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# Cloning, Expression, and Isolation of the Mannitol Transport Protein from the Thermophilic Bacterium *Bacillus stearothermophilus*

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**A mannitol phosphotransferase system (PTS) was identified in *Bacillus stearothermophilus* by in vitro complementation with *Escherichia coli* EI, HPr, and IIA<sup>Mtl</sup>. Degenerate primers based on regions of high amino acid similarity in the *E. coli* and *Staphylococcus carnosus* EII<sup>Mtl</sup> were used to develop a digoxigenin-labeled probe by PCR. Using this probe, we isolated three overlapping DNA fragments totaling 7.2 kb which contain the genes *mtlA*, *mtlR*, *mtlF*, and *mtlD*, encoding the mannitol IICB, a regulator, IIA, and a mannitol-1-phosphate dehydrogenase, respectively. The *mtlA* gene consists of 1,413 bp coding for a 471-amino-acid protein with a calculated mass of 50.1 kDa. The amino acid sequence shows high similarity with the sequence of IICB<sup>Mtl</sup> of *S. carnosus* and the IICB part of the IICBA<sup>Mtl</sup>s of *E. coli* and *B. subtilis*. The enzyme could be functionally expressed in *E. coli* by placing it behind the strong *tac* promoter. The rate of thermal inactivation at 60°C of *B. stearothermophilus* IICB<sup>Mtl</sup> expressed in *E. coli* was two times lower than that of *E. coli* IICB<sup>Mtl</sup>. IICB<sup>Mtl</sup> in *B. stearothermophilus* is maximally active at 85°C and thus very thermostable. The enzyme was purified on Ni-nitrilotriacetic acid resin to greater than 95% purity after six histidines were fused to the C-terminal part of the transporter.**

Many bacteria transport D-mannitol and other carbohydrates via a phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS). Two general cytoplasmic proteins, EI and HPr, are responsible for the transfer of the phosphoryl group from PEP to different sugar-specific PTS proteins (15, 18, 22). In the mannitol PTSs of *Staphylococcus carnosus* and *Enterococcus faecalis* (7, 8), the phosphoryl group of HPr is transferred first to the soluble IIA<sup>Mtl</sup> and then to the B domain of IICB<sup>Mtl</sup>. (The terms A domain, B domain, C domain, etc., are used for domains which are covalently attached. Enzymes or domains which have been cloned and expressed separately are designated IIA<sup>Mtl</sup> for the A domain of the mannitol-specific enzyme II and IICB<sup>Mtl</sup> for the CB domain of the mannitol-specific enzyme II. The genes encoding IICB<sup>Mtl</sup> or IICBA<sup>Mtl</sup>, IIA<sup>Mtl</sup>, mannitol-1-phosphate dehydrogenase, and a regulatory protein are designated *mtlA*, *mtlF*, *mtlD*, and *mtlR*, respectively.) This B domain is responsible for the phosphorylation of mannitol that is transported by the transmembrane C domain of IICB<sup>Mtl</sup>. In the case of the mannitol PTS of *Escherichia coli* (13), IIA is covalently linked by a flexible peptide chain to the B domain to form a IICBA<sup>Mtl</sup> protein. In general, the genes of proteins involved in the uptake and phosphorylation of a specific sugar are located in a single operon. To date, the *mtlA* genes of *E. coli* (13), *S. carnosus* (7), and *Bacillus subtilis* (1) and the cryptic *mtlA* gene of *E. coli* (29), all encoding homologous mannitol transporters, have been cloned. Our attempts to crystallize the *E. coli* protein have been unsuccessful, possibly because of limited stability. Since membrane pro-

teins in thermophilic bacteria were found to be more stable than their mesophilic counterparts, a search for a mannitol PTS was done in these organisms. A mannitol PTS had been found in the obligate anaerobic thermophile *Clostridium thermocellum* (21). This paper reports the identification of a mannitol PTS in the aerobic thermophile *Bacillus stearothermophilus* and presents the cloning of the mannitol operon, sequencing of the *mtlA* gene, functional expression of *B. stearothermophilus* IICB<sup>Mtl</sup> in *E. coli*, and purification and partial characterization of the mannitol transporter.

## MATERIALS AND METHODS

Strains and plasmids used in this study are listed in Table 1. Restriction enzymes, *Taq* DNA polymerase, Klenow enzyme, T4 DNA ligase, digoxigenin (DIG) labeling mix, and DIG nucleic acid detection kit were purchased from Boehringer Mannheim. The DNA sequencing kit was obtained from Pharmacia. Sodium deoxycholate (DOC), *n*-decyl-β-D-maltopyranoside (d-maltopyranoside), PEP, and mannitol-1-phosphate were obtained from Sigma. Decylpolyethylene glycol 300 (d-PEG) was synthesized by B. Kwant, Department of Chemistry, University of Groningen. D-[1-<sup>14</sup>C]mannitol (2.04 GBq/mmol) was obtained from Amersham. D-[1-<sup>3</sup>H(N)]mannitol (976.8 GBq/mmol) was from NEN Research Products. Ni-nitrilotriacetic acid (NTA) resin was obtained from Diagen. EI, HPr, and IIA<sup>Mtl</sup> were purified as described previously (25, 32, 35). Membrane vesicles with *E. coli* IICB<sup>Mtl</sup> were obtained from strain LGS322 expressed with plasmid pMaIICBPr (4).

