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Cloning, Nucleotide Sequencing, and Characterization of the *ptsG* Gene Encoding Glucose-Specific Enzyme II of the Phosphotransferase System from *Brevibacterium lactofermentum*

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Abstract A *Brevibacterium lactofermentum* gene coding for a glucose-specific permease of the phosphoenolpyruvate-dependent phosphotransferase system (PTS) was cloned, by complementing an *Escherichia coli* mutation affecting a *ptsG* gene with the *B. lactofermentum* genomic library, and completely sequenced. The gene was identified as a *ptsG*, which enables an *E. coli* transformant to transport non-metabolizable glucose analogue 2-deoxyglucose (2DG). The *ptsG* gene of *B. lactofermentum* consists of an open reading frame of 2,025 nucleotides encoding a polypeptide of 674 amino acid residues and a TAA stop codon. The 3' flanking region contains two stem-loop structures which may be involved in transcriptional termination. The deduced amino acid sequence of the *B. lactofermentum* enzyme II^{Glc} specific to glucose (EII^{Glc}) has a high homology with the *Corynebacterium glutamicum* enzyme II^{Man} specific to glucose and mannose (EII^{Man}), and the *Brevibacterium ammoniagenes* enzyme II^{Glc} specific to glucose (EII^{Glc}). The 171-amino-acid C-terminal sequence of the EII^{Glc} is also similar to the *Escherichia coli* enzyme IIA^{Glc} specific to glucose (IIA^{Glc}). It is interesting that the arrangement of the structural domains, IIBCA, of the *B. lactofermentum* EII^{Glc} protein is identical to that of EIIs specific to sucrose or β-glucoside. Several *in vivo* complementation studies indicated that the *B. lactofermentum* EII^{Glc} protein could replace both EII^{Glc} and $EIIA^{Glc}$ in an *E. coli ptsG* mutant or *crr* mutant, respectively.

Key words: *Brevibacterium lactofermentum*, glucose-specific enzyme II, phosphotransferase system, *ptsG* gene, cloning, nucleotide sequence

In bacteria, the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) is responsible for the uptake and phosphorylation of a number of carbohydrates [15]. The system consists of two general constitutive cytoplasmic phosphoproteins, enzyme I (EI) and histidine-containing phosphocarrier protein (HPr) that are both required for the transport of PTS carbohydrates, as well as sugar-specific permeases occurring either as protein subunits or as domains in a single polypeptide chain, commonly designated as an enzyme II (EII). The cytoplasmic phosphoproteins sequentially transfer a phosphoryl group from a phosphoenolpyruvate (PEP) to a membrane-bound EII, which catalyzes the concomitant transport and phosphorylation of the carbohydrates.

The EIIs contain three or four structural domains, IIA, IIB, IIC, and IID [22], and are divided into four classes [19] according to their amino acid sequence similarities. Among them, EII proteins, specific to glucose, sucrose or β-glucoside, and belonging to the glucose-PTS class, contain three domains, IIA, IIB, and IIC. The domain IIA, that interacts with a phosphorylated-HPr, exists as a separate protein or as another domains-linked protein. The IIC domain comprises between six and eight putative transmembrane segments showing high hydrophobicity. Recently, the genes encoding EII specific to both glucose and mannose were cloned from *Brevibacterium flavum* [10], *Brevibacterium ammoniagenes* [31], and *Corynebacterium glutamicum* [13], and the nucleotide sequences of the *C. glutamicum* EII gene [14] and *B. ammoniagenes* EII gene were determined [31]. It has been thought that the mannose-specific enzyme II (EII^{Man}) of *C. glutamicum* belongs to the sucrose/β-glucoside subgroup of the glucose-PTS class on the basis of its sequence alignments, even though its sugar specificities are similar to those of the EII^{Man} from bacteria including *Escherichia coli* [9], *Lactobacillus curvatus* [28], and *Vibrio furnissii* [3]. The EII^{Man} proteins are composed of four domains, IIA, IIB, IIC, and IID, and their amino acid sequences are not

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homologous to those of glucose-specific enzyme II (EII^{Glc}) proteins while they do have a specificity toward glucose. The EIIs of both *B. ammoniagenes* and *C. glutamicum* contain IIA, IIB, and IIC domains in a single polypeptide with an arrangement of structural domains, IIBCA, corresponding to the sucrose-specific enzyme II^{Scr} (EII^{Scr}) of *Streptococcus mutans* [24], and β-glucoside-specific enzyme II^{Bgl} (EII^{Bgl}) of *E. coli* [4, 25] and *Clostridium longisporum* [5]. Furthermore, they show no sequence similarity with the EIIs of the mannose-PTS class as reported so far.

