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BetS Is a Major Glycine Betaine/Proline Betaine Transporter Required for Early Osmotic Adjustment in *Sinorhizobium meliloti*

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Hybridization to a PCR product derived from conserved betaine choline carnitine transporter (BCCT) sequences led to the identification of a 3.4-kb *Sinorhizobium meliloti* DNA segment encoding a protein (BetS) that displays significant sequence identities to the choline transporter BetT of *Escherichia coli* (34%) and to the glycine betaine transporter OpuD of *Bacillus subtilis* (30%). Although the BetS protein shows a common structure with BCCT systems, it possesses an unusually long hydrophilic C-terminal extension (169 amino acids). After heterologous expression of *betS* in *E. coli* mutant strain MKH13, which lacks choline, glycine betaine, and proline transport systems, both glycine betaine and proline betaine uptake were restored, but only in cells grown at high osmolarity or subjected to a sudden osmotic upshock. Competition experiments demonstrated that choline, ectoine, carnitine, and proline were not effective competitors for BetS-mediated betaine transport. Kinetic analysis revealed that BetS has a high affinity for betaines, with K_ms of 16 ± 2 μM and 56 ± 6 μM for glycine betaine and proline betaine, respectively, in cells grown in minimal medium with 0.3 M NaCl. BetS activity appears to be Na^+ driven. In an *S. meliloti betS* mutant, glycine betaine and proline betaine uptake was reduced by about 60%, suggesting that BetS represents a major component of the overall betaine uptake activities in response to salt stress. β-Galactosidase activities of a *betS-lacZ* strain grown in various conditions showed that *betS* is constitutively expressed. Osmotic upshock experiments performed with wild-type and *betS* mutant cells, treated or not with chloramphenicol, indicated that BetS-mediated betaine uptake is the consequence of immediate activation of existing proteins by high osmolarity, most likely through posttranslational activation. Growth experiments underscored the crucial role of BetS as an emerging system involved in the rapid acquisition of betaines by *S. meliloti* subjected to osmotic upshock.

Osmoregulation is a fundamental phenomenon developed by bacteria, fungi, plants, and animals to overcome osmotic stress. The most widely distributed strategy of response to hyperosmotic stress is the accumulation of compatible solutes, which protects the cells and allows growth. One of the most effective compatible solutes widely used by bacteria is glycine betaine, the *N*-trimethyl derivative of glycine (8, 27), which can be accumulated intracellularly at high concentration through either synthesis, uptake, or both.

Transporters for glycine betaine have been extensively investigated at the molecular level in the gram-negative enteric bacteria *Escherichia coli* and *Salmonella enterica* serovar Typhimurium and in the gram-positive soil bacteria *Bacillus subtilis* and *Corynebacterium glutamicum*. In *E. coli* and serovar Typhimurium, two transport systems, ProP and ProU, are primarily responsible for the uptake of glycine betaine. ProP, a secondary transporter, functions as an H^+ symporter and is regulated mainly at the activity level (5, 31). ProU is a binding protein-dependent transporter, a member of the ABC superfamily, and is regulated at the level of both transcription and activity (6, 28).

In *B. subtilis*, three high-affinity effective glycine betaine transporters have been characterized so far. Two systems, OpuA and OpuC, are members of the ABC superfamily of transporters (19, 21, 22), and one, OpuD, is a secondary transporter (20). OpuA and OpuC present identity to the periplasmic binding protein ProU transporter from *E. coli*, but as a gram-positive bacterium, *B. subtilis* lacks the periplasm, and the binding proteins are anchored in the cytoplasm membrane to prevent their loss in the surrounding medium (22). Whereas OpuC can transport a wide variety of compatible solutes such as glycine betaine, choline, ectoine, and carnitine, OpuA and OpuD exhibit a restricted substrate specificity for glycine betaine.