**Preparation of membrane vesicles of *B. stearothermophilus*.** A *B. stearothermophilus* preculture was grown overnight at 45°C on TY medium (27). The preculture was diluted 25-fold into M9 minimal medium (27) with 1 mM MgSO<sub>4</sub>, 0.1 mM CaCl<sub>2</sub>, 1 ml of trace solution (33) per ml, and 50 mM mannitol as the only carbon source and grown to an optical density at 660 nm of 0.8 at 65°C. Four 5-liter flasks with 500 ml of the same minimal medium were inoculated with this culture (1:25) and grown in a New Brunswick G25 incubator shaker at 65°C and 250 rpm to an optical density at 660 nm of 1. The cells were spun down and resuspended in 50 ml of 25 mM Tris (pH 7.5)–5 mM dithiothreitol (DTT)–1 mM phenylmethylsulfonyl fluoride–1 mM MgSO<sub>4</sub>–0.2 mg of DNase and RNase per ml. The cells were ruptured with a French press at 10,000 lb/in<sup>2</sup>, and 2 mM EDTA was added. After removal of whole cells by centrifugation at 20,000 × *g*, *B. stearothermophilus* membrane vesicles were collected by ultracentrifugation at 200,000 × *g* for 1 h. The vesicles were washed in 20 ml of buffer and collected by

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
<b>Strains</b>		
<i>B. stearrowthermophilus</i>		ATCC 7954
<i>E. coli</i> LGS322	$\Delta(mtlA'p)$ <i>mtlD</i> (Con) $\Delta(gutR'$ <i>MDBA-recA</i> )	9
<i>E. coli</i> JM101		36
<b>Plasmids</b>		
pSK <sup>-</sup>	Amp <sup>r</sup> <i>lacZ</i>	Stratagene
pTAQI	Amp <sup>r</sup> <i>Ptac lacI'</i>	Laboratory collection
pSK408	pSK + 408-bp PCR fragment of <i>B. stearrowthermophilus mtlA</i>	This study
pSKH1.9	pSK + 1.9-kb <i>Hind</i> III <i>B. stearrowthermophilus</i> fragment	This study
pSKEB1.7	pSK + 1.7-kb <i>Eco</i> RI- <i>Bam</i> HI <i>B. stearrowthermophilus</i> fragment	This study
pSKEB5.5	pSK + 5.5-kb <i>Eco</i> RI- <i>Bam</i> HI <i>B. stearrowthermophilus</i> fragment	This study
pSKMtl-A	pSK + complete <i>B. stearrowthermophilus mtlA</i> gene	This study
pSKIIBC	pSK + <i>B. stearrowthermophilus mtlA</i> with <i>Xba</i> I site prior to the ATG start	This study
pSKIIBC1	Intermediate pSKIIBC	This study
pTAQIIBC	Expression system of <i>B. stearrowthermophilus</i> IICB <sup>Mtl</sup>	This study
pSKIIBC2	Intermediate pQEIIBC-his	This study
pQE-12	His tag expression vector	Diagen
pQEIIBC-his	IICB + His tag in pQE-12	This study
pSKIIBC-his	Intermediate pTAQIIBC-his	This study
pTAQIIBC-his	Expression system for <i>B. stearrowthermophilus</i> IICB <sup>Mtl</sup> -His	This study
pMacBPr	Expression system for <i>E. coli</i> IICB <sup>Mtl</sup>	4

a second ultracentrifugation. Finally, the membrane vesicles were resuspended in 1 ml of 25 mM Tris (pH 8.4)–5 mM DTT per g of cells with a glass potter and stored at  $-80^{\circ}\text{C}$ .

**PEP-dependent mannitol phosphorylation assay.** PEP-dependent mannitol phosphorylation activity was measured as described by Robillard and Blaauw (25). The assays were performed in a 100- $\mu\text{l}$  assay mixture containing 25 mM Tris (pH 7.5), 5 mM  $\text{MgCl}_2$ , 0.25% d-PEG, 5 mM DTT, 5 mM PEP, and 33  $\mu\text{M}$  mannitol, plus 0.3  $\mu\text{M}$  EI, 16.8  $\mu\text{M}$  HPr, and 5.9  $\mu\text{M}$  IIA<sup>Mtl</sup>, all from *E. coli*.

**Mannitol/mannitol-1-phosphate exchange assay.** The mannitol/mannitol-1-phosphate exchange activity was measured in a reaction mixture of 100  $\mu\text{l}$  containing 50 mM sodium phosphate (pH 6), 5 mM DTT, 5 mM  $\text{MgCl}_2$ , and 2 mM mannitol-1-phosphate. The reaction was started by the addition of 200 nM [<sup>3</sup>H]mannitol. The formation of phosphorylated [<sup>3</sup>H]mannitol was determined as described by Lolkema et al. (17).

**General methods.** Standard DNA manipulations were performed as described by Sambrook et al. (27). *B. stearrowthermophilus* chromosomal DNA was isolated essentially as described by Leenhouts et al. (14) except that mutanolysine was omitted. Plasmid DNA was isolated as described by Birnboim and Doly (3). Protein concentration was determined by the method of Lowry et al. (19). Southern blots and colony blots were made on GeneScreen nylon membranes as described in the GeneScreen manual. Detection of the *B. stearrowthermophilus mtlA* gene was performed with a DIG nucleic acid detection kit as instructed by the manufacturer (Boehringer Mannheim).