Brevibacterium lactofermentum has been used in the industrial production of various amino acids along with *B. flavum* and *C. glutamicum*. Their sugar utilities have been regarded to be very important because sugars, including glucose and sucrose, are used as major carbon sources for amino acid production. This work describes the cloning, identification, and sequencing of a complete *B. lactofermentum ptsG* gene that codes for EII^{Glc}, and compares the deduced amino acid sequence with those of *C. glutamicum* EII^{Man} and *B. ammoniagenes* EII^{Glc}.

MATERIALS AND METHODS

Chemicals, Enzymes, and Isotopes

Restriction endonucleases, DNA polymerase (Klenow fragment), T4 DNA ligase, and RNase were obtained from Boehringer Mannheim (Mannheim, Germany) and were used as recommended by the manufacturers. Sequenase, a modified T7 DNA polymerase, was obtained from the U.S. Biochemical Co. (Cleveland, U.S.A.). [α-^{35}S] dATP (1,000 Ci/mmol) was purchased from Amersham (Little Chalfont, U.K.) and 2-deoxy-D-[U-^{14}C]Glucose (300-350 mCi/mmol) from DuPont, NEN Research Products (Boston, U.S.A.). Ampicillin and agarose were purchased from Sigma Chemical Co. (St. Louis, U.S.A.), and the bacterial medium was obtained from Difco (Detroit, U.S.A.).

Bacterial Strains, Plasmids, and Media

B. lactofermentum ATCC 13869 (L-glutamic acid producer) was used as a source of the gene coding for the EII^{Glc} of the PTS. *E. coli* JM109 (*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi* Δ(*lac-proAB*) F'[*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]) was used as the host for the cloning experiments and M13 phage growth [30]. For the *in vivo* complementation studies, *E. coli* mutant strains ZSC113 [7], carrying the *ptsG, manA,* and *glk* mutations, and JLV86 [29], with *crr, nagE, manI,* and *manA* mutations, were used. The plasmids pUC9 and M13 mp18/19 were used for all the cloning and sequencing experiments. MacConkey indicator plates containing glucose (1%) were used to investigate the sugar-fermenting capacities of the *E. coli* transformants.

Transport Experiment

E. coli cells were grown to the late logarithmic phase in an M9 minimal medium (6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$ per l) supplemented with a 0.4% non-PTS carbohydrate (glycerol) as the sole carbon source. After centrifugation, the cells were washed twice with an M9 minimal medium that did not contain the carbon source, and were then resuspended in the same medium at $O.D._{600}$=0.5 (0.3 mg dry weight/ml). To decrease the uptake rate of 2DG in the cells, the cell suspension was incubated in ice for 30 min. [^{14}C]2DG (0.15 Ci/mol) was then added to 5 ml of the ice-cold cell suspension, and 0.5 ml samples were taken at 10 sec intervals, filtered through a membrane filter (0.45 μm porosity), and washed quickly with an ice-cold suspension medium. The filters with cells were placed in 10 ml of a scintillation fluid and counted in a liquid scintillation spectrometer (Beckman, Fullerton, U.S.A.).

DNA Preparations and Manipulations

For the rapid isolation of the plasmids from the *E. coli* cells, the alkaline lysis method was employed as described by Birnboim and Doly [2]. Single-stranded DNAs of the M13 derivatives were purified as described by Messing [16]. The chromosomal DNA was isolated from the cells of *B. lactofermentum* grown exponentially in an LB medium supplemented with glycine (1%) according to the preparative method described by Rodriquez and Tait [20].