With respect to osmotic adaptation, *C. glutamicum* is another well-studied gram-positive soil bacterium. In this organism, two secondary carriers for the uptake of glycine betaine have been characterized: the high-affinity, Na^+-coupled glycine betaine uptake system BetP, and EctP, which prefers ectoine to glycine betaine (10, 36, 38). Both systems are regulated by the external osmolarity on the level of activity. BetP and EctP are closely related to each other and to other prokaryotic carriers for compatible solutes, such as the glycine betaine transporter OpuD from *B. subtilis*, the choline transporter BetT and the carnitine transporter CaiT from *E. coli*, the glycine betaine transporter BetL from *Listeria monocytogenes* (49), and the putative BetP proteins from *Mycobacterium tuberculosis* (39) and from *Haemophilus influenzae* (14). These proteins constitute a small family of structurally and functionally related systems, all belonging to the betaine choline car-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli		
DH5α	F$^-$ *supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *hsdR17*(r_K^- m_K^+) *recA1*	47
MT616	*pro-82 thi-1 hsdR17 supE44 recA56*	12
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flb-5301 deoC1 ptsF25 rbsR* Δ*bet* Sm^r	18
MKH13	MC4100 Δ(*putPA*)*101* Δ(*proP*)*2* Δ(*proU*)*608* Sm^r	18
S. meliloti		
102F34	Wild-type derivative, Rif^r	43
GMI211	Derivative of Rm2011, Sm^r Lac$^-$	33
UNA315	GMI211 *betS*::*lacZ betS*$^+$ Km^r	This work
UNA316	GMI211 *betS*::*lacZ* Km^r	This work
Plasmids		
pRK600	ColE1 replicon with RK2 transfer region, Cm^r	12
pKOK5	pSUP202 derivative; source of *lacZ*-Km^r cartridge, Ap^r Km^r	23
pUC119	Sequencing vector, ColE1 *oriV*, Ap^r	53
pSUP202	Replicon ColE1, Mob$^+$ Tc^r Ap^r Cm^r	48
pLAFR3	Cosmid cloning vector, Tc^r	15
pBT47	pLAFR3, *S. meliloti* 20-kb cosmid clone with *betS* gene, Tc^r	This work
pBT58	3.4-kb *Sal*I fragment from pBT47 with *betS* into pUC119 vector, Ap^r	This work
pSBI39	3.4-kb *Sal*I fragment from pBT47 with *betS* into pSUP202 vector, Ap^r Cm^r	This work
pSBA15	pSBI39, *betS*::*lacZ* Km^r (insertion into *Bam*HI)	This work
pSBA16	pSBI39, *betS*::*lacZ* Km^r (insertion into *Bam*HI), insertion in reverse orientation	This work

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Rif^r, rifampin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance.

nitine transporters (BCCT) or trimethylammonium transporters (20).

Previous works have demonstrated the crucial role of glycine betaine for osmotic stress resistance in the gram-negative soil bacterium *Sinorhizobium meliloti*, the alfalfa (*Medicago sativa* L.)-symbiotic species, and have led to investigations of glycine betaine synthesis and transport (25, 50). The biosynthetic pathway, the enzymatic oxidation of choline or choline-*O*-sulfate to glycine betaine, has been well characterized at the molecular level (34, 43) and involves four genes, *betICBA*, organized in one operon. In addition to the genes encoding a presumed regulatory protein (*betI*), the betaine aldehyde dehydrogenase (*betB*), and the choline dehydrogenase (*betA*), enzymes also found in *E. coli* (24), *S. meliloti*, unlike other bacteria, possesses an additional gene (*betC*) which encodes a choline sulfatase catalyzing the conversion of choline-*O*-sulfate and, to a lesser extent, phosphorylcholine into choline (34).

The genes encoding transport systems for glycine betaine in *S. meliloti* are largely unexplored, although it has been demonstrated previously that glycine betaine transport activity is strongly stimulated in cells grown at high osmolarity (2) and that a glycine betaine-binding protein exists in the periplasm of such cells (26). In addition to glycine betaine, *S. meliloti* uses proline betaine, also known as stachydrine or dimethylproline, as an osmoprotectant (17). This quaternary ammonium derivative of proline occurs widely in *Medicago* species (42) and has been identified as an inducer of *S. meliloti* nodulation genes (40), although the proline betaine transport system has not been characterized. Nevertheless, recently we have described an ATP-binding cassette histidine transporter (Hut), also involved in high-affinity proline and proline betaine uptake and low-affinity glycine betaine transport (3). Expression analysis of the *hut* operon revealed induction by histidine but not by osmotic stress, suggesting that this uptake system has a catabolic role rather than being involved in osmoprotection.