**Cloning of an internal region of *B. stearrowthermophilus mtlA*.** Two highly conserved regions within the amino acid sequence of *S. carnosus* IICB<sup>Mtl</sup> (7) and *E. coli* IICB<sup>Mtl</sup> (13) were selected to design degenerate primers (5). A 17-bp-long forward primer [5'-CA(T/C) GA(A/G) AT(T/C) TA(T/C) TT(T/C) CC-3'] was directed against a conserved area in the *E. coli* sequence extending from the H-256 codon to the first two bases of the P-261 codon. The reverse primer [3'-AC(A/G) CT(A/G) CG(T/C/A/G) CC(T/C/A/G) TAC CC-5'] was derived from the C-384 to G-398 codons. A 408-bp fragment was amplified by 30 PCR cycles in a 100- $\mu\text{l}$  reaction mixture containing 0.2 mg each of the forward and reverse primers per ml, 200  $\mu\text{M}$  deoxynucleoside triphosphate, 2 mM  $\text{MgCl}_2$ , 10 mM Tris (pH 8.3), 50 mM KCl, 2.5 U of *Taq* DNA polymerase, and 500 ng of *B. stearrowthermophilus* chromosomal DNA. One PCR cycle consisted of 1 min of denaturation at  $94^{\circ}\text{C}$  followed by a decrease in temperature to  $20^{\circ}\text{C}$  within 2 min. After 1 min of annealing at  $20^{\circ}\text{C}$ , the temperature was raised within 5 min to  $70^{\circ}\text{C}$ ; after 1 min of extension at  $70^{\circ}\text{C}$ , the temperature was raised to  $94^{\circ}\text{C}$  for the next cycle. The amplified 408-bp fragment was cloned into the *Eco*RV site of pSK<sup>-</sup>, yielding pSK408, and was sequenced.

**Cloning of the *B. stearrowthermophilus mtlA* and flanking regions.** The 408-bp fragment, as the template, and the degenerate primers were used to synthesize a DIG-labeled DNA probe (probe I) by PCR as described by Lion and Haas (16). This probe was used to identify a 1.9-kb fragment from a *Hind*III digest and a 5.5-kb fragment from an *Eco*RI-*Bam*HI digest of *B. stearrowthermophilus* chromosomal DNA. The two fragments were cloned into pSK<sup>-</sup>, yielding pSKH1.9 and pSKEB5.5 (Fig. 1). Both fragments contained the area encoding the C-terminal part of IICB<sup>Mtl</sup> and the downstream region of the gene. To obtain the N-terminal part of the *mtlA* gene, a second DIG-labeled probe (probe II) was synthesized by PCR (16) using 5'-AATCATCGGCGGGCTGT-3' and 5'-CGCGGATTGCTTTGCCAT-3' as primers and pSKH1.9 as the template. With this probe, a 1.7-kb

fragment was identified in an *Eco*RI-*Bam*HI digest of *B. stearrowthermophilus* chromosomal DNA and was cloned into pSK<sup>-</sup>, yielding plasmid pSKEB1.7 (Fig. 1).

**DNA sequencing.** Subclones of the initial cloned fragments were sequenced as described by Sanger et al. (28), using a Pharmacia T7 sequence kit or sequenced with a Pharmacia A.L.F. automatic sequencer. DNA sequences were analyzed with the program PC-GENE. The EMBL database was screened for similar proteins, using the National Center for Biotechnology Information BLAST electronic mail server (2).

**Expression system for *B. stearrowthermophilus* IICB<sup>Mtl</sup> and histidine-tagged IICB (IICB-His) in *E. coli*.** Figure 1 lists the constructs necessary for the cloning of the *mtlA* gene in the expression vector pTAQI behind the *tac* promoter and a ribosome binding site. An *Xba*I site was created prior to the ATG start codon by using PCR and a forward primer containing the necessary mutations (5'-AATC TAGAATGACTCATAAC-3') (underlining indicates the mutated bases; italics

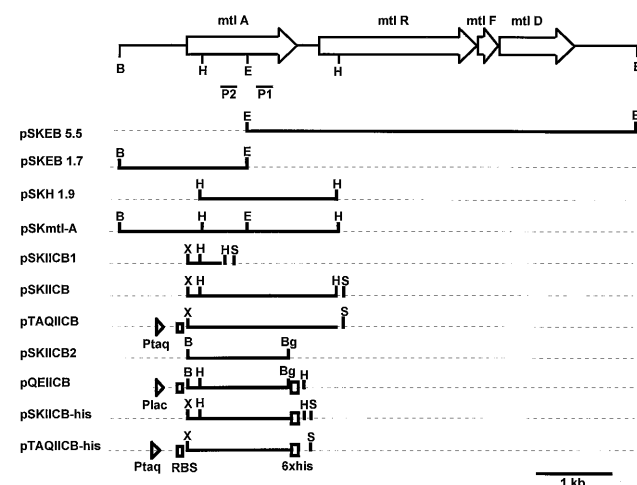


FIG. 1. Scheme of the *B. stearrowthermophilus* mannitol operon and the constructs used to create the IICB<sup>Mtl</sup> expression systems pTAQIIBC and pTAQIIBC-His. The open arrows indicate the genes identified, *mtlA*, *mtlR*, *mtlF*, and *mtlD*. The lengths and the positions of the three cloned fragments and subclones are indicated below. The locations of the DNA regions recognized by probes I and II are indicated by P1 and P2, respectively. Promoters are represented by triangles. RBS, ribosome binding site; 6xhis, the six-histidine tag. Only restriction sites used in the previous and subsequent cloning steps of a construct are indicated (B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; S, *Sal*I; X, *Xba*I). A detailed description of the construction is given in the Materials and Methods.

