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# Characterization of the Proton/Glutamate Symport Protein of *Bacillus subtilis* and Its Functional Expression in *Escherichia coli*

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Transport of acidic amino acids in *Bacillus subtilis* is an electrogenic process in which L-glutamate or L-aspartate is symported with at least two protons. This is shown by studies of transport in membrane vesicles in which a proton motive force is generated by oxidation of ascorbate-phenazine methosulfate or by artificial ion gradients. An inwards-directed sodium gradient had no (stimulatory) effect on proton motive force-driven L-glutamate uptake. The transporter is specific for L-glutamate and L-aspartate. L-Glutamate transport is inhibited by  $\beta$ -hydroxyaspartate and cysteic acid but not by  $\alpha$ -methyl-glutamate. The gene encoding the L-glutamate transport protein of *B. subtilis* (*gluP*<sub>Bsu</sub>) was cloned by complementation of *Escherichia coli* JC5412 for growth on glutamate as the sole source of carbon, energy, and nitrogen, and its nucleotide sequence was determined. Putative promoter, terminator, and ribosome binding site sequences were found in the flanking regions. UUG is most likely the start codon. *gluP*<sub>Bsu</sub> encodes a polypeptide of 414 amino acid residues and is homologous to several proteins that transport glutamate and/or structurally related compounds such as aspartate, fumarate, malate, and succinate. Both sodium- and proton-coupled transporters belong to this family of dicarboxylate transporters. Hydrophathy profiling and multiple alignment of the family of carboxylate transporters suggest that each of the proteins spans the cytoplasmic membrane 12 times with both the amino and carboxy termini on the inside.

The amino acid transporters in the thermophile *Bacillus stearothermophilus* studied to date facilitate an electrogenic symport reaction in which Na<sup>+</sup> is used as the coupling ion. The apparent affinity constants for Na<sup>+</sup> are in the range of 0.5 to 1 mM (14). The transport of glutamate and aspartate is driven by the proton motive force ( $\Delta p$ ) but also by an inwardly directed Na<sup>+</sup> gradient ( $\Delta p_{Na}$ ). The transport of glutamate occurs most likely in symport with one H<sup>+</sup> and one Na<sup>+</sup> (7); the apparent affinity constant for Na<sup>+</sup> is <10  $\mu$ M. So far, sodium/proton/glutamate transporters have been found in the thermophiles *Bacillus* sp. strain IS1 (*gluT*<sub>Bi</sub>) (42), *B. stearothermophilus* (*gluT*<sub>Bs</sub>), and *Bacillus caldotenax* (*gluT*<sub>Bc</sub>). The genes encoding *GluT*<sub>Bs</sub> and *GluT*<sub>Bc</sub> have been cloned and functionally expressed in *Escherichia coli* (43).

Studies on the transport of L-glutamate and L-aspartate in whole cells of *B. subtilis* W23, 60015, 6GM, and 8G5 suggested that  $\Delta p_{Na}$  is not involved as a driving force in this mesophilic *Bacillus* species (41). The glutamate transporter of *B. subtilis* is likely to differ from those of thermophilic bacilli with respect to not only cation selectivity but also thermostability. In order to compare the sodium/proton/glutamate symport protein of the thermophile *B. stearothermophilus* with the glutamate transport protein of the closely related mesophile *B. subtilis*, the latter system was studied at the molecular level. This study confirms that glutamate uptake in *B. subtilis* is indeed coupled to the  $\Delta p$ . The primary sequence of the glutamate transporter of *B. subtilis* is highly similar to that of the glutamate transport proteins of *B. stearothermophilus* and *B. caldotenax*. In addition, the substrate specificity of these proteins is similar but the cation selectivity is different.

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## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains, plasmids, and phages used are listed in Table 1. *B. subtilis* 6GM was grown at 37°C with vigorous aeration in Luria-Bertani medium (LB) adjusted to pH 7.0 (33). *E. coli* strains were grown at 37°C with vigorous aeration in LB, M9, M9G (M9 in which ammonium chloride was replaced by L-glutamate at a final concentration of 10 mM), or M9CA medium (33, 43). The mineral media were supplemented with essential nutrients as indicated by the auxotrophic markers. When needed, carbenicillin and isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) were added to a final concentration of 100  $\mu$ g/ml and 100  $\mu$ M, respectively.

**DNA manipulations.** Mini- and large-scale preparations of plasmid DNA were obtained by the alkaline lysis method (4, 15). Chromosomal DNA was isolated essentially as described previously (25), except that mutanolysin was omitted. The strains were transformed after rubidium chloride treatment of the cells (33) or by electrotransformation (8). Other DNA techniques were performed as described previously (33).

**Cloning of the glutamate transport gene.** The *gluP*<sub>Bsu</sub> gene was cloned essentially as described previously (43). Partially *EcoRI*-, *HindIII*-, *PstI*-, or *Sau3A*-digested chromosomal DNA of *B. subtilis* was fractionated by agarose gel (1%, wt/vol) electrophoresis. Fragments of 2 to 10 kb were electroeluted from the gel and ligated into linearized and dephosphorylated pKK223-3. The resulting hybrid plasmids were used to transform *E. coli* JC5412 by electrotransformation. This strain does not grow on glutamate as the sole source of energy, nitrogen, and carbon. Transformants able to grow on M9G plates (supplemented with carbenicillin and IPTG) were analyzed with respect to their plasmid content. Purified plasmids were used to retransform *E. coli* JC5412 in order to distinguish between *Glu*<sup>+</sup> revertants and true transformants.