Construction of *B. lactofermentum* Gene Library

Fifty micrograms of the purified *B. lactofermentum* chromosomal DNA was partially digested with *Bgl*II, and DNA fragments ranging from 2 to 10 kb were isolated by sucrose gradient centrifugation for 20 h at 25,000 rpm in a Beckman SW40 rotor. The *Bgl*II-generated chromosomal DNA fragments were ligated to *Bam*HI-digested, dephosphorylated pUC9. A ligation mixture was used to transform the *E. coli* mutant strain ZSC113.

DNA Sequencing and Analysis

The DNA fragments generated by the restriction endonucleases were cloned into either pUC9 or M13mp18/19. Both strands of the subcloned fragments were completely sequenced according to the dideoxy-chain termination method [23] using the T7 sequencing kit and single- or double-stranded DNA as templates. A reverse primer from Phamacia was used for sequencing the double-stranded DNAs. The DNA and protein sequences were analyzed using the DNASIS (Hitachi Software Engineering, Japan) program.

RESULTS AND DISCUSSION

Cloning of the *ptsG* Gene from *B. lactofermentum*

The *ptsG* gene of *B. lactofermentum* was cloned by *in vivo* complementation of an *E. coli* mutant strain lacking EII^{Glc}. The identification of the *E. coli* ZSC113 transformants which expressed the EII^{Glc} enzyme was based on the ability of the cells to ferment glucose on a MacConkey plate supplemented with glucose (1%) as an additional carbon source, as described by Yoon *et al.* [31]. A library of *B. lactofermentum* *Bgl*II DNA fragments was constructed in the vector pUC9, and transformed into *E. coli* ZSC113. The cells were plated on a MacConkey-glucose plate containing ampicillin. From approximately 5,000 transformants, two glucose-fermenting colonies were identified based on their deep-red color. The plasmid DNAs from the two clones were found to carry the same 6.2-kb *Bgl*II insert, and the plasmid was named pBSBG2.

To determine whether the 6.2-kb *B. lactofermentum* DNA in pBSBG2 complements the function of $EIIA^{Glc}$ in an *E. coli crr* mutant, the *E. coli crr* mutant strain JLV86 was transformed with pBSBG2 and pUC9, respectively. The resulting JLV86 strain carrying the pBSBG2 formed red colonies on MacConkey-glucose plates, while the JLV86 with the pUC9 formed white colonies, suggesting that the *B. lactofermentum* DNA of pBSBG2 contains a glucose transporter gene. However, it has been reported that glucose can be transported by the fructose-PTS as well as by the glucose-PTS in *B. flavum* and *C. glutamicum*. In addition, glucose-PTS is active on 2DG, whereas fructose-PTS is not [17]. In order to determine whether the *B. lactofermentum* gene in pBSBG2 included glucose-PTS or fructose-PTS, the sugar uptake of the *E. coli* cells carrying plasmids was tested using [^{14}C]2DG as described previously. Since 2DG is a non-metabolizable glucose analogue, its accumulation was determined by measuring the radioactivity of the cells as shown in Fig. 1. The *E. coli* ZSC113 cells could effectively transport the [^{14}C]2DG with the plasmid pBSBG2 whereas it could barely do so with pUC9. It was also found that the strain ZSC113 (pBSBG2) could grow in an M9 minimal medium containing glucose or mannose as the sole carbon source yet the ZSC113 (pUC9) could not (data not shown).

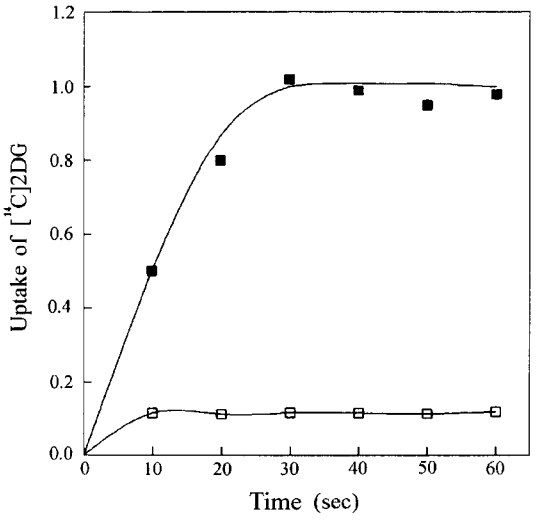

Fig. 1. Uptake of [^{14}C]2DG in *E. coli* ZSC113 cells carrying plasmids pBSBG2 (-■-) and pUC9 (-□-).
Uptake activity is expressed in nanomoles per mg of cells dry weight.