indicate a newly constructed restriction site). The reverse primer was directed against a sequence downstream of the *Hind*III site at position 788 (3'-CGACC TTAGTAGCCGCCCC-5'). A 439-bp fragment was amplified by PCR using both primers and pSKEB1.7 as the template and cloned in a T-tailed *EcoRV* site (20) of pSK<sup>-</sup>, yielding pSKIICB1. The part of the fragment downstream of the *Hind*III site was deleted by digestion of pSKIICB1 with *Hind*III and replaced by the 1.9-kb *Hind*III fragment of pSKH1.9, yielding pSKIICB. Finally, the gene was removed from pSKIICB and placed in pTAQI with the *Xba*I and *Sal*I restriction sites, creating the expression vector pTAQIICB.

A six-histidine tag was fused to the C terminus of the protein for rapid purification. To place the gene into the His tag expression vector pQE-12, a *Bam*HI site at the start and a *Bgl*II site at the end of the gene were created by PCR. Two primers containing the necessary mutations were designed (forward, '5-CTCATGGATCCGAAAATCAGGCT-3'; reverse, '5-CCTTTTAAGATCTT AACATTTTCG-3'). First, the PCR fragment was cloned in the *EcoRV* site of pSK<sup>-</sup> and sequenced. The vector created, pSKIICB2, was used to clone the gene into the *Bam*HI and *Bgl*II sites of pQE-12. Since IICB-His was poorly expressed from the pQEIICB-his construct, the 5' part of the gene with the six histidine codons was transferred to pSKIICB after digestion with *Hind*III. The entire gene, including the His tag-coding region from the resulting plasmid, pSKIICB-his, was placed into pTAQI by using the *Xba*I and *Sal*I sites. For the expression of IICB<sup>Mtl</sup> and IICB-His, pTAQIICB or pTAQIICB-his was transformed to the *E. coli* mannitol deletion mutant LGS322 (9). Cells were grown on LB medium (27) to an optical density at 660 of 0.6 and induced with 3 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). After 2 h, the cells were harvested and membrane vesicles were prepared as described below. The membrane vesicles were resuspended in 1 ml of buffer per g (wet weight) of cells.

**Purification of IICB<sup>Mtl</sup>.** IICB-His was solubilized from membrane vesicles of *E. coli* by extraction with acetone-recrystallized DOC. While being stirred, 0.5 ml membrane vesicles was slowly added to 10 ml of extraction buffer (25°C) containing 0.5% DOC, 25 mM Tris (pH 8.4), 3 mM  $\beta$ -mercaptoethanol, 35 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride. The solution was stirred for 30 min, after which unsolubilized membranes were removed by ultracentrifugation for 45 min at 200,000  $\times$  g. The supernatant was brought to final concentrations of 20 mM imidazole and 300 mM NaCl to reduce nonspecific binding to the resin. Then 0.4 ml of Ni-NTA resin was added, and the mixture was incubated with permanent agitation for 1 h at 4°C. The Ni-NTA resin was collected in a small column and washed successively with 1 column volume of 0.5% (wt/vol) DOC, 1 column volume of 0.35% (vol/vol) d-PEG, and 2 column volumes of 6 mM d-maltopyranoside, all in buffer containing 25 mM Tris (pH 8.5), 300 mM NaCl, 20 mM imidazole, and 3 mM  $\beta$ -mercaptoethanol. The enzyme was eluted in 2 column volumes with washing buffer containing 6 mM d-maltopyranoside and 200 mM imidazole. During the purification, 100  $\mu$ M mannitol was present in all solutions to stabilize the protein.

**Nucleotide sequence accession number.** The GenBank nucleotide sequence accession number for the *B. stearothersophilus mtl* locus is U18943.

## RESULTS

### Identification of the mannitol PTS in *B. stearothersophilus*.

A mannitol PTS has been found in *B. subtilis* (24), and since it was possible to grow *B. stearothersophilus* on minimal medium with mannitol as the only carbon and energy source, a mannitol PTS was also expected to be present in *B. stearothersophilus*. To identify a mannitol PTS, *B. stearothersophilus* membrane vesicles were isolated and used in PEP-dependent phosphorylation assays in combination with the *E. coli* proteins EI and HPr and the soluble A domain of the *E. coli* IICBA<sup>Mtl</sup> (35). PEP-dependent phosphorylation was stimulated by a factor of 4 upon addition of *E. coli* IIA<sup>Mtl</sup> to the reaction mixture containing the *E. coli* EI and HPr (Table 2).