**Sequence determination.** The nucleotide sequences of both strands of the *HindIII* fragment of pGTU100, or subclones derived thereof in pUC18/19 or M13mp18/19 (*AccI*, *AluI*, *HaeIII*, *HincII*, *HindIII*, *PstI*, *RsaI*, *Sau3A*, and *SphI* fragments), were determined by using the dideoxy-chain termination method (34). Single- or double-stranded DNA was sequenced with a T7 sequencing kit (Pharmacia). Micro Genie (release 5.0; Beckman, Palo Alto, Calif.) and PCGene (release 6.26; Genofit, Geneva, Switzerland) were used for computer-assisted sequence analysis. Amino acid sequences homologous to *GluP*<sub>Bsu</sub> in the EMBL data bank were located with the TBLASTN program (1).

**Transport assays with whole cells.** Cells (15 ml) of strain JC5412 harboring plasmid pKK223-3 or pGTU100, grown for 14 h in LB (supplemented with carbenicillin and IPTG), were harvested, washed three times in 50 mM potassium phosphate (pH 6.0) plus 5 mM MgSO<sub>4</sub>, and resuspended to a final *A*<sub>660</sub> of approximately 10 in the same buffer. Uptake of L-[<sup>14</sup>C]glutamate by the *E. coli* cells was assayed at 37°C, upon 100-fold dilution of the cells into 200  $\mu$ l of 50 mM potassium phosphate (pH 6.0)–5 mM MgSO<sub>4</sub>–10 mM glucose. This mixture was incubated for 1 min at 37°C under continuous aeration. To initiate the uptake

TABLE 1. Bacterial strains, plasmids, and phages used

Strain, plasmid, or phage	Relevant characteristics	Source or reference
<b>Bacteria</b>		
<i>B. stearothermophilus</i>		ATCC 7954
<i>B. subtilis</i> 6GM		Laboratory collection
<i>E. coli</i>		
JM101	$\Delta(lac-proAB)$ F' <i>lacI</i> <sup>q</sup> $\Delta$ M15	50
JC5412	No growth on L-glutamate as the sole source of carbon, nitrogen, and energy	49
BK9MDG	GltP <sup>-</sup>	47
<b>Plasmids</b>		
pUC18/19	Ap <sup>r</sup>	
pKK223-3	Ap <sup>r</sup> , expression vector	Pharmacia
pGTU100	pKK223-3 carrying <i>gltP</i> of <i>B. subtilis</i> on a 2,122-bp <i>Hind</i> III- <i>Hind</i> III fragment	This study
pGTU2000	pUC18 carrying <i>gltP</i> of <i>B. subtilis</i> on a 2,122-bp <i>Hind</i> III- <i>Hind</i> III fragment	This study
<b>Phage M13mp18/19</b>		
		50

experiment, L-[<sup>14</sup>C]glutamate was added to a final concentration of 1.9  $\mu$ M. The uptake reactions were terminated by adding a 10-fold excess of ice-cold 0.1 M potassium chloride and immediately filtering over cellulose nitrate filters (pore size, 0.45  $\mu$ m). The filters were washed once with 2 ml of ice-cold potassium chloride.

**Isolation of membrane vesicles.** For studies of transport in membrane vesicles, cells of *B. subtilis* 6GM were grown in LB (33) to an  $A_{660}$  of 1.0. Cells were harvested and membrane vesicles were isolated as described previously by Konings et al. (23).

Cells of *E. coli* BK9MDG harboring plasmid pKK223-3 or pGTU100 were grown to an  $A_{660}$  of 1.0 in LB (supplemented with 100  $\mu$ g of carbenicillin per ml and 100  $\mu$ M IPTG) and membrane vesicles were isolated as described previously by Kaback (18). Cytoplasmic membranes of *B. subtilis* and *E. coli* were finally resuspended to 15 mg of protein per ml in 50 mM potassium phosphate, pH 6.0, and stored in liquid nitrogen.

**Transport assays. (i) Sodium- and proton motive force-driven uptake.** The electron donor system potassium-ascorbate (K-asc)-phenazine methosulfate (PMS) was used to generate a  $\Delta p$ . Membrane vesicles were diluted 100-fold into 50 mM potassium phosphate (pH 6.0)–5 mM MgSO<sub>4</sub>–10 mM K-asc–100  $\mu$ M PMS. The effect of the sodium motive force ( $\Delta s$ ) was assessed by adding 10 mM NaCl to the assay buffer. When appropriate, valinomycin (2 nmol per mg of protein), nigericin (1  $\mu$ M), or carbonyl cyanide *m*-chlorophenylhydrazone (10  $\mu$ M) (CCCP) was added to abolish the transmembrane electrical potential ( $\Delta\psi$ ), the transmembrane proton gradient ( $\Delta p$ H), or the  $\Delta p$ , respectively. After a 1-min incubation, uptake was initiated by adding L-[<sup>14</sup>C]glutamate to a final concentration of 1.9  $\mu$ M. The uptake reaction was terminated as described above.