From these results, it can be concluded that the 6.2-kb *Bgl*II insert of pBSBG2 carries the *ptsG* gene encoding the glucose-specific permease of *B. lactofermentum* that is capable of complementing the function of glucose uptake in *E. coli* mutants defective in EII^{Glc} or $EIIA^{Glc}$ activities. However, it is not clear whether the gene product is a single polypeptide or not.

Subcloning, Complementation, and Sequencing

In order to determine the minimal size of the DNA containing the *B. lactofermentum ptsG* gene in the plasmid pBSBG2, a new recombinant plasmid pBSG2 complementing the *E. coli* mutants lacking EII^{Glc} and $EIIA^{Glc}$ was obtained by subcloning a 3.2-kb *Hin*dIII fragment into pUC9, which included the *B. lactofermentum* DNA and a part of mutiple cloning sites derived from pUC9 (Fig. 2).

To locate the *ptsG* structural gene on the 3.2-kb *B. lactofermentum* DNA fragment of pBSG2, five subclones were constructed and used for the transformation of both *E. coli* strains ZSC113 and JLV86. The plasmid pBSG2 was partially or completely digested with restriction enzymes *Hin*dIII for pBST3, *Pst*I for pBSP9, *Bam*HI for pBSB6, and *Ava*I for pBSA7 and pBST5, respectively. The restricted DNA fragments, which contained the *B. lactofermentum* DNA, were self-ligated or ligated with pUC9 to obtain the plasmids pBST3, pBSP9, pBSB6, pBSA7, and pBST5. Maps of the resulting plasmids and phenotypes of the *E. coli* ZSC113 or JLV86 transformed with them onto the MacConkey-glucose plates are shown in Fig 2. In the *B. lactofermentum* DNA of pBSG2, the DNA fragments corresponding to the right regions could be deleted without affecting the capability of the recombinant plasmids (pBST3, pBSA7, pBSP9) to complement the EII^{Glc} activity of *E. coli* ZSC113. It appears that the *B. lactofermentum ptsG* gene in the three recombinant plasmids, pBST3, pBSA7, and pBSP9, can be expressed in *E. coli* regardless of its orientation with respect to the *lacZ* promoter in the vector. This may indicate that the *ptsG* gene uses its own promoter in *E. coli*. The plasmids pBST5 and pBSB6 could not complement the EII^{Glc} activity of *E. coli* ZSC113. It was also found that the $EIIA^{Glc}$ activity of *E. coli* JLV86 could not be complemented with any one of the pBSG2 derivatives, suggesting that the right region on *B. lactofermentum* DNA located in pBSG2 corresponds to the *crr* gene of *E. coli*.