**Cloning and sequencing of the *mtlA* gene of *B. stearothersophilus*.** Degenerate primers were designed on the basis of the amino acid homology between the *mtlA* genes of *E. coli* and *S. carnosus* (7). A fragment of 408 bp of the *B. stearothersophilus mtlA* gene was amplified by PCR using these primers and *B. stearothersophilus* chromosomal DNA as the template for the synthesis of a DIG-labeled DNA hybridization probe (probe I). This probe enabled us to identify, by Southern blotting, a 1.9-kb fragment in a *Hind*III digest of *B. stearothersophilus* chromosomal DNA. Fragments ranging from 1.8 to 2.0 kb were isolated from an agarose gel, ligated to pSK<sup>-</sup>, and cloned in JM101. Four of 800 clones screened hybridized with the probe, and all contained the same insert. One of these clones, pSKH1.9, was used for further study. Upon sequencing

TABLE 2. In vitro complementation of IICB<sup>Mtl</sup> and IICB-His expressed in *B. stearothersophilus* and in *E. coli* LGS322 with *E. coli* EI, HPr, and IIA<sup>Mtl</sup>

Source of membranes	Complementation of <i>E. coli</i> IIA <sup>Mtl</sup> (5.9 $\mu$ M)	Phosphorylation rate (nmol $\cdot$ min <sup>-1</sup> $\cdot$ mg <sup>-1</sup> ) <sup>a</sup>
<i>B. stearothersophilus</i>	+	11.1
	-	2.7
<i>E. coli</i> LGS322	+	0.5
	-	0.1
<i>E. coli</i> LGS322/pTAQIICB	+	31.2
	-	1.2
<i>E. coli</i> LGS322/pTAQIICB-his	+	45.9
	-	5.3

<sup>a</sup> Vesicles were prepared and PEP-dependent phosphorylation was measured as described in Materials and Methods.

the clone, we found an open reading frame encoding the C-terminal part of IICB<sup>Mtl</sup>. Using the 1.9-kb fragment as the template, we synthesized a second DIG-labeled probe (probe II) by PCR to clone the part encoding the N-terminal part of IICB<sup>Mtl</sup>. This second probe yielded a 1.7-kb *EcoRI*-*Bam*HI fragment containing the rest of the *mtlA* gene. This fragment was isolated from an *EcoRI*-*Bam*HI digest and cloned into pSK<sup>-</sup>, yielding plasmid pSKEB1.7. In addition, a 5.5-kb fragment from the same *EcoRI*-*Bam*HI digest, identified by using probe I, was isolated and cloned into pSK<sup>-</sup>, yielding pSKEB5.5. This 5.5-kb fragment contains the C-terminal encoding part of the *mtlA* gene and the 5-kb downstream region of the gene.

Plasmids pSKH1.9 and pSKEB1.7 were used to sequence both strands of the entire *mtlA* gene and its flanking regions. In several *mtl* operons, the *mtlA* gene is followed by *mtlF*, *mtlD*, and in some cases *mtlR* genes, encoding a IIA protein, a mannitol-1-phosphate dehydrogenase, and a regulatory protein, respectively (8). Since a similar order is expected for the *B. stearothersophilus mtl* operon, 4.9 kb of the sequence downstream of the *mtlA* gene was determined to localize these genes, using plasmid pSKEB5.5. Genes *mtlF* and *mtlD* and a gene with similarity to transcriptional regulators were identified downstream of the *mtlA* gene. The *mtl* operon is mapped in Fig. 1; all positions mentioned in the text refer to the *mtlA* sequence deposited in GenBank.

**Sequence of the *mtlA* gene of *B. stearothersophilus*.** The *mtlA* gene encodes a 471-amino-acid protein with a calculated mass of 50,153 Da. A putative ribosome binding site (positions 348 to 355) is located 11 bp upstream of the ATG start codon. This ribosome binding site (AAAGGGGG) provides a good match with the 3' terminus of the 16S rRNA of *B. stearothersophilus* (UUUCCUCC) (6). Positions 302 to 315 show a sequence (TGTAAGCGCTTTAA) matching the catabolite-responsive element consensus sequence [(T/A)GNAA(C/G)CGN(T/A)(T/A)NCA] (11). This finding indicates that catabolic repression by glucose via phosphoserine-HPr and CcpA may be involved in the regulation of this operon (10). This assumption is strengthened by the observation that *B. stearothersophilus* grown on minimal medium with glucose or rich LB medium even with added mannitol shows low expression levels of the mannitol transporter (data not shown). An inverted repeat that could be involved in the regulation of the operon as a binding site for a regulator protein is found upstream of the catabolite-responsive element box at positions 146 to 179. Directly downstream of the *mtlA* gene, at positions 1773 to 1846, are two partially overlapping inverted repeats that possibly form stable stem-loop RNA structures.

