The molecular weights (ca. 120,000) of the subunits of the two enzymes were almost the same (Fig. 4A). (iv) Single fragments of 2.7, 10, 9.5, 4.5, and 14 kbp among the chromosomal DNA fragments obtained by *Eco*RI, *Cla*I, *Eco*RV, *Sst*II, and *Stu*I digestion, respectively, were detected by Southern hybridization with the nick-translated 2.7-kbp *Eco*RI fragment of pHG10 as the probe, suggesting a single locus of the *bgaA* gene on the chromo-

some (Fig. 5). (v) The size of the inserted fragment in pHG32 (3.8 kbp) agreed well with that expected from the molecular weight of the enzyme subunit. (vi) The isoelectric points of the enzymes (pI 5.2 for β-gal II and pI 5.1 for β-gal III) were almost the same. (vii) The molecular weights of the native enzymes estimated from the mobilities on four different concentrations of polyacrylamide (nondenatured) gel (see above) were 240,000 for β-gal II and 480,000 for β-gal III. These results indicate that the difference in electrophoretic mobility is the result of different assembly of the same gene product (β-gal II is a dimer, and β-gal III is a tetramer), although the possibility remains of minor differences in the processing of the proteins.

Presence of another β-galactosidase gene (*bgaB*). β-Galactosidase activity was also detected in *E. coli* harboring pHG1 (a hybrid plasmid consisting of pACYC177 and the 9.1-kbp *Xho*I fragment of the chromosomal DNA; see Fig. 2) but not in *E. coli* harboring pHG11 (a derivative plasmid of pHG1, lacking the 4.8-kbp *Bam*HI fragment of pHG1 [the 10.7- to 14.2-kbp region on the map and the 1.3-kbp fragment of pACYC177]; see Fig. 2). This result suggests the existence of another β-galactosidase gene located at least partly in the *Bam*HI fragment. This was supported by the detection of β-galactosidase activity in *E. coli* harboring pHG12 (a hybrid plasmid consisting of pBR322 and the 4.8-kbp *Bam*HI fragment of pHG1) or pHG2 (pBR322 and the 2.95-kbp *Pst*I fragment [the 10.7- to 13.7-kbp region on the map]). These results indicate that a different β-galactosidase gene (*bgaB*) is present in the 10.7- to 13.7-kbp region on the map. To confirm this result, we characterized the *bgaB* gene product in *B. subtilis* and compared it with the β-galactosidases of *B. stearothermophilus*. We recloned the 2.95-kbp *Pst*I fragment of pHG2 (the 10.7- to 13.7-kbp map region, after changing the *Pst*I sites to *Eco*RI sites; see the legend to Fig. 2) into the *Eco*RI site of pUB110. The resultant hybrid plasmid, pHG5, was used to transform *B. subtilis*. Figure 6 shows that the β-galactosidase produced in *B. subtilis*(pHG5) had electrophoretic mobility similar to the mobility of β-gal I protein and that heat treatment (70°C for 15 min) did not affect the intensities of the bands, in contrast to β-gal II and β-gal III bands, which disappeared after the same treatment. Thus, the *bgaB* gene product is analogous to β-gal I protein in its
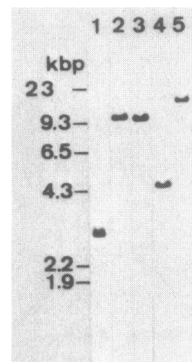

FIG. 5. Southern blot hybridization of restriction fragments. After chromosomal DNAs of *B. stearothermophilus* IAM11001 had been digested with *Eco*RI (lane 1), *Cla*I (lane 2), *Eco*RV (lane 3), *Sst*II (lane 4), and *Stu*I (lane 5), the DNAs were fractionated by agarose gel (1%) electrophoresis. DNA fragments homologous to the *bgaA* gene were detected by Southern blotting and hybridization with the ^{32}P-labeled 2.7-kbp *Eco*RI fragment of pHG10 (see Fig. 2) as probe.
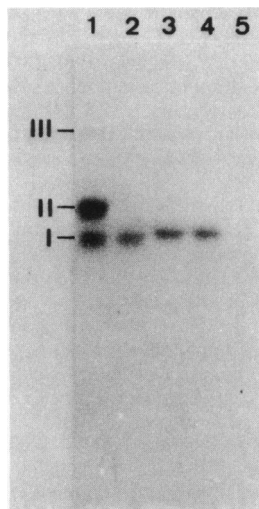
FIG. 6. Polyacrylamide gel electrophoresis of β-galactosidases. After β-galactosidases had been subjected to electrophoresis on polyacrylamide gel (pH 9.0), the gels were incubated in 6-bromo-2-naphthyl-β-D-galactopyranoside for the activity staining. In some experiments, the cell extracts of *B. stearothermophilus* IAM11001 (8.5 U/ml, 4.5 mg of protein per ml) and *B. subtilis* MI111(pHG5) (30 U/ml, 3 mg of protein per ml) were incubated at 70°C for 15 min and centrifuged at 10,000 × *g* for 5 min, and the resulting supernatants were applied. Lane 1, Cell extracts of *B. stearothermophilus* (0.1 U, 54 μg of protein); lane 2, heat-treated cell extract of *B. stearothermophilus* (0.018 U, 22 μg of protein); lane 3, cell extract of *B. subtilis*(pHG5) (0.02 U, 2 μg of protein); lane 4, heat-treated cell extract of *B. subtilis* MI111(pHG5) (0.019 U, 0.5 μg of protein); lane 5, cell extract of *B. subtilis*(pUB110) (6 μg of protein).

thermostability. These results suggest that the structural gene of the β-gal I protein is *bgaB*, located in the 10.7- to 13.7-kbp region. SDS-polyacrylamide gel electrophoresis gave a molecular weight for the subunit of the *bgaB* gene product of 70,000 (Fig. 4B).

