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Structure of a β-Galactosidase Gene of *Bacillus stearothermophilus*

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The nucleotide sequence of the *bgaB* gene, which encodes the thermostable β-galactosidase I of *Bacillus stearothermophilus*, and its flanking region was determined. A 2,016-base-pair open reading frame observed was concluded to be for β-galactosidase I (M_r 78,051) from observations that the amino acid composition of the enzyme and the sequence of 14 amino acids from the amino-terminus of the enzyme coincided with those deduced from this open frame. A 107-base-pair *Hae*III-*Alu*I fragment just upstream of the estimated Shine-Dalgarno sequence of the *bgaB* gene had promoter activity toward *cat-86* (chloramphenicol acetyltransferase gene) and produced the enzyme at a level equivalent to 7% of the total cellular protein of *B. subtilis*. From the base sequence of this DNA region and the transcriptional start site determined by S1 nuclease mapping, the −35 and −10 sequences are estimated to be TTGACA and TAATTT, respectively, which are similar to the consensus sequence of *B. subtilis* σ^{43} RNA polymerase.

Although extensive enzymatic and genetic studies have been made on the β-galactosidases of *Escherichia coli*, little information is available on thermophilic β-galactosidases. We have cloned two β-galactosidase genes (*bgaA* and *bgaB*) of a thermophilic bacterium, *Bacillus stearothermophilus* IAM11001, and revealed that two of the three β-galactosidases (β-galactosidases II and III) produced by this bacterium are coded on the same gene (*bgaA*) but differ in quaternary structure (11). The other β-galactosidase (β-galactosidase I [β-galI]) coded on *bgaB* is the most thermostable of the three, being stable up to 70°C (11), so its amino acid sequence will provide an example of the primary structure of a thermostable protein.

Bacillus subtilis harboring a hybrid plasmid containing the *bgaB* gene produced about 50 times more β-galI than *B. stearothermophilus*, and β-galI accounted for 6% of the total protein of the host bacterium (12). This high production suggests that the promoter of the *bgaB* gene is effective in *B. subtilis*.

In this paper we determined the nucleotide sequence of the *bgaB* gene, including its promoter region, and analyzed the amino acid sequence of β-galI. The high expression of this promoter was confirmed by using the *cat-86* gene.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. *B. stearothermophilus* IAM11001 (ATCC 8005), *B. subtilis* MI111 (*arg-15 leuA8* r_M m_M), and the plasmid pHG5 (a 2.9-kilobase [kb] *Eco*RI fragment containing the *bgaB* gene plus the 4.5-kb *Eco*RI fragment of pUB110) have been described previously (11). *B. subtilis* BR151, harboring the promoter cloning plasmid pPL603, was obtained from the *Bacillus* Genetic Stock Center (Ohio State University) (31). *E. coli* JM103 was used as host strain for the M13 phage derivatives mp10 and mp11 (20). Bacteria were grown on LL medium (11) or Penassay broth (Difco Laboratories). When necessary, kanamycin (5 μg/ml) or chloramphenicol (5 μg/ml) was added to the medium.

Electrophoresis. Gel electrophoresis of plasmid DNAs digested with restriction endonucleases was done as described previously (23). Sodium dodecyl sulfate (SDS)-polyacrylamide gel (10%) electrophoresis of proteins was performed by the method of Laemmli (16).

Isolation of DNA, transformation, and enzymes. Preparation of plasmid DNAs and restriction endonuclease digestions were performed as described previously (23). *E. coli* JM103 was transformed as described by Messing (20), and *B. subtilis* MI111 was transformed by the protoplast procedure (6). Restriction endonucleases, T4 DNA ligase, and T4 DNA polymerase were obtained from Takara Shuzo Co. (Kyoto) or from New England Biolabs, Inc. (Beverly, Massachusetts).