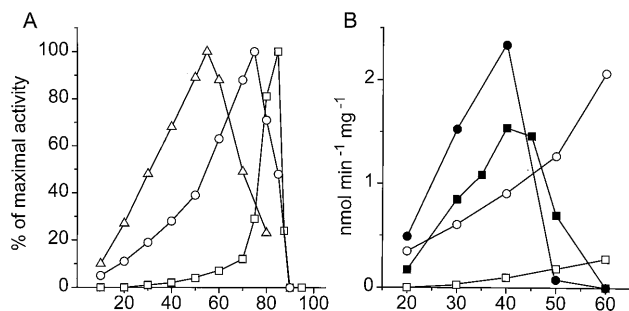


FIG. 2. Temperature dependence of the mannitol/mannitol-P exchange activity of IICB<sup>Mtl</sup> of *B. stearrowthermophilus* and *E. coli* membrane preparations. Membrane vesicles were prepared, and activity was measured as described in Materials and Methods. Before the reaction was started with [<sup>3</sup>H]mannitol, the samples were incubated for 5 min at the specified temperature. The reaction was monitored for 4 min at the same temperature by determining the amount [<sup>3</sup>H]mannitol-1-phosphate formed at 1-min intervals. (A) Activity of the enzyme as a percentage of the maximally measured specific activity. *B. stearrowthermophilus* IICB<sup>Mtl</sup> expressed in *B. stearrowthermophilus* (squares) and in *E. coli* LGS322 (circles) and *E. coli* IICB<sup>Mtl</sup> expressed in *E. coli* LGS322 (triangles) (2, 0.27, and 0.3 μg of total protein, respectively) were added; these amounts gave maximal specific activities of 4.0, 3.3, and 10 nmol · min<sup>-1</sup> · mg<sup>-1</sup>, respectively. (B) Influence of detergent on the exchange activity of *B. stearrowthermophilus* IICB<sup>Mtl</sup>. The exchange activity of *B. stearrowthermophilus* IICB<sup>Mtl</sup> in *B. stearrowthermophilus* membrane vesicles (squares) and *E. coli* LGS322 membrane vesicles (circles) was determined at different temperatures in the absence (open symbols) and presence (filled symbols) of 0.25% d-PEG. Total amounts of membrane protein added to the reaction were as in the experiment presented in panel A.

**Expression of *B. stearrowthermophilus* IICB<sup>Mtl</sup> and IICB-His in *E. coli*.** Two expression systems, pTAQIICB expressing the wild-type IICB<sup>Mtl</sup> and pTAQIICB-his expressing IICB<sup>Mtl</sup> with a six-histidine tag fused at the C-terminal end of the protein, were created. IICB<sup>Mtl</sup> and IICB-his<sup>Mtl</sup> expression by these plasmids was tested in the EII<sup>Mtl</sup>-deficient *E. coli* LGS322. Membrane vesicles of induced cells were prepared, and PEP-dependent phosphorylation rates were determined and compared with wild-type activities in *B. stearrowthermophilus* grown on mannitol as the only carbon and energy source (Table 2). In all cases, PEP-dependent phosphorylation was observed only when *E. coli* EI, HPr, and IIA<sup>Mtl</sup> were added to the reaction mixture. The activity of IICB<sup>Mtl</sup> expressed in *E. coli* was 2.8-fold higher and that of the His-tagged protein was 4.1-fold higher than that found in *B. stearrowthermophilus* membrane vesicles.

**Stability of the *B. stearrowthermophilus* IICB<sup>Mtl</sup>.** The thermostability of the *B. stearrowthermophilus* mannitol transporter was examined by monitoring the mannitol/mannitol-1-phosphate exchange activity at temperatures ranging from 10 to 95°C. The exchange activity rather than the phosphorylation activity was monitored to avoid effects due to the thermal instability of the HPr, EI, and IIA<sup>Mtl</sup> required for the phosphorylation activity measurements. After a 5-min incubation of the reaction mixture at the designated temperature, [<sup>3</sup>H]mannitol was added and the reaction was monitored for 4 min. The effects of temperature on the exchange activity of *B. stearrowthermophilus* IICB<sup>Mtl</sup> expressed in *B. stearrowthermophilus* and *E. coli* were compared with results for *E. coli* IICB<sup>Mtl</sup> expressed in *E. coli* (Fig. 2A). The optimal temperature for the exchange reaction,  $T_{max}$ , depends on the organism in which IICB<sup>Mtl</sup> is expressed. The  $T_{max}$ s for the *B. stearrowthermophilus* IICB<sup>Mtl</sup> were 85°C in *B. stearrowthermophilus* membrane vesicles and 75°C in *E. coli* membrane vesicles. For comparison, the  $T_{max}$  of the *E. coli* IICB<sup>Mtl</sup> in *E. coli* membrane vesicles was 55°C. The effects of detergent on the exchange reaction of *B. stearrowthermophilus* IICB<sup>Mtl</sup> in *B. stearrowthermophilus* and *E. coli* membrane vesicles

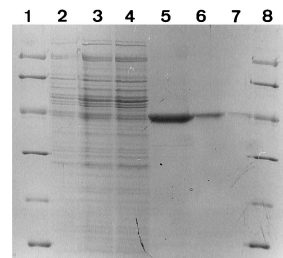


FIG. 3. Overview of the purification of the *B. stearrowthermophilus* IICB<sup>Mtl</sup> on SDS-PAGE. Lanes: 2, isolated membrane vesicles of *E. coli* with pTAQIICB-his; 3, DOC extract of the isolated membrane vesicles; 4, flow through of the Ni-agarose column; 5 to 7, with 25, 5, and 1 μl of the Ni-agarose pool, comparable to 15, 3.0, and 0.61 μg of protein, respectively; 1 and 8, protein standards (phosphorylase *b* [94 kDa], bovine serum albumin [67 kDa], ovalbumin [43 kDa], carbonic anhydrase [30 kDa], soybean trypsin inhibitor [20 kDa], and α-lactalbumin [14.5 kDa]). Samples were electrophoresed on an SDS-15% polyacrylamide gel, and the gel was stained with Coomassie blue.

were also studied (Fig. 2B). The addition of the detergent d-PEG to the membrane vesicles resulted in an increase of the exchange activity at temperatures below 50°C. This effect was maximal at 40°C, a temperature at which the exchange activities of the *B. stearrowthermophilus* IICB<sup>Mtl</sup> in *B. stearrowthermophilus* and *E. coli* membrane vesicles increased by factors 16 and 2.6, respectively.