**(ii) Artificial ion gradients.** The buffers used to generate artificial gradients are listed in Table 2. Membrane vesicles were washed twice in buffer 1 and subsequently incubated for 2 h at 4°C in the same buffer. After centrifugation for 5 min

at 200,000  $\times$  g the membranes were resuspended in buffer 1 to a concentration of approximately 40 mg of protein per ml. Uptake driven by specific ion gradients was initiated by diluting the membrane vesicles 100-fold into the appropriate buffer (Table 2) containing L-[<sup>14</sup>C]glutamate (1.9  $\mu$ M). The reaction was terminated as described above. Care was taken to avoid contamination of buffers with sodium ions; disposable plastic materials and ultrapure chemicals were used in all experiments. The uptake experiments were performed at 37°C unless stated otherwise. The kinetic parameters for transport, apparent  $K_m$ , and  $V_{max}$ , were estimated from the initial rates of uptake of the labeled amino acid determined after 10 s. Results were analyzed by fitting the data to the Michaelis equation.

**Protein determination.** Protein was measured by the method of Lowry et al. (26), using bovine serum albumin as the standard.

**Nomenclature.** In order to discriminate between Na<sup>+</sup>/glutamate, H<sup>+</sup>/glutamate and Na<sup>+</sup>/H<sup>+</sup>/glutamate transport proteins, the gene designations *gltS*, *gltP*, and *gltT* are used. Additionally, the subscripts Bs, Bc, Bsu, and Ec (B or K-12) are used to discriminate between the genes or proteins of *B. stearothermophilus*, *B. caldotenax*, *B. subtilis*, and *E. coli* (B or K-12), respectively.

**Nucleotide sequence accession numbers.** The nucleotide sequence accession numbers for the proteins discussed in this article are as follows: GltP<sub>Bsu</sub>, U15147; GltT<sub>Bs</sub>, M86508 (43); GltT<sub>Bc</sub>, M86509 (43); GltP<sub>Ec</sub>, M84805 (44); DctA<sub>Rm</sub>, J03683 (48), M26399 (10), and M26531 (16); DctA<sub>Rl</sub>, S38912 (45); DctA<sub>Rl</sub>, Z11529 (31); ASCT1<sub>Hs</sub>, L14595 (3); SATT<sub>Hs</sub>, L19444 (36); GluA<sub>Hs</sub>, U03504, D26443, and L19158 (2, 21, 37); GluB<sub>Hs</sub>, U03505 (2); GluC<sub>Hs</sub>, U03506 (2); GLAST (GLUT-1), X63744 (38) and S59158 (39); GLT-1, X67857 (29); EAAC1, L12411 (20).

## RESULTS

**Glutamate transport. (i) Effects of ionophores on glutamate transport.** Membrane vesicles of *B. subtilis* accumulate L-glutamate at a high rate and at high steady-state levels in the presence of K-asc–PMS (Fig. 1). L-Glutamate in/out ratios of approximately 700 are reached when a specific internal volume of 3  $\mu$ l/mg of protein is assumed (22, 24). Nigericin, which dissipates the transmembrane pH gradient (electroneutral K<sup>+</sup>/H<sup>+</sup> exchange), inhibited the uptake of L-glutamate partially. Valinomycin, which dissipates the membrane potential (K<sup>+</sup> ionophore), decreased the uptake of L-glutamate even further (Fig. 1). Complete inhibition of glutamate uptake was observed in the presence of nigericin and valinomycin (Fig. 1). These findings, and the observation that NaCl (10 mM) did not affect transport (Fig. 1), suggest that L-glutamate is transported in *B. subtilis* by an electrogenic process in symport with protons. Similar results were obtained with membrane vesicles of *E. coli* BK9MDG/pGTU2000 in which the glutamate transport protein of *B. subtilis* was functionally expressed (data not shown).

**(ii) Artificial gradients.** Since L-glutamate is an anionic species at a physiological pH, the electrogenic nature of the transport process suggests that at least two cations are symported with the substrate. To specify the nature of the cotransported cations more precisely, experiments in which glutamate uptake

TABLE 2. Buffers used to generate artificial ion gradients and resulting forces

Buffer <sup>a</sup>	Composition <sup>b</sup>		Valinomycin <sup>c</sup>	Force(s)
	MES (mM)	Other ingredient(s) <sup>d</sup>		
1 <sup>e</sup>	20	HAc, KOH	–	None
2 <sup>e</sup>	120	Mglu	+	$\Delta p$
3 <sup>e</sup>	20	HAc, Mglu	+	$\Delta\psi$
4 <sup>e</sup>	120	KOH	+	$\Delta p$ H
5	20	HAc, NaOH	–	$\Delta p$ Na
6	20	HAc, NaOH	+	$\Delta p$ Na + $\Delta\psi$
7	120	NaOH	–	$\Delta p$ Na + $\Delta p$ H
8	120	NaOH	+	$\Delta p$ Na + $\Delta p$

<sup>a</sup> Adjusted to pH 6.0 with methylglucamine or H<sub>2</sub>SO<sub>4</sub>; 5 mM MgSO<sub>4</sub> was present in all cases.