DNA sequencing. DNA sequencing was carried out by the method of Maxam and Gilbert (18) or by the dideoxy chain termination method (25). For the chain termination method, the sequencing kit was obtained from Takara Shuzo Co., and [α-^{32}P]dCTP (Amersham Corp.) was used.

Purification of β-galactosidase. β-GalI (the *bgaB* gene product) was purified from the heat-treated (70°C) cell extracts of *B. subtilis*(pHG5) by DEAE-Sephadex A-50 column chromatography (12). The enzyme was further purified by gel permeation chromatography on a Toyopearl HW55 column (2.5 by 95 cm; 50 mM sodium phosphate buffer [pH 7.0], 0.1 M NaCl).

Amino-terminal sequence analysis of β-galI. The first 14 amino acid residues of the purified β-galI were determined by automated Edman sequencing with a 470A sequenator (Applied Biosystems) (13). The phenylthiohydantoin derivatives were identified by high-performance liquid chromatography as described previously (30).

Amino acid composition of β-galI. The purified β-galI was hydrolyzed at 110°C for 24, 48, or 72 h with 6 N HCl, and hydrolysates were analyzed by use of an amino acid analyzer (Hitachi 835).

Assays. β-Galactosidase activity was measured at 55°C (pH 7.0) with *o*-nitrophenyl-β-D-galactopyranoside as substrate as described previously (11). One unit of enzyme activity was defined as the amount of the enzyme hydrolyzing 1 μmol of the substrate in 1 min. Chloramphenicol acetyltransferase (CAT) activity was measured at 37°C by the spectrophotometric method of Shaw (26). One unit of

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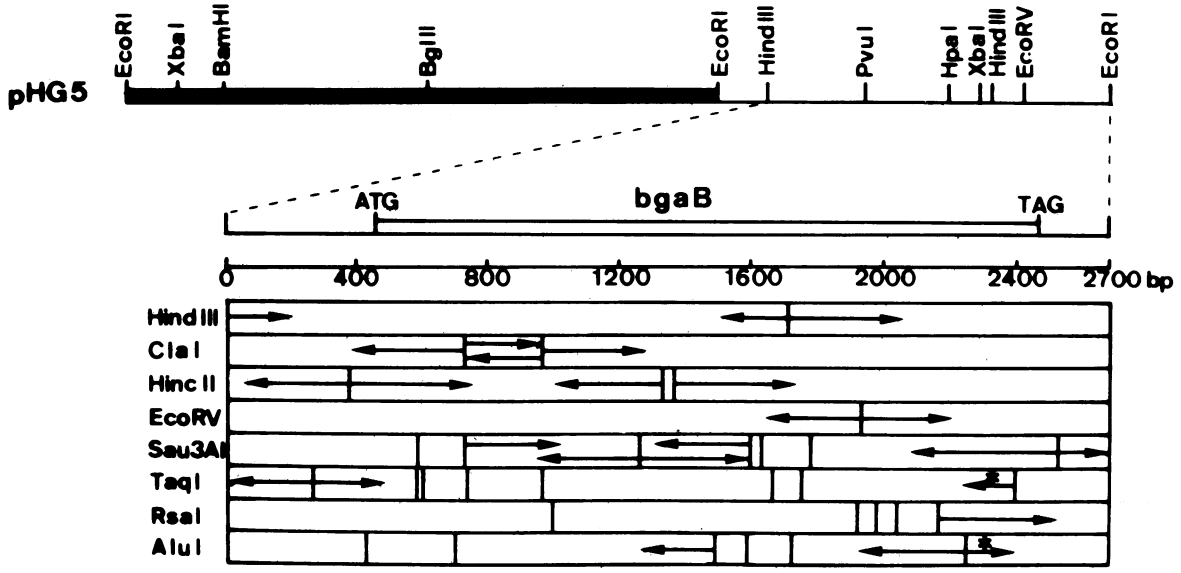