Southern hybridization experiments revealed that the ^{32}P-labeled 2.7-kbp *Eco*RI fragment of pHG10 (*bgaA* gene probe) did not hybridize with the 3.5-kbp *Bam*HI-*Xho*I fragment of pHG1 (*bgaB* region). Neither could the 5.6-kbp *Bam*HI-*Xho*I fragment (*bgaA* region) be detected with the ^{32}P-labeled 2.95-kbp *Pst*I fragment (*bgaB* probe) (Fig. 7). No immunoreaction was observed between the anti-β-gal II serum and the *bgaB* gene product of *B. subtilis*(pHG5), even when the enzyme with twofold higher β-galactosidase activity than β-gal II protein was applied on the Ouchterlony plate (Fig. 3). These results suggest that the two β-galactosidases have no homology.

DISCUSSION

This study revealed that two β-galactosidase structural genes (*bgaA* and *bgaB*) are present in *B. stearothermophilus* IAM11001. The most thermostable β-galactosidase, β-gal I, is estimated to be a product of the *bgaB* gene, and the other two less stable isozymes (β-gal II and β-gal III) are estimated to be *bgaA* gene products. When cell extracts of *E. coli* harboring pHG3 or pHG32, which encodes the whole *bgaA* gene, were subjected to polyacrylamide (nondenatured) gel electrophoresis followed by activity staining, three β-galacto-sidase bands (relative mobilities, 0.23, 0.29, and 0.34), which differed in mobility from β-gal II (0.32) and β-gal III (0.15),

were observed. Heterologous products of the *bgaB* gene were also detected in *E. coli*. These results indicate that different processing of the parental proteins or different starting sites of translation between *B. stearothermophilus* and *E. coli* strains result in alteration of the isozyme formation patterns. The *bgaA* gene was poorly expressed in *B. subtilis* harboring a hybrid plasmid consisting of the 3.8-kbp *Bam*HI-*Eco*RI fragment of pUB110 and the 3.8-kbp *Bgl*II-*Eco*RI fragment of pHG32 (the 4.5- to 8.25-kbp region containing the *bgaA* gene; see Fig. 2). For these reasons we could not confirm that the cloned *bgaA* gene produced both β-gal II and β-gal III enzymes.

Two different β-galactosidase structural genes, *lacZ* (2) and *ebgA* (6), are reported to be present on the chromosome of *E. coli* K-12 at 8- and 68-min positions on its chromosomal map. In contrast to the large separation of these two genes, those of *B. stearothermophilus* IAM11001 (*bgaA* and *bgaB*) are located very close to each other on the chromosome. The subunit molecular weight of the *bgaA* gene products is very similar to that of *lacZ* β-galactosidase (116,400) (13) and *ebgA* β-galactosidase (120,000) (19). Despite the size similarity, no precipitin lines were formed on the Ouchterlony plate between the anti-β-gal II serum and the cell extracts of *E. coli* 294, suggesting that the *bgaA* gene products and the enzymes of *E. coli* are immunologically different. The subunit molecular weight of the *bgaB* gene product is also analogous to β-gal III of *Klebsiella* species (20). Comparative analyses of amino acid sequences between these β-galactosidases would provide important information for understanding the mechanisms of thermostability of proteins.
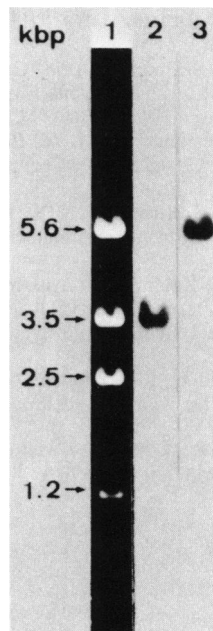

FIG. 7. Southern hybridization of plasmid pHG1 digested with *Bam*HI plus *Xho*I. DNA fragments of pHG1 digested with *Bam*HI plus *Xho*I were fractionated by 1% agarose gel electrophoresis, and then the DNA was transferred to a nitrocellulose filter by the method of Southern. The filter containing the blotted DNA was hybridized with the ^{32}P-labeled 2.95-kbp *Pst*I fragment of pHG2 (*bgaB* gene probe) (lane 2) or with the ^{32}P-labeled 2.7-kbp *Eco*RI fragment of pHG10 (*bgaA* gene probe) (lane 3). Lane 1, Ethidium bromide staining of the agarose gel.

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