Here, we report the molecular characterization and disruption of *betS*, a gene which plays an important role in high-affinity Na^+-coupled glycine betaine and proline betaine transport in *S. meliloti*. Furthermore, we show that *betS* is constitutively expressed, whereas BetS activity depends on posttranslational activation by high osmolarity and is most likely the emergency system transporting betaines for immediate osmotic protection.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and β-galactosidase assays. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were routinely grown in Luria-Bertani (LB) medium (47) at 37°C, and *S. meliloti* strains were routinely grown in LB supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (LBmc) at 30°C. When required, antibiotics were added at the final concentrations described previously (11, 43). For transport assays, *E. coli* cells were grown in M63 minimal medium supplemented with 0.2% glucose as the carbon source (7) or in M63 modified medium containing Na_2HPO_4 (40.8 mM) instead of K_2HPO_4. *S. meliloti* strains were grown in MCAA medium (50) for transport assays or in LAS minimum medium containing 10 mM sodium lactate and 10 mM sodium aspartate (2) for β-galactosidase assays. The osmotic strength of the media was increased by the addition of 0.3 or 0.5 M NaCl, 0.3 M KCl, or 0.48 M mannitol. β-Galactosidase activity was determined by the method of Miller (30) using overnight induced cultures at an optical density at 600 nm (OD_{600}) of 0.4.

PCR amplification of the *S. meliloti betS* gene. PCR mixtures contained 50 pmol of each degenerate primer; 200 ng of *S. meliloti* strain 102F34 genomic DNA; a 200 μM concentration each of dATP, dTTP, dGTP, and dCTP; 1× *Taq* polymerase buffer (Appligene, Illkirch, France); and 1 U of *Taq* DNA polymerase (Appligene) in a final volume of 50 μl. Reaction mixtures were cycled automatically using a Biometra thermocycler (T gradient model; Biometra GmbH, Göttingen, Germany) through temperature and time cycles as follows: denaturation, 95°C for 1 min; annealing, variable from 46 to 56°C for 1 min; and extension, 72°C for 1 min. The denaturation time of the first cycle was prolonged to 5 min to ensure a single-stranded template for the PCR, and the final extension time was increased to 10 min to ensure completion of strand synthesis. Twenty microliters of reaction mixture was analyzed by electrophoresis on 1.5% agarose gels. The sequences of the two degenerate primers used were 5′-TTY GCN GGN ATN GG-3′ (BT1) and 5′-CCA CCA NGC CCA RWA NA-3′ (BT3R).