FIG. 1. Restriction map of and sequencing strategy for the *bgaB* gene. Plasmid pHG5 is a hybrid plasmid consisting of pUB110 (thick line) and the 2.9-kb *Eco*RI fragment containing the *bgaB* gene of *B. stearothermophilus* (thin line) (11). The unique open reading frame for β-gall, which starts from the ATG codon and terminates at the TAG codon, is also shown. The DNA sequence was determined by the dideoxy method and the Maxam-Gilbert method (indicated by asterisks on the arrows). The arrows indicate the direction and extent of the DNA sequencing.

enzyme activity was defined as the amount of the enzyme acetylating 1 μmol of chloramphenicol in 1 min. Protein concentration was measured by the method of Lowry et al. (17), with bovine serum albumin as a standard.

S1 nuclease mapping. The transcriptional start site was determined by S1 nuclease (Boehringer Mannheim) mapping as described by Berk and Sharp (2). RNA was extracted and purified from *B. subtilis*(pHG5) grown on Penassay broth at 37°C for 20 h as described previously (9).

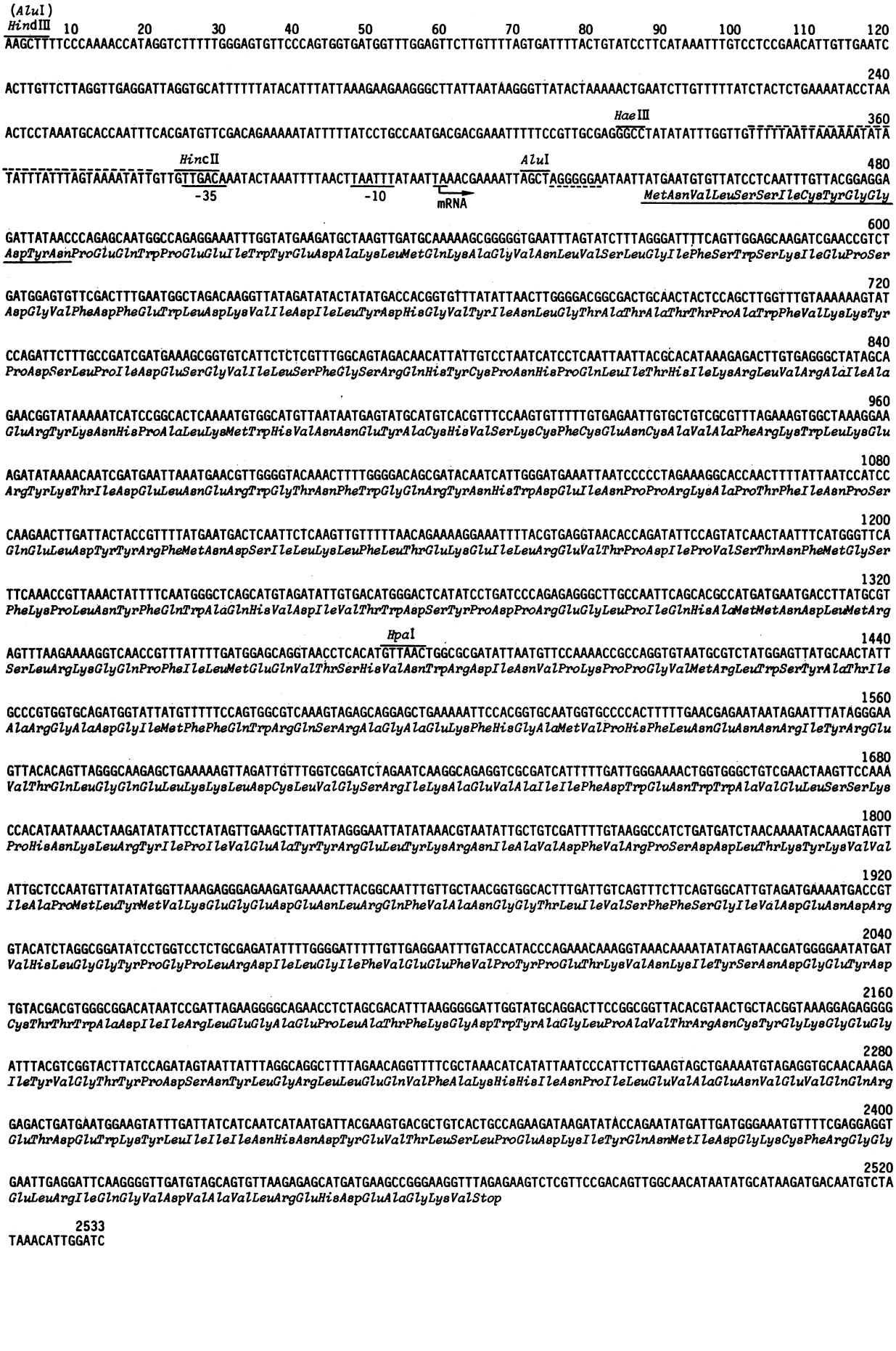
RESULTS

Nucleotide sequence of *bgaB*. The restriction map of and sequencing strategy for the *bgaB* gene are shown in Fig. 1. The DNA sequencing was carried out by the dideoxy chain termination method, except where this method was inapplicable; that part was sequenced with overlapping at the restriction sites used for the sequencing start. The DNA base sequence and amino acid sequence deduced are shown in Fig. 2. A 2,016-base-pair (bp) open reading frame