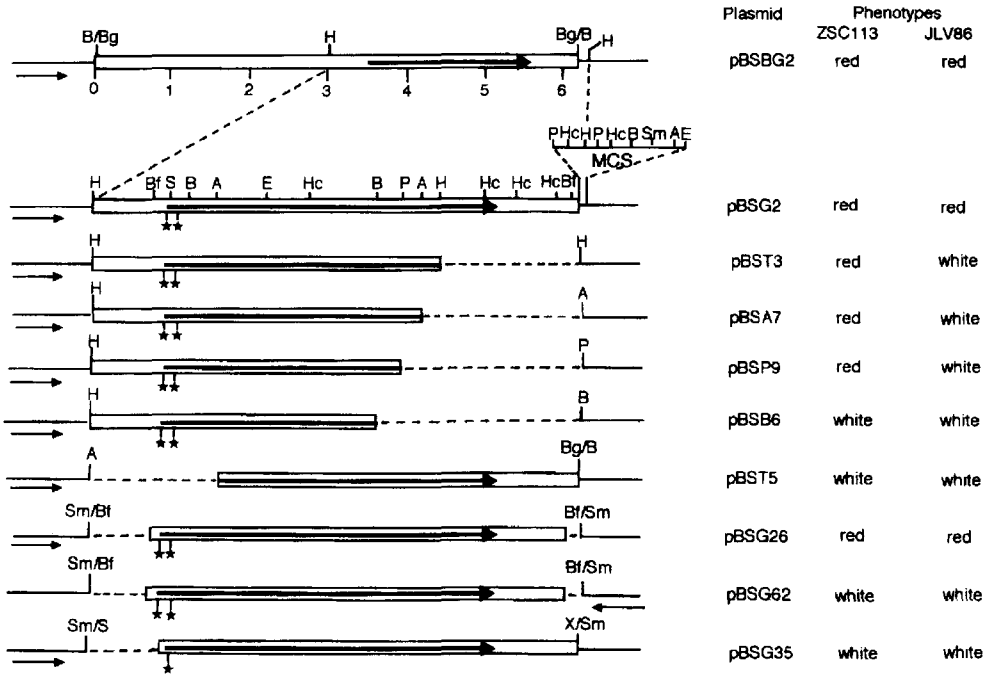

Fig. 2. Restriction endonuclease map and characterization of deletion derivatives of pBSG2 containing the *ptsG* gene of *B. lactofermentum*.

The construction of the plasmids is described in the text. The phenotypes of the *E. coli* mutant strains ZSC113 and JLV86 transformed with these plasmids were determined by checking the color of their colonies grown overnight on MacConkey-glucose plates. MCS refers to the multiple cloning sites of pUC9 which was used as a vector DNA for subcloning. The boxes and lines indicate the DNA regions corresponding to *B. lactofermentum* DNA and pUC9, respectively. In the box of recombinant plasmids, the bold lines denote the 2,025 nucleotides encoding the *ptsG* gene with the arrow indicating the direction of the transcription. Under the bold line, the asterisks correspond to the two putative ATG start codons of the *ptsG* gene. The thin arrow indicates the *lacZ* promoter of pUC9. The dashed lines correspond to the deleted regions in the *B. lactofermentum* DNA. The restriction site abbreviations are as follows: A, *Aval*; B, *Bam*HI; Bf, *Bfr*I; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; P, *Pst*I; S, *Ssp*I; Sm, *Sma*I.

For sequencing the complete *B. lactofermentum* DNA of pBSG2, various restricted fragments were subcloned into either pUC9 or M13mp18/19. Overlapping sequences of the 3.2-kb insert of pBSG2 were obtained throughout and the entire sequence was thus determined from both strands.

Nucleotide and Amino Acid Sequences

The nucleotide sequence of a complete *B. lactofermentum* gene and deduced amino acid sequence are both shown in Fig. 3. Two putative ATG initiation codons (positions 506 and 578) are present at the beginning of a coding sequence of 2,022 bp or 1,950 bp, both of which terminate at position 2,527 with the ochre stop codon TAA.

In order to determine the translation initiation site as one of the two putative ATG initiation codons of the coding sequence for the *ptsG* gene, recombinant plasmids were constructed with the deletion of different nucleotides upstream from the ATG codons using the restriction endonucleases *Bfr*I or *Ssp*I of which the cleavage sites are indicated in Figs. 2 and 3. This was followed by complementing the function of the glucose uptake in both *E. coli* ZSC113 and JLV86. From the plasmid pBSG2, *Bfr*I and a *Ssp*I-*Sma*I fragment that included the *ptsG* gene were subcloned into pUC9. From the resulting plasmids, pBSG26, pBSG62, and pBSG35, the plasmid pBSG26 conferred a glucose-utilizing activity on the *E. coli* mutants, whereas the others could not (Fig. 2). Accordingly, the *ptsG* gene of pBSG26 includes the two putative ATG start codons, and is expressed with the *lacZ* promoter of pUC9. The results strongly suggest that the translation of the *ptsG* gene starts at position 506. The AAGG sequence element, six bases upstream from the first ATG start codon at nucleotide position 496-499, could be the ribosome binding site for the mRNA.