<sup>b</sup> MES, morpholineethanesulfonic acid; Mglu, methylglucamine.

<sup>c</sup> Final concentration, 2 nmol/mg of protein.

<sup>d</sup> 100 mM each.

<sup>e</sup> Sodium ion contamination,  $\leq$ 10  $\mu$ M.

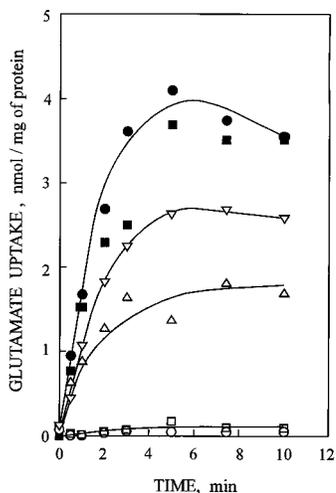


FIG. 1. Effects of ionophores and sodium ions on the uptake of L-glutamate in membrane vesicles of *B. subtilis*. Uptake of L-glutamate (1.9  $\mu\text{M}$ ) was measured at 37°C in oxygen-saturated 50 mM potassium phosphate (pH 6.0) plus 5 mM  $\text{MgSO}_4$ . Uptake was performed in the absence (○) or presence (●) of the electron donor system K-asc-PMS either without ionophores or in the presence of nigericin (1  $\mu\text{M}$ ) (▽), valinomycin (2 nmol/mg of protein) (△), nigericin plus valinomycin (□), or 10 mM NaCl (■).

was driven by artificial ion gradients were carried out. The  $\Delta p$  as well as its components  $\Delta\Psi$  and  $\Delta\text{pH}$  were able to drive L-glutamate uptake (Fig. 2). A sodium gradient, whether or not in addition to an artificially generated  $\Delta p$ ,  $\Delta\Psi$ , or  $\Delta\text{pH}$ , had no effect on glutamate uptake (data not shown). The sodium gradients were generated by varying the external sodium concentration among 0, 0.2, 0.5, 1, 5, 10, 50, and 100 mM; the initial internal concentrations were less than 10  $\mu\text{M}$ . These data strongly suggest that only protons are cotransported with glutamate.

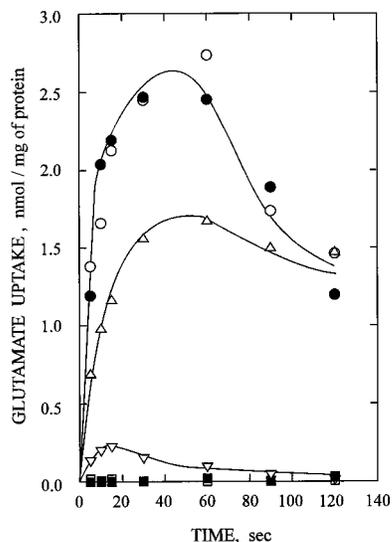


FIG. 2. Uptake of L-glutamate in membrane vesicles of *B. subtilis* driven by artificially imposed ion gradients. Glutamate uptake was performed in the presence of a  $\Delta p$  (○),  $\Delta p$  plus  $\Delta p\text{Na}$  (●),  $\Delta\text{pH}$  (△),  $\Delta\Psi$  (▽), or sodium motive force (□) as described in Materials and Methods. Control experiments were performed by diluting the membrane vesicles 100-fold into the buffer in which the membranes were resuspended (■).

TABLE 3. Inhibition of the initial uptake rate of L-glutamate in membrane vesicles of *B. subtilis* and *E. coli* BK9MDG/pGTU2000

Inhibitor <sup>a</sup>	% Inhibition	
	<i>B. subtilis</i>	BK9MDG/pGTU2000
L-Glutamate	91	64
D-Glutamate	27	19
L-Glutamine	12	8
L-Aspartate	84	86
L-Asparagine	5	18
$\beta$ -Hydroxyaspartate	84	83
Cysteic acid	80	64
$\alpha$ -methyl-Glutamate	1	0

<sup>a</sup> Added in a 50-fold excess. Final L-[<sup>14</sup>C]glutamate concentration, 1.9  $\mu\text{M}$ .

(iii) **Substrate specificity.** The substrates used to examine the substrate specificity of the L-glutamate transporter are listed in Table 3. The effects of a 50-fold excess of unlabeled substrates on the initial rate of L-glutamate uptake indicate that the transport system is specific for L-glutamate, L-aspartate,  $\beta$ -hydroxyaspartate, and cysteic acid but not for D-glutamate,  $\alpha$ -methyl-glutamate, L-glutamine, or L-asparagine (Table 3).