starting at ATG (position 448) and terminating at TAG (position 2,464) was found which was capable of coding a peptide of 672 amino acid residues of 78,051 daltons. Though this molecular weight is 10% larger than that of the β-galI subunit estimated by SDS-polyacrylamide gel electrophoresis (70,000), we concluded that this open reading frame encoded β-galI from the following observations. (i) This is the only reading frame capable of coding a peptide of more than 110 amino acid residues among the six frames of the normal and reverse strands of this DNA region. (ii) Fourteen amino acid residues from the amino-terminus of β-galI purified to homogeneity from *B. subtilis*(pHG5) (specific activity of the enzyme, 110 U/mg of protein) were determined by sequential Edman degradation to be Met-Asn-Val-Leu-Ser-Ser-Ile-Cys-Tyr-Gly-Gly-Asp-Tyr-Asn, which are identical to those deduced for this open reading frame. (iii) Except for the unstable amino acids cysteine and tryptophan, the number of amino acid residues per subunit predicted from this open reading frame agreed with that calculated from the result of amino acid analysis of the hydrochloride hydrolysate of β-galI on the basis of a molecular weight of 78,051. The values obtained (calculated) were: Lys, 38 (38.4); His, 19 (17.5); Trp, 21 (7.0); Arg, 36 (35.9); Asx, 78 (75.2); Thr, 25 (25.7); Ser, 29 (29.3); Glx, 72 (73.8); Pro, 37 (32.6); Gly, 43 (45.8); Ala, 36 (36.9); Cys, 10 (2.5); Val, 49 (49.4); Met, 15 (14.2); Ile, 48 (46.5); Leu, 52 (53.0); Tyr, 35 (33.6); and Phe, 29 (27.9).