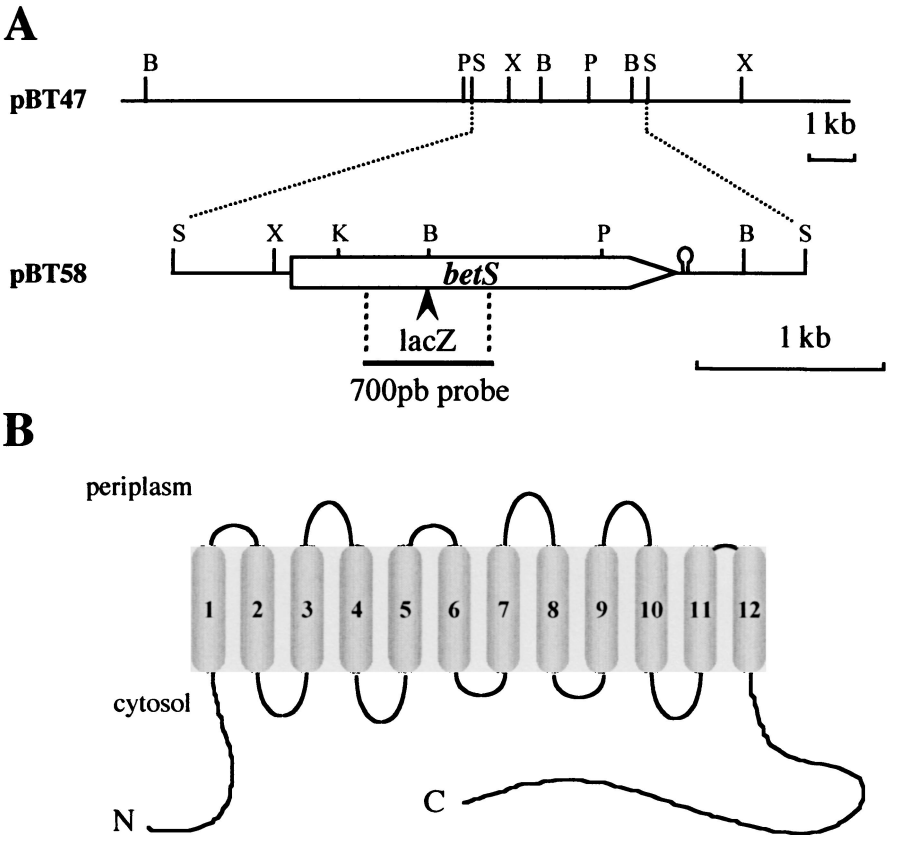

FIG. 1. (A) Physical and genetic maps of the *S. meliloti betS* region. The *betS* gene is represented by an open arrow. The positions of both the *lacZ* fusion and the 700-bp probe are indicated below. The hairpin indicates the position of a stem-loop element able to form a secondary structure. Restriction sites: B, *Bam*HI; P, *Pst*I; S, *Sal*I; K, *Kpn*I; and X, *Xho*I. (B) Putative secondary structure of BetS.

DNA manipulations, sequencing, and plasmid and mutant constructions. Restriction analysis, ligation, transformation, plasmid DNA extraction, and Southern hybridization were performed by standard methods (47). PCR-purified fragments were subcloned in the pGEM-T cloning vector (Promega, Charbonnières, France). DNA probes were labeled using the Prime-a-Gene random priming system (Promega) and [α-^{32}P]dCTP (purchased from Amersham Corp., Little Chalfont, United Kingdom). Total DNA from *S. meliloti* was isolated as described previously (29). The genomic library of *S. meliloti* 2011, made up of an *Sau*3A partial DNA digest cloned into pLAFR3, was kindly provided by D. Kahn (Laboratoire CNRS-INRA, Castanet-Tolosan, France). Screening of the genomic library was performed by colony hybridization using the 700-bp PCR product (Fig. 1) as the probe, according to standard procedures (47).

The 3.4-kb *Sal*I fragment from the identified cosmid (pBT47) which contained the *betS* gene was transferred into the sequencing vector pUC119 and suicide vector pSUP202 to yield pBT58 and pSBI39, respectively. Derivatives of pSBI39 were obtained after partial digestion with *Bam*HI and ligation with a 4.7-kb *Bam*HI fragment carrying a *lacZ*-Km^r cartridge from pKOK5 (23). The location and orientation of the cartridge at the correct *Bam*HI site in *betS* were verified by restriction analysis. pSBA15 contains a transcriptional *lacZ* fusion in *betS*, and pSBA16 carries the *lacZ* coding sequence in the opposite orientation of *betS*.

In order to construct recombinant *betS-lacZ* fusion and mutant strains, these plasmids were transferred by triparental mating, using *E. coli* strain MT616 (pRK600) as the helper, into *S. meliloti* GMI211 (12). A single recombinant clone, selected as Sm^r, Nm^r, and Cm^r and containing pSBA15, was named UNA315, while a double recombinant clone, selected as Sm^r, Nm^r, and Cm^s and containing pSBA16, was designated UNA316. The location of the cartridge at the correct *Bam*HI site in *betS* was verified by Southern hybridization. The nucleotide sequence of both strands of pBT58 *Sal*I fragment was established using the fluorescent ABI dye-labeled deoxy terminator method of MWG Biotech (Edersberg, Germany). The DNA and derived protein sequences were analyzed by using ClustalW (51) and Blast (1) software programs.