The ORF defined by the nucleotide sequence is 2,022 nucleotides long and encodes a polypeptide of 674 residues with a calculated M_r of 71,543. This value is comparable to those of large EII proteins consisting of domains IIA, IIB, and IIC. In the 3'-flanking region of the *ptsG* gene, there are two palindromic regions beginning 11 nucleotides and 74 nucleotides downstream of the TAA stop codon, respectively. These elements could be involved in the rho-independent termination of the *ptsG* gene transcription.

A hydropathy of the deduced amino acid sequence was calculated using the method of Kyte and Doolittle [11] (data not shown). From the result, it was predicted that the EII^{Glc} would contain a hydrophobic region (IIC domain)

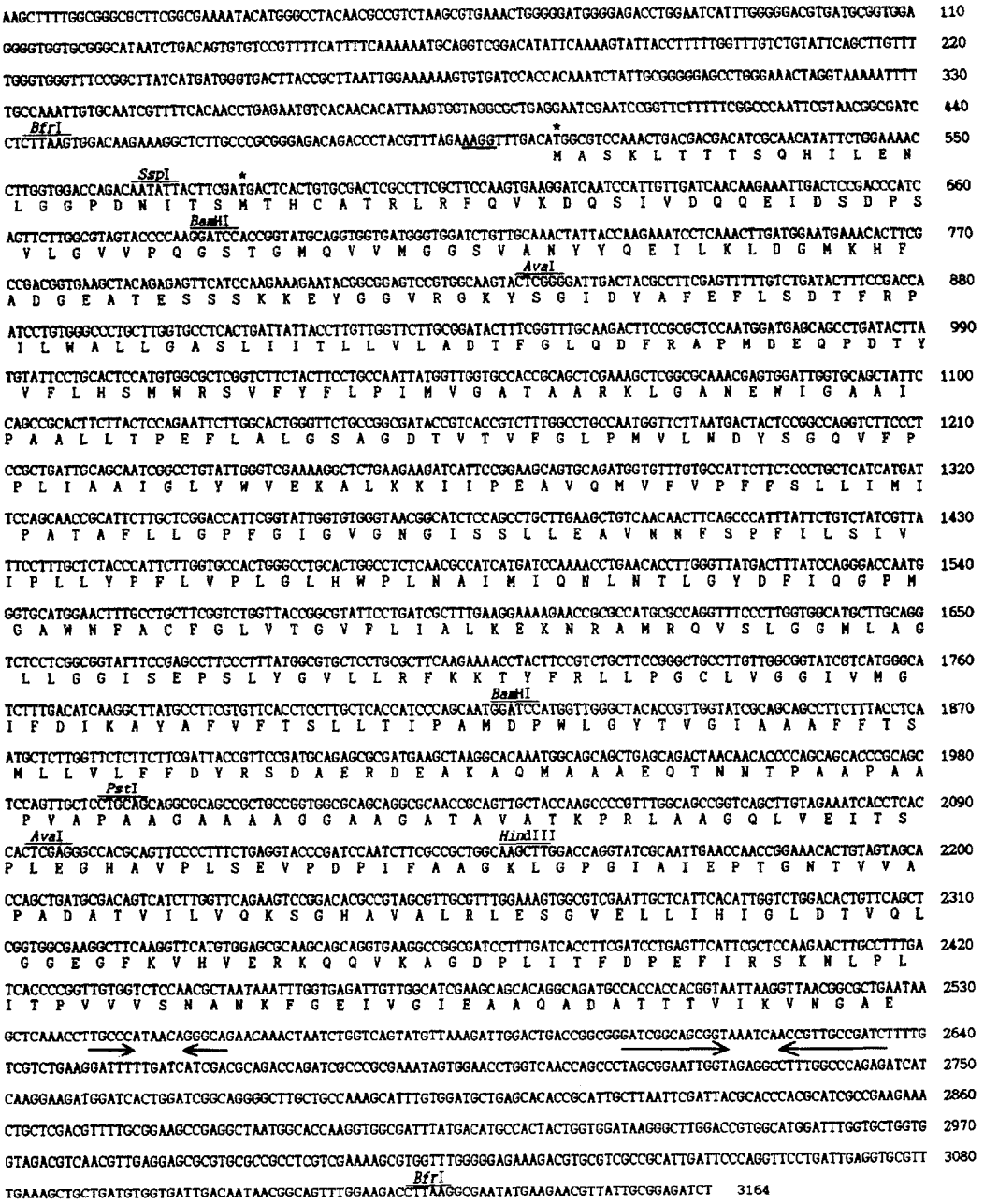

Fig. 3. Nucleotide sequence of 3,164-nucleotide *Hin*dIII-*Bgl*II fragment and deduced amino acid sequence.
The underlined sequence preceding the ATG start codon of the *ptsG* gene is a putative ribosome-binding site. The overlined sequences are the restriction sites used for constructing the recombinant plasmids as shown in Fig. 2. The two putative ATG start codons are indicated with asterisks above the sequences. The two inverted repeats are underlined by horizontal arrows. The numbers at the end of each line correspond to the nucleotide positions. The sequence has been deposited with GenBank under accession number L18875.

corresponding to amino acid residues from 120 to 470 with six sub-regions of an average hydropathy exceeding 1.0, each comprised of 23 amino acid residues capable of spanning the cytoplasmic membrane. The amino-terminal part (1-120 aa) of the protein was relatively hydrophilic (IIB domain) and the carboxy-terminal part (470-674 aa) was relatively hydrophobic (IIA domain), indicating that the EII^{Glc} of *B. lactofermentum* has an arrangement of IIBCA domains identical to those of *C. glutamicum* EII^{Man} and *B. ammoniagenes* EII^{Glc}.

Comparison of *B. lactofermentum* EII^{Glc} with Other EIIs

When the deduced amino acid sequence of *B. lactofermentum* EII^{Glc} was compared with the sequences of other EIIs in the NCBI database using the BLAST search program [1], the EII^{Glc} showed the highest score of a 90.9%