**Cloning of the glutamate transport gene of *B. subtilis*.** The *gluP<sub>Bsu</sub>* gene was cloned as outlined in Materials and Methods. A  $\text{Glu}^+$  transformant, originating from the *Hind*III chromosomal digest, was grown in liquid M9G medium, and plasmid DNA was isolated. The isolated plasmid pGTU100 (pKK223-3 harboring a 2.1-kb *Hind*III insert) conferred upon retransformation a  $\text{Glu}^+$  phenotype on *E. coli* JC5412. Uptake of L-glutamate by whole cells (*E. coli* JC5412) harboring pGTU100 (*GluP<sub>Bsu</sub>*) was severalfold higher than that in cells harboring pKK223-3 (data not shown). Membrane vesicles derived from strain BK9MDG/pGTU100 also showed significantly higher L-glutamate uptake than membrane vesicles derived from strain BK9MDG/pKK223-3 (data not shown). The kinetics of glutamate uptake in membrane vesicles of *E. coli* BK9MDG/pGTU100 and BK9MDG/pKK223-3 as well as that of *B. subtilis* 6GM was determined. The apparent  $K_m$  and  $V_{max}$  for glutamate uptake in membrane vesicles of *B. subtilis* 6GM were 9  $\mu\text{M}$  and 65  $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$ , respectively (Fig. 3). The data for *E. coli* BK9MDG/pGTU100 were 9  $\mu\text{M}$  and 12.4  $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$ , respectively (Fig. 3, inset).

**Nucleotide sequence and coding regions.** From sequencing data of the 3' and 5' ends of the cloned DNA fragment, it became clear that the multiple cloning site of pKK223-3 had been partly duplicated, resulting in its presence at both ends of the cloned DNA fragment. Southern blot analysis revealed that the 1,820-bp *Sph*I-*Hind*III fragment, together with a 275-bp fragment upstream of the *Sph*I site of pGTU100, originates from *B. subtilis* 6GM. The sequencing strategy for this 2,095-bp fragment is presented in Fig. 4; the sequence is shown in Fig. 5 (the duplicated 27-bp vector sequence GCATGCAAGCTT GGCTGCAGGTCGACG upstream of the *B. subtilis* sequence is not included in this figure). Between positions 499 and 1741 an open reading frame of 1,242 bp was found. The deduced polypeptide contains 414 amino acid residues (molecular mass, 44,707 Da).

**Amino acid composition and hydrophathy.** The amino acid composition of *GluP<sub>Bsu</sub>* is typical of an integral membrane protein; it contains 68.9% nonpolar and 31.1% polar residues (5). Of the 414 residues, 31 (7.5%) are basic (His residues not taken into account) and 27 (6.5%) are acidic. The hydrophathy

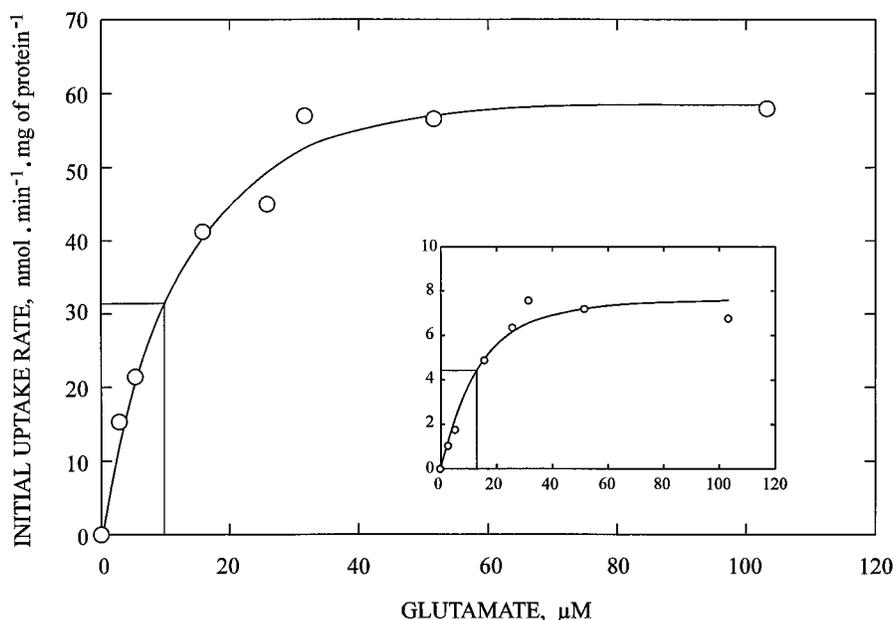


FIG. 3. Saturation kinetics of glutamate transport in membrane vesicles of *B. subtilis* 6GM and *E. coli* BK9MDG/pTU100 (inset). The kinetic parameters for transport, apparent  $K_m$  and  $V_{max}$ , were estimated from the Michaelis-Menten equation that was used to fit the experimental data. Uptakes were performed at 37°C by diluting membrane vesicles 100-fold into 50 mM potassium phosphate (pH 6.0)–5 mM  $MgSO_4$ –10 mM K-asc–100  $\mu M$  PMS. After a 1-min incubation,  $L$ - $[^{14}C]$  glutamate (0 to 100  $\mu M$ ) was added, and initial rates of uptake were determined after 10 s. Samples were further handled as described in Materials and Methods.

profile of  $GltP_{Bsu}$ , calculated by the method of Eisenberg et al. (9), predicts a minimum of 10 membrane-spanning regions (data not shown).