The time-dependent stability of the enzyme at 60°C was determined for the *B. stearrowthermophilus* enzyme expressed in *B. stearrowthermophilus* and *E. coli* and compared with the stability of the *E. coli* IICB<sup>Mtl</sup>. The enzyme mixtures were incubated for various times at 60°C in the presence of 15 mM DTT, to prevent inactivation by oxidation, and assayed for exchange activity immediately at 60°C. The exchange activity decreased exponentially. Half-times of 366, 333, and 172 min were found for the *B. stearrowthermophilus* IICB<sup>Mtl</sup> in *B. stearrowthermophilus* and *E. coli* membranes and the *E. coli* IICB<sup>Mtl</sup> in *E. coli* membrane vesicles, respectively (data not shown).

The pH dependence of the exchange reaction for the *B. stearrowthermophilus* IICB<sup>Mtl</sup> expressed in *E. coli* or *B. stearrowthermophilus* was also analyzed. Optimal activity was found around pH 6 for both membrane preparations (data not shown). A similar value was found for the purified *E. coli* enzyme (12).

**Purification of IICB-his<sup>Mtl</sup>.** Membrane vesicles of *E. coli* LGS322 containing *B. stearrowthermophilus* IICB-his<sup>Mtl</sup> were solubilized by extraction with 0.5% DOC. After removal of unsolubilized membranes by ultracentrifugation, the extract was mixed with Ni-NTA resin and incubated for 90 min. The resin was subsequently washed with extraction buffer and buffer with 0.35% d-PEG instead of DOC, to remove impurities. The enzyme was eluted with 200 mM imidazole after replacement of d-PEG by 6 mM d-maltopyranoside. During the isolation, the enzyme inactivated rapidly; this could be prevented by minimizing the number of washing steps and by adding 100 μM mannitol or 0.1 mg of *E. coli* lipids per ml to the DOC extract and to the washing and elution buffers. The isolation procedure described above, in the presence of mannitol, yield IICB<sup>Mtl</sup> with >95% purity (Fig. 3), with a final yield of 46% of the original PEP-dependent phosphorylation activity of the membrane vesicles (Table 3). The extraction of the enzyme from the membranes appeared to be the most inefficient step of the isolation; more than 40% of the total activity was lost in this step. The purified protein has a mass of approximately 43 kDa on a sodium dodecyl sulfate (SDS)-polyacrylamide gel, whereas a mass of 50.2 kDa was deduced from the DNA sequence. An increase in mobility on SDS-polyacrylamide gel electrophoresis (PAGE) has also been

TABLE 3. Overview of the purification based on PEP-dependent mannitol phosphorylation

Step	Total activity (nmol · min <sup>-1</sup> )	Yield (%)
Membrane vesicles	5.7	100
DOC extract	3.1	54
Flowthrough	0.6	11
Ni-NTA pool	2.6	46

observed for *E. coli* EI<sup>Mtl</sup> and is probably caused by binding of extra SDS to the hydrophobic membrane protein (13). Three milligrams of purified IICB<sup>Mtl</sup> protein could be isolated from 1 liter of cultured cells.

DISCUSSION

**Cloning of the mannitol operon of *B. stearothermophilus*.** A mannitol PTS was identified in *B. stearothermophilus*, since PEP-dependent phosphorylation of mannitol by membrane vesicles of *B. stearothermophilus* was observed after the addition of *E. coli* EI, HPr, and IIA. The entire mannitol operon could be cloned by using degenerate primers directed against conserved areas within the *E. coli* and *S. carnosus* *mitA* sequences. The four open reading frames of the operon show similarity to mannitol IICBs, transcriptional regulators, mannitol IIAs, and mannitol-1-phosphate dehydrogenases.

**Sequence alignment of the IICB<sup>Mtl</sup>.** Multiple sequence alignments of *B. stearothermophilus* IICB<sup>Mtl</sup> with the IICB part of IICBA<sup>Mtl</sup> of *E. coli* and the IICB<sup>Mtl</sup> of *S. carnosus* (Fig. 4) reveal 64 and 56% identical amino acids, respectively, with 41% identical amino acids among all three sequences. A putative *mitA* gene of *B. subtilis* was identified in the sequence database; it resulted from the systematic sequencing of the *B. subtilis* genome (1). The *B. subtilis* IICBA<sup>Mtl</sup> has, in some parts, a high identity and, in other parts, no similarity to the *B. stearothermophilus* IICB<sup>Mtl</sup>. Detailed analysis of the *B. subtilis* DNA sequence indicates that the dissimilarity in some parts is probably caused by sequence errors in the *B. subtilis* sequence. If the sequence is altered (see the legend to Fig. 4) by inserting or deleting a base at five positions in the GenBank/EMBL/DDBJ databank sequence (accession number D38161), the identity with the *B. stearothermophilus* IICB<sup>Mtl</sup> is 70%. Since the sequence of a putative cryptic *E. coli* IICB<sup>Mtl</sup> (29) appears to be unreliable in its C-terminal part (according to a note to the sequence P32059 deposited at the Swiss Prot databank), it was not included in the alignment in Fig. 4.