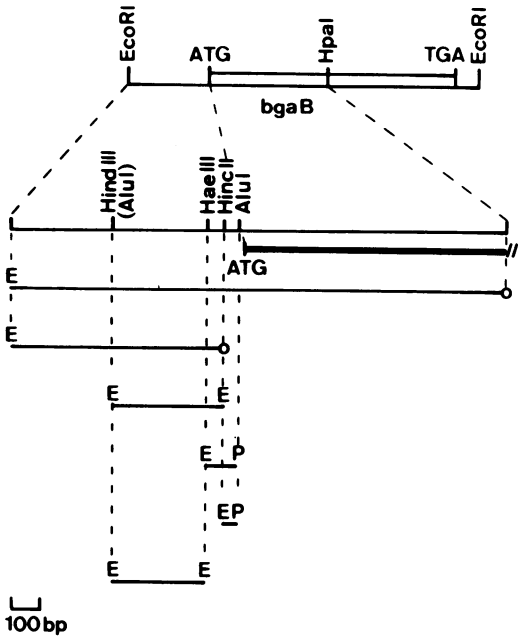
A Shine-Dalgarno sequence, the ribosome binding site for translation, AGGGGGA, which is complementary to the 3' end of 16S rRNAs of *B. subtilis* (22) and *B. stearothermophilus* (27), was observed 6 bp upstream from the initiation codon (Fig. 2). The free-energy change of the most stable Shine-Dalgarno pairing calculated for this sequence was −18 kcal/mol (29), which is in the range reported (−11.6 to −21 kcal/mol) for *B. subtilis* 16S rRNA (19).

Isolation of the *bgaB* promoter. To study the molecular basis of the high expression of *bgaB*, the promoter region was analyzed by using a promoter cloning plasmid of *B. subtilis*, pPL603 (31). It consists of pUB110 and the *cat-86* gene coding CAT of *B. pumilus* without a vegetative promoter (7). Since pPL603 has unique *Eco*RI and *Pst*I sites just upstream of the *cat-86* gene, we inserted the 1.7-kb *Eco*RI-*Hpa*I fragment of pHG5 between these two restriction sites after converting the *Pst*I site (3' cohesive end) to a flush end by use of T4 DNA polymerase. Instead of the 1.7-kb *Eco*RI-*Hpa*I fragment, various restriction fragments derived from it were introduced at the same position, if necessary with suitable modification.

Plasmids pTF3 and pTF4 were constructed from the 4.6-kb *Eco*RI-*Pst*I fragment (after conversion of the *Pst*I site to a flush end by use of T4 DNA polymerase) of pPL603 and the 1.7-kb *Eco*RI-*Hpa*I fragment of pHG5 (for pTF3) or the 0.73-kb *Eco*RI-*Hin*cII fragment of pHG5 (for pTF4). Plasmids pTF5 and pTF8 were constructed from the 4.8-kb *Eco*RI fragment of pPL603 and the 382-bp *Alu*I-*Hin*cII fragment (after converting both ends to *Eco*RI sites by synthetic linkers [GGAATTCC]) of pHG5 (for pTF5) or the



2533
TAAACATTGGATC



	CAT activity (U/mg)
pTF3	4.5
pTF4	0.77
pTF5	0.24
pTF6	17
pTF7	0.072
pTF8	0.085
pPL603	0.052

FIG. 3. Relationship between the restriction fragments cloned in pPL603 and CAT activities in *B. subtilis* harboring the hybrid plasmids. See the text for details of plasmid construction. E and P, Restriction sites for *Eco*RI and *Pst*I, respectively. An open circle at the end of a fragment (for pTF3 and pTF4) indicates the flush end. *B. subtilis* cells harboring the hybrid plasmids were grown on 50 ml of Penassay broth containing 5 μg of chloramphenicol per ml at 37°C for 20 h. The cells were suspended in 5 ml of 0.1 M Tris hydrochloride buffer (pH 7.8) and sonicated for 4.5 min at 20 kHz. Supernatant obtained by centrifugation was used as the crude enzyme solution for CAT assay. CAT activities are expressed per milligram of cellular protein.

322-bp *Alu*I-*Hae*III fragment (after converting both ends to *Eco*RI sites) of pHG5 (for pTF8). Plasmids pTF6 and pTF7 were constructed from the 4.6-kb *Eco*RI-*Pst*I fragment of pPL603 and the 107-bp *Hae*III-*Alu*I fragment (*Hae*III and *Alu*I sites were converted to *Eco*RI and *Pst*I sites by *Eco*RI linker and *Pst*I linker [GCTGCAGC], respectively) of pHG5 (for pTF6) or the 47-bp *Hin*cII-*Alu*I fragment (*Hin*cII and *Alu*I sites were converted to *Eco*RI and *Pst*I sites, respectively) of pHG5 (for pTF7). The nucleotide sequences of the 119-bp *Eco*RI-*Pst*I fragment of pTF6 and the 59-bp *Eco*RI-*Pst*I fragment of pTF7 were confirmed by the dideoxy method.