## DISCUSSION

The studies of uptake in *B. subtilis* membrane vesicles demonstrate that  $L$ -glutamate is transported electrogenically in symport with at least two protons. This is shown by the effect of ionophores on K-asc–PMS-energized transport as well as  $L$ -glutamate transport in the presence of artificially imposed ion gradients. The  $\Delta\Psi$  and  $\Delta pH$  alone can drive transport but not an inwardly directed  $Na^+$  gradient. Also,  $\Delta pNa$  does not stimulate  $\Delta p$ -,  $\Delta pH$ -, or  $\Delta\Psi$ -driven uptake. Our results are in accordance with previous observations of  $L$ -glutamate transport in whole cells of *B. subtilis* (41). Thus, it appears that  $L$ -glutamate transport in *B. subtilis* is coupled to protons whereas sodium ions and protons are used in the related thermophiles *B. stearothermophilus* and *B. caldotenax* (7, 14).

Studies in membrane vesicles of *B. subtilis* and *E. coli*

BK9MDG/pGTU2000, in which  $GltP_{Bsu}$  was functionally expressed, revealed that the  $L$ -glutamate transporter is specific for the substrates  $L$ -glutamate and  $L$ -aspartate.  $L$ -Glutamate transport is inhibited by the inhibitors  $\beta$ -hydroxyaspartate and cysteic acid, which also inhibit the  $H^+$ /glutamate symport protein ( $GltP$ ) of *E. coli* (35). The inhibitor of the  $Na^+$ /glutamate symporter ( $GltS$ ) of *E. coli*,  $\alpha$ -methyl-glutamate (35), did not affect  $GltP_{Bsu}$ .

$GltP_{Bsu}$  is homologous to various carboxylate transport proteins (see below). On the basis of the similarities between  $GltP_{Eck12}$ ,  $GltT_{Bs}$ , and  $GltT_{Bc}$ , the putative start codon is located at positions 499 to 501, which correspond to the leucine codon UUG. UUG is not commonly used as a translation initiation codon, but its use has been described before (11). A putative ribosome binding site is located upstream of the UUG codon, which shows extensive similarity to the 3' end of *B. subtilis* 16S rRNA (13, 28). The stop codon (positions 1741 to 1743) is immediately followed by an inverted repeat ( $\Delta G^\circ$ ,  $-23.4$  kcal [ca.  $-97.9$  kJ]/mol, calculated by the method of Tinoco et al. [40]) and has features typical of a putative

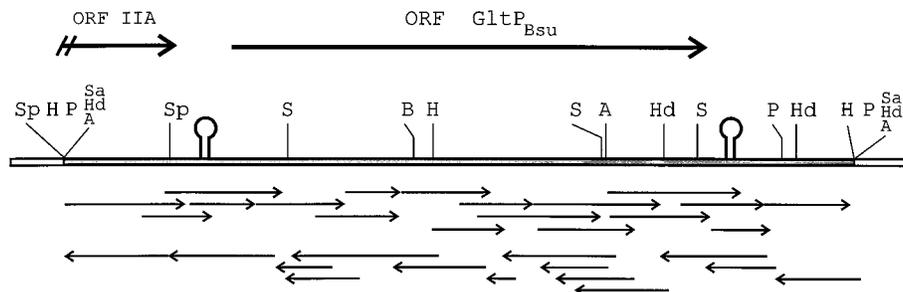


FIG. 4. Sequencing strategy. Part of the vector sequence (open box), the cloned fragment (shaded box), and the position and direction of transcription of the putative gene upstream of  $gltP_{Bsu}$  and  $gltP_{Bsu}$  are shown. ORF, open reading frame. The regions sequenced are indicated below (arrows). Sp, *Sph*I; S, *Stu*I; Sa, *Sal*I; B, *Bal*I; H, *Hind*III; A, *Acc*I; Hd, *Hind*II; P, *Pst*I.