A comparison of the secondary structure of *B. stearothermophilus* IICB<sup>Mtl</sup> with the model of Sugiyama et al. for *E. coli* IIC<sup>Mtl</sup> (30) reveals stretches of high overall identity in the first two transmembrane helices (64%) and in the second cytoplasmic loop between helices 4 and 5 (54%). Also, 44% identity is found in the B domain, especially around the putative phosphorylation site (C-389), with a row of 12 identical amino acids in all sequences. The amino acid identity in the other four predicted transmembrane helices is relatively low (~25%). Also, a low homology (10%) exists in the area which connects the B domain with the last transmembrane helix of the C domain, amino acids 341 to 382. Hydrophobicity analysis by the method of von Heijne (34) predicts transmembrane segments in all four sequences. The locations of most of the predicted transmembrane segments have been confirmed by PhoA fusions of *E. coli* IICBA<sup>Mtl</sup> (30).

**Stability of IICB<sup>Mtl</sup>.** As described in Results, the *B. stearothermophilus* IICB<sup>Mtl</sup> expressed both in *B. stearothermophilus*

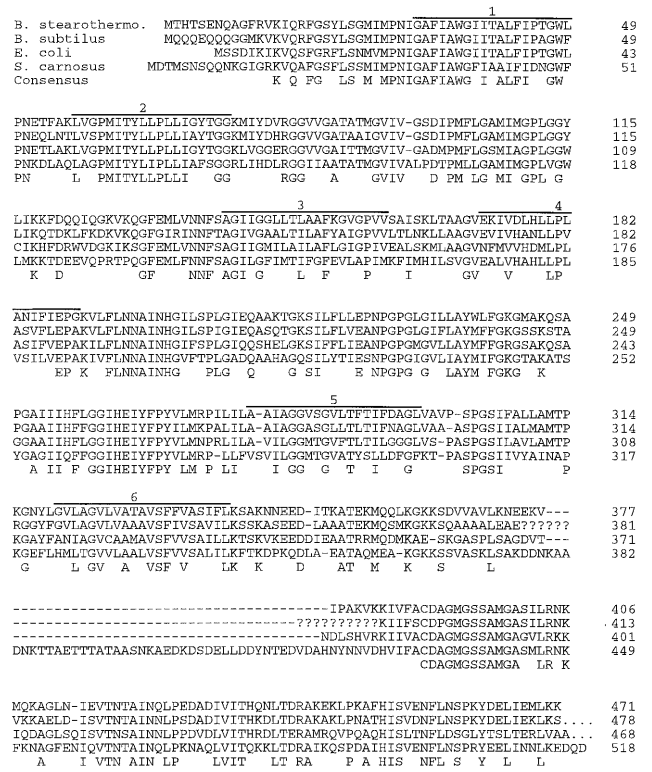


FIG. 4. Multiple amino acid sequence alignment of the *B. stearothermophilus* IICB<sup>Mtl</sup>, the IICB part of the IICBA<sup>Mtl</sup>s of *B. subtilis* (1) and *E. coli* (13), and the *S. carnosus* IICB<sup>Mtl</sup> (7). Identical amino acids are given in the consensus sequence. Membrane-spanning regions of the *E. coli* enzyme predicted by PhoA fusions (30) are indicated by numbered lines above the sequence. Probable sequence errors in the *B. subtilis* sequence (accession number D38161) were corrected by a deletion of a guanine at position 1068 and an adenine at position 1490 and an insertion of an alanine at position 1119. Also, in the linker region between the C and B domains, a frame shift probably caused by a deletion occurs. Because of the low similarity in this part of the sequence, the position of this deletion is not clear. This part is indicated with question marks.

and *E. coli* appears to be more resistant to elevated temperatures than the *E. coli* IICB<sup>Mtl</sup> expressed in *E. coli*. However, expression of the *B. stearothermophilus* IICB<sup>Mtl</sup> in *E. coli* instead of *B. stearothermophilus* lowers its *T*<sub>max</sub> from 85 to 70°C, indicating that thermostability is caused not only by the amino acid composition but also by the lipid environment.

The activity of the enzyme expressed in *E. coli* increases gradually with temperature to its maximum, while the activity of the enzyme expressed in *B. stearothermophilus* increases drastically only above 70°C. Since the gel-to-liquid crystalline phase transition temperature of membranes from *B. stearothermophilus* grown at 65°C was found to be 53°C (23), the transition from the gel to liquid crystalline phase cannot be the major reason for the dramatic increase of the enzyme activity at temperatures above 70°C. The decrease of the viscosity of the membrane at these elevated temperatures accounts for part of the acceleration. The observation that solubilization of the *B. stearothermophilus* membrane vesicles by detergents (Fig. 2B) resulted in a 16-fold stimulation of the enzyme activity at 40°C, while the same enzyme expressed in *E. coli* was stimulated only by a factor 2.6 upon solubilization, is consistent with this interpretation. Effects of the membrane composition on the properties of transport proteins expressed *B. stearothermophilus* and *E. coli* have been described before. For example, the substrate specificity of the L-glutamate transport of *B.*

*stearothermophilus* seems to be influenced by the nature of the membrane. Na<sup>+</sup>- and H<sup>+</sup>-dependent symport of glutamate was observed when the enzyme was investigated in *B. stearothermophilus* membrane vesicles, while in *E. coli* membrane vesicles, only H<sup>+</sup>-driven transport was found (31).

Experiments are in progress to determine the influence of detergents and lipids on EII<sup>Mtl</sup>'s stability and activity.

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