After transformation of *B. subtilis* with these derivative plasmids, CAT activity in the cell extract was measured (Fig. 3). *B. subtilis* harboring pTF3 or pTF6, both of which contained the 107-bp *Hae*III-*Alu*I fragment (positions 324 to 431 in Fig. 2) of pHG5, produced 90 and 340 times more CAT, respectively, than *B. subtilis*(pPL603). Other hybrid plasmids (pTF4, pTF5, pTF7, and pTF8) which partly or completely lacked this 107-bp region, had very small CAT productivity. Some of the CAT activity encoded by pTF5 and pTF8 could be due to the postexponential promoter activity residing on the 203-bp *Eco*RI-*Pst*I fragment of pPL603 (7). From these results we concluded that the 107-bp fragment contains the whole DNA region necessary for promoter activity and that the *Hin*cII site (position 384) is located in the region essential for activity. To estimate the amount of CAT protein in *B. subtilis*, the total protein in a soluble-cell extract of *B. subtilis*(pTF6) was fractionated by SDS-polyacrylamide gel electrophoresis. Densitometric analysis of the gel showed that CAT accounted for 7% of the total soluble protein in the cell extract (data not shown). This result suggests that the 107-bp *bgaB* promoter fragment is effective not only for high expression of the *bgaB* gene but also for that of other genes.

Transcriptional start site of the *bgaB* promoter. The transcriptional start site of the *bgaB* gene was determined by S1 nuclease mapping. RNA prepared from *B. subtilis*(pHG5) was hybridized with the 588-bp *Hin*dIII-*Sau*3A1 fragment (positions 1 through 588 in Fig. 2) of pHG5 labeled with ^{32}P at the 5′ end of the *Sau*3A1 site, and the mixture was subjected to S1 nuclease digestion followed by electrophoresis on a 5% polyacrylamide gel in the presence of 7 M urea. A single 174-nucleotide fragment was found to be protected from S1 nuclease digestion. This protected fragment was electrophoresed on an 8% polyacrylamide DNA-sequencing gel. Sequencing ladders of the probe DNA were used as the size markers (Fig. 4). From this result, the transcriptional start site was assigned to the T residue (position 420) or the A residue (position 421).

FIG. 2. Nucleotide and amino acid sequence of the β-galactosidase gene of *B. stearothermophilus*. The nucleotide sequence is presented from the *Hin*dIII site to the *Sau*3A1 site. The arrow shows the *bgaB* mRNA start sites. Sequences constituting the −10 and −35 regions of the *bgaB* promoter are underlined. The possible Shine-Dalgarno sequence of the *bgaB* gene is underlined with a dashed line. The deduced amino acid sequence of the β-galI is also shown. The underlined amino acid sequence was identical with the sequences determined by automated Edman sequencing of the purified β-galI. The adenosine- and thymine-rich region is overlined with a dashed line. The restriction sites used for cloning the promoter in pPL603 are overlined.