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GATCAGGCTCTTTTCAGAAAAATGATGGCGAGGGATTTGCGATTATACCATCAGAAGGA 60
D Q V F S E K M M G E G F A I I P S E G
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K V V A P A D G E I V S I F P T K H A I
GGCTTTATGAGCGCGCGGCACTGAAATCCTGATTCATGTCGGCATCGATACCGTCAAA 180
G F M S A G G T E I L I H V G I D T V K
CTGAATGGGGAAGGCTTTGAAGCACATGTCACAAGCGGCAAGCCGTCAAACAAGCGGAA 240
L N G E G F E A H V T S G Q A V K Q G E
CTGCTTCTCACCTTTGATCTCAATACATCAAGCAGCATGCGGCTTCAGCCATAACACCG 300
L L L T F D L N Y I K Q H A A S A I T P
GTTATTTTCAAAATACCTCTGAAGAAGATCTAAAGCACATTCAAATGAAGTAAAAAAGT 360
V I F T N T S E E D L K H I Q M K - - - - -
TERMINATOR
CCCCCTGCTGCGGGGACTTTTTCCGCCAACATAATGAAAGCGCTGTCAAATAAAATAA 420
- - - - -
-35 -10
CTTGA AAAAGGTTTTCGCAATTGAAATCACGAATGATGAATATGCCTACATTCTATCG 480
RBS START
TTCCTAAGGGGAGTTCATTTGAAAAAATAATCGCGTTTCAAATTTAATATCGCTTTGGCT 540
L K K L I A P Q I L I A L A
GTCGGCGCGGTGATCGGCCACTTTTTCTGATTTGCGGATGGCACTGAGGCCCTGTCCGA 600
V G A V I G H F F P D F G M A L R P V G
GATGATTTATCCGCGCTGATTAATAAGTGTGTGCGGATGATTTTCTACCATTTGTC 660
D G F I R L I K M I T T G T G C C G A T T G A T T T T T C T A C C A T T G C
ATTGGAGCCGCGAGGAGCGGATGAAATAAGTGGCAGTCTCGGCATCAAGACGATG 720
I G A A G S G S G C A T G A A A A A A T G G C A G T C T C G G C A T C A A G A C G A T
ATTTGGTTCGAAGTATTACAACCTTGGTATTAGGCTTGGACTTTTATTAGCAATGTC 780
I W F E V I T T L V L G L G L L L A N V
TTAAAACAGGTTGCGGCTTGACCTCTCCATTTGGCAAAAAGATATTCATGAACCT 840
L K P G V G L D L S H L A K K D I H E L
TCCGGCTACACAGAAAGTTGTGATTTCAAGCAATGATCCCTGGATATATCCCTACA 900
S G Y T D K K V I D F K Q M I L D I I P T
AACATATATGATGTCATGGCCAGAAATGATTTGCTTGCCTTATTTTCTGCCATTTTA 960
N I I D V M A R N D L L A V I F F A I L
TTTGGTGTGCGCGAGCTGATCGGCAAGCTTCTGAACAGTAATGAAGTTTITGAA 1020
F G V A A A G I G K A S E P V M K F F E
TCTACAGCTCAGATTATGTTCAAGCTCAGCAAAATCGTCATGGTCACTGCTCTATCGGC 1080
S T A Q I M P K L T Q I V M V T A P I G
GTGTTAGCCCTGATGGCTCTTCGTAGGCCAATATGGCATTTGAATGATCCCTCCCTATG 1140
V L A L M A A S V G Q Y G I E L L L P M
TTTAAGCTGTTGGCACCGTATTCCTTGGCCCTGTTCTGATCCCTTTGCTCTCTTCCG 1200
F K L V G T V L G L F V L F V L F P
CTTCTCGGCTCATCTTTAGATTAAGTATTTCGAAGTATTGAAAATGATATGGGATCTG 1260
L V G L I F Q I K Y F E V L K M I W D L
TTTTTAATGCAITTTCCACCAAGAGTGAACCAATTCCTCCCTCAGCTGATGGATAGA 1320
F L I A F S T T S T E T I L P Q L M D R
ATGGAAATACCGCTGTCCCAAAGAGTGTATCATTGTGCTTCCCTCCGGCTTGTCA 1380
M E K Y G C P K R V V S F V V P S G L S
TTGAAGTGTGACGGCTCCAGCTTATATTATCGGTTTCTGTATCTTTCTCGCACAGGCC 1440
L N C D G S S L Y L S V S C I F L A Q A
TTTCAAGTAGACATGACATTTCCAGCAGCTGCTCATGATGCTTGTGCTTGTGATGACC 1500
F Q V D M T L S Q Q L L M M L V L V M T
AGTAAAGGCATTCAGCTGATCCCTCAGGATCGCTTGTAGTCTTCTCGGCGACTGCCAAT 1560
S K G I A A V P S G S G L V V L L A T A N
GCTGTAGGACTGCGGCTGAAGGCGTGGCCATTATCGGGTGTGACCGCGCTCATGGAC 1620
A V G L P A E G V A I I A G V D R V M D
ATGGCGAAGACGGCGTGAACGTACCGGACATGCGATCGCTGATCGGTATCAAAA 1680
M A R T G V N V P G H A I A C I V S K
TGGGAAAAGGCTTCCGCCAAAAGATGGGTTTCCGCAACAGCCAAACTGAAAGCATA 1740
W E K A P R Q K E W V S A N S Q T E S I
STOP
TAGAAAAAAGAACACCTCAATACGAGGTGTTCTTTTTTTTATTTCAGCAGCAGCGTATCT 1800
- - - - -
TCTGTTCATCGCTGCCAGTCTTCCGTTTACTTCTTCTCTGTCATATTATGCTCTTTC 1860
ACATACGGGTTTCTTGGGTTTGGTCCGCGCATTCGTGGCTGCAGCTCGCATATATTATA 1920
CTCATTTTCCGGTGTGATATCATTTTTTTTATTTGCAAGATGGATTTGGCGCAGTTCACAT 1980
AACGCTCACACGGTCTCCTGTAAAGTAGTCTTGCACAATCACATGCTCGACAACGGT 2040
TGACAGGCAGCGTATTCTTTCATCAATACGTAGCACTGGCCGTCCCAAAGCTT 2095