Fig. 4. Comparison of *B. lactofermentum* EII^{Glc} (BLA) with *C. glutamicum* EII^{Man} (CGL) and *B. ammoniagenes* EII^{Glc} (BAM).
The amino acid sequences of the three polypeptides are shown by one-letter code and have been aligned by introducing gaps (hyphens) to maximize the similarities. The residues identical to the EII^{Glc} amino acid sequence of *B. lactofermentum* are indicated by asterisks in all other sequences. The numbers at the beginning of each line correspond to the amino acid positions in the protein.

sequence homology with the EII^{Man} of *C. glutamicum* and a 44.8% sequence identity with the EII^{Glc} of *B. ammoniagenes* (Fig. 4). Gaps were introduced to optimize the alignment. In addition, *B. lactofermentum* EII^{Glc} was found to be similar to the EII^{Bgl} proteins from *E. coli* [4, 27], *B. subtilis* [27], *Erwinia chrysanthemi* [8], *Klebsiella oxytoca* [12], and *C. longisporum*, [5], and the EII^{Scr}proteins from *S. mutans* [24] and *Streptococcus sobrinus* [6]. The intact EII^{Glc} shows an approx. 30% sequence similarity with the above, whereas the 171-amino-acid C-terminal region of EII^{Glc} exhibits a 38% similarity with the EIIA^{Glc} of *E. coli* [21].

The IIA domain of *B. lactofermentum* EII^{Glc} is connected to the hydrophobic region of the protein by a PA linker consisting of Pro-Ala repeats (487-503 aa), which is similar to those of *C. glutamicum* EII^{Man}, and EII^{Man} and EII^{Nag} (*N*-acetylglucosamine-specific protein) from *E. coli* [18]. However, the amino acid sequence of the PA linker region exhibits the lowest similarity between the EII proteins of *B. lactofermentum* and *C. glutamicum*, while the amino acid sequences of the remaining regions have over 90% homology with each other. As shown in Figs. 2 and 3, the 178-amino-acid carboxy-terminal region including the EIIA portion could be deleted from *B. lactofermentum* EII^{Glc} without affecting the remaining portion of the EII^{Glc} capability of complementing the function of glucose uptake in *E. coli* ZSC113. This suggests that the EII^{Glc} of *B. lactofermentum* interacts efficiently with the EIIA^{Glc} in the cells of *E. coli*, as demonstrated previously by Vogler *et al.* [29] and Sondej *et al.* [26].

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