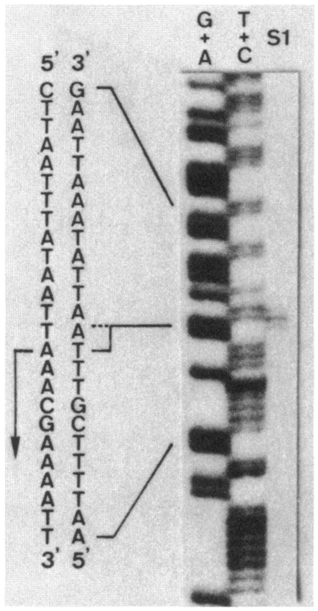


FIG. 4. S1 nuclease mapping of the transcription start site of the *bgaB* gene. The RNA preparation (20 μg) was hybridized in 80% formamide at 45°C with the 588-bp *Hin*dIII-*Sau*3A1 fragment (from position 1 to 588 in Fig. 2) labeled at the *Sau*3A1 5' terminus (40,000 cpm). The mixture was treated with 1,500 U of S1 nuclease, and digested products were analyzed on an 8% polyacrylamide gel in the presence of 7 M urea. Probe DNA was subjected to the chemical reactions specific for purines (G+A) or pyrimidines (T+C) by the method of Maxam and Gilbert (18). A 1.5-nucleotide correction has been made between the sequence ladder and the digested products (4).

DISCUSSION

Although significant homology has been reported among the amino acid sequences of *lacZ* (*E. coli*) (15), *ebgA* (*E. coli*) (28), *lacZ* (*Klebsiella pneumoniae*) (5), and *LAC4* (*Kluyveromyces lactis*) (3) β-galactosidases, no overall homology was observed between the amino acid sequence of β-galI and those sequences. The highest homology between β-galI and the *lacZ* (*E. coli*) β-galactosidase was between residues 94 through 100 of β-galI and residues 457 through 463 of *lacZ* β-galactosidase. The homologous regions are Ser-Leu-Ile-Pro-Asp-Glu-Ser-Gly (β-galI) and Ser-Leu-Gly-Asn-Glu-Ser-Gly (*lacZ* β-galactosidase). The active site of *lacZ* β-galactosidase, Glu-461, estimated by esterification with conduritol C *cis*-epoxide, in included in this homologous region (10). Homology has also been reported in the active region between *lacZ* and *ebgA* β-galactosidases (8).

Various type of intramolecular bonding may contribute to the thermostability of an enzyme molecule. One type, aliphatic bonding, can be evaluated by the aliphatic index proposed by Ikai, which is defined as the relative volume of a protein occupied by aliphatic side chains (alanine, valine, leucine, and isoleucine) (14). The index of thermostable proteins is reportedly significantly higher than that of mesophilic proteins. The aliphatic index of β-galI calculated from the formula presented by Ikai was 84.5, significantly higher than that of *lacZ* β-galactosidase (77.1). Though the size of its contribution to the thermostability of β-galI cannot be evaluated, aliphatic bonding may contribute at least in part.

The results indicate that the *bgaB* promoter activity is coded in the DNA sequence stretching from base 324 to 431 and that the start site of transcription is at position 420 or 421 (Fig. 2). From this we estimated the −10 sequence to be TAATTT (positions 409 through 414), which has three of six bases in common with the consensus sequence (TATAAT) recognized by σ^{43} RNA polymerase, and the −35 sequence to be TTGACA (positions 385 through 390), which is identical to the consensus sequence. Another candidate for the −10 region was TATAAT, which is identical to the consensus sequence located at positions 414 through 419, but this is too close to the transcriptional start site. The distance between the −35 and −10 regions, 17 bp, which is the preferred spacing of σ^{43} RNA polymerase (21), also supports the inference that the −10 region is TAATTT. Upstream of the promoter region, a 39-bp region rich in adenine and thymine (Fig. 2) was observed. This region might help the transcriptional start by destabilizing the DNA helix, as has been observed in the *celA* gene of thermophilic *Clostridium thermocellum* (1).

In addition to the *bgaB* promoter and the consensus sequence of *B. subtilis* promoter recognized by σ^{43}, the preference in the codon usage of the *bgaB* gene might help its high expression in *B. subtilis*. The preference tendency observed in 6,121 codons in *B. subtilis* (24) is similar to that in the *bgaB* gene (eg. GAU > GAC, GAA > GAG, UAU > UAC, CAU > CAC).

ACKNOWLEDGMENTS

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