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FIG. 5. Nucleotide sequence of the 2,095-bp fragment comprising the *gluP<sub>Bsu</sub>* gene of *B. subtilis* and flanking regions. The start and stop codons, a putative promoter (-35/-10), a possible ribosome binding site (RBS), and possible terminator sequences (-> <-) are indicated. The amino acid sequence deduced from the DNA sequence of the *gluP<sub>Bsu</sub>* gene and the putative open reading frame upstream of *gluP<sub>Bsu</sub>* are shown below the DNA sequence.

rho-independent transcription terminator sequence (32). A transcription termination sequence is also found at positions 352 to 384, i.e., immediately upstream of *gluP<sub>Bsu</sub>*. Several promoter elements can be identified between this terminator sequence and the start codon. For none of the sequences is the expected distance of 16 to 18 bp observed (13, 28). A putative promoter with a 15-bp spacing between the -35 and -10 regions is indicated in Fig. 5. Upstream of the *gluP<sub>Bsu</sub>* gene the 3' end of a putative open reading frame was found (Fig. 5). This region encodes 117 amino acid residues of a polypeptide which is homologous to IIA proteins of several phosphoenolpyruvate-sugar-phosphotransferase systems as well as the IIA domain of the lactose transport protein (LacS) of *Streptococcus thermophilus* (30).

Sequence comparison of the H<sup>+</sup>/glutamate symport protein of *B. subtilis* and sequences in the EMBL data bank revealed a number of homologous proteins. All these proteins transport one or more of the structurally related compounds glutamate, aspartate, fumarate, malate, and/or succinate. The systems comprise sodium as well as proton-coupled transporters. Extensive similarity was found between *GluP<sub>Bsu</sub>* and the thermophilic Na<sup>+</sup>/H<sup>+</sup>/glutamate symport proteins of *B. stearothermophilus* and *B. caldotenax* (43) and between *GluP<sub>Bsu</sub>* and the mesophilic H<sup>+</sup>/glutamate symport protein of *E. coli* K-12 (44) (in each case the identity was approximately 44%). The similarity between *GluP<sub>Bsu</sub>* and the C<sub>4</sub>-dicarboxylate carriers of *Rhizobium meliloti* (10, 16, 48) and *Rhizobium leguminosarum* (31, 45) corresponds to approximately 34% identical residues. *GluP<sub>Bsu</sub>* also is approximately 26% identical with a third group of proteins: the *Homo sapiens* Na<sup>+</sup>/alanine/serine/cysteine/threonine transporter (ASCT1<sub>Hs</sub> [adult motor brain]) (3), Na<sup>+</sup>/alanine/serine/cysteine transporter (SATT<sub>Hs</sub> [hippocampus]) (36), excitatory glutamate transporters 1 to 3 (motor cortex) (2, 21, 37), and glutamate transporter (GLTRp1 [brain and pancreas]) (27); the *Rattus norvegicus* Na<sup>+</sup>/glutamate/aspartate transporters GLAST (brain) (38) and GLUT-1 (brain) (39) and Na<sup>+</sup>/glutamate transporter GLT-1 (brain glial cells) (29); and the *Oryctolagus cuniculus* Na<sup>+</sup>/glutamate transporter (EAAC1 [rabbit small intestine]) (20). On the other hand, no significant similarity exists between *GluP<sub>Bsu</sub>* and the Na<sup>+</sup>/glutamate symport proteins of *E. coli* B (6) and K-12 (19) (data not shown).

Alignment of the homologous proteins reveals that identical and similar residues are distributed along the entire amino acid sequence (Fig. 6). Also, when the proton- and sodium-dependent symporters are compared, it appears that differences are not confined to one or a few regions, making it difficult to predict whether a given residue or protein segment determines the cation selectivity. Moreover, changes such as substituting a single amino acid may already alter the cation selectivity, as has been observed for the melibiose transport protein of *Klebsiella pneumoniae* (12). The number of membrane-spanning helices of the proteins indicated in Fig. 6 as predicted according to the method of Eisenberg et al. (9) ranges from 9 to 12. In the case of the C<sub>4</sub>-dicarboxylate carrier of *R. meliloti*, the secondary-structure predictions are substantiated by a limited number of PhoA and LacZ gene fusions, and the number of membrane-spanning helices was proposed to be 12 (17). In addition, indicative of similar secondary and tertiary structures are the gene fusions between *gluP<sub>Ec</sub>* and *gluT<sub>Bs</sub>* that result in fully functional glutamate transport proteins (41). On the basis of the similarity between the proteins, the hydropathy profiles of the individual sequences, and the topology rules proposed by Von Heijne (46), we propose that the transporters shown in Fig. 6 may have a similar secondary structure with 12 putative transmembrane-spanning α-helices. The locations of these putative



helices coincide with those predicted by the DctA-PhoA and DctA-LacZ gene fusions in the DctA protein of *R. meliloti* (17) (Fig. 6). The proposed transmembrane segment 4 is highly amphipathic in GltP<sub>BSU</sub>, GltT<sub>BC</sub>, GltT<sub>BS</sub>, SATT<sub>HS</sub>, ASCT1<sub>HS</sub>, GLT-1, GluA<sub>HS</sub>, EAAC1, and GluC<sub>HS</sub>.

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