Transport assays. Radioactive [*methyl*-^{14}C]glycine betaine was prepared from [*methyl*-^{14}C]choline (2.04 GBq/mmol; Amersham Corp.) as previously described (35). [U-^{14}C]proline betaine (4.6 GBq/mmol) was obtained from the Commissariat à l'Energie Atomique (Gif-sur-Yvette, France). *E. coli* cells were harvested at an OD_{420} of 0.8 to 1.0 and washed twice in the medium used for the culture. All assays were carried out at 37°C with 1 ml of the cell suspension and radioactive substrates (100,000 dpm). Uptake was determined by rapid filtration through GF/F glass microfiber filters (Whatman) rinsed with 3 ml of the corresponding medium. The radioactivity remaining on the filters was determined with a liquid scintillation spectrometer (model LS6000SC; Beckman Instruments, Villepinte, France). For kinetics studies with *E. coli* cells, the radioactive substrates were used at final concentrations of 1 to 100 μM. For competition experiments, unlabeled choline, carnitine, ectoine, histidine, proline, and proline betaine were added at a final concentration of 100 μM or 1 mM to a 10 μM [*methyl*-^{14}C]glycine betaine solution. Competition uptake assays were run on a 5-min incubation period before filtration.

Transport assays with *S. meliloti* cells were realized in the same conditions as described for *E. coli* except that [*methyl*-^{14}C]glycine betaine and [U-^{14}C]proline betaine were used at 40 μM. Upshock effects on betaine transport activities in *S. meliloti* were measured from cells grown in low-osmolarity MCAA medium to the late exponential phase (OD_{420} of 0.8). The culture was then divided into two parts, and one was treated with 100 μg of chloramphenicol per ml. After 30 min, the chloramphenicol-treated culture and the untreated culture were subjected to a sudden osmotic upshock by adding NaCl to the growth medium at a final concentration of 0.3 M. Measurements of [*methyl*-^{14}C]glycine betaine uptake were done at various times before and after the upshock.

Nucleotide sequence accession number. The nucleotide sequence of the *betS* gene has been deposited in the GenBank database under accession number AF323271.

RESULTS

Identification and sequence analysis of the *betS* gene of *S. meliloti*. A PCR strategy was used to isolate a trimethylammonium transporter in *S. meliloti*. Alignment of various amino acid sequences of BCCT transporters, such as the glycine betaine uptake systems OpuD of *B. subtilis* (accession number GI 1524397) (20), BetP of *C. glutamicum* (accession number GI 1325948) (36), and BetL of *L. monocytogenes* (accession number GI 11279740) (49), the choline transporter BetT of *E. coli* (accession number GI 1786506) (24), and the BetT-like protein of *H. influenzae* (accession number GI 6626252) (14), revealed blocks of well-conserved amino acid residues. Strongly conserved regions are found in the putative transmembrane α-helix 8 and the small adjacent cytoplasmic connecting loop.

Two stretches of amino acids, FAAGM/IG and F/YWAWW, were defined to synthesize degenerate primers called BT1 and BT3R (see Materials and Methods). PCR amplification using total genomic DNA of *S. meliloti* 102F34 as the template with these primers resulted in a 700-bp amplified fragment of the expected size. This fragment was subcloned in pGEM-T, amplified, and used as a probe to screen a genomic DNA library of *S. meliloti* 2011. Ten positive clones were detected, and one was analyzed further. It contained a recombinant cosmid, pBT47, carrying a 20-kb *Sau*3A insert. By restriction analysis and Southern hybridization, the region homologous to the PCR-amplified fragment was restricted to a 3.4-kb *Sal*I fragment and subcloned in pBT58 (Fig. 1A). The nucleotide sequence of the 3,375-bp DNA fragment carried by pBT58 led to the identification of one major open reading frame (ORF) of 2,121 nucleotides which encoded a polypeptide of 706 residues with a calculated molecular mass of 77.6 kDa. This ORF, hereafter referred to as the *betS* gene, is preceded by a potential ribosome-binding site (GAGGA) adjacent to the putative ATG start codon. An inverted repeat sequence is present downstream of the stop codon and might serve as a transcription terminator. Analysis of the annotated genomic sequence of the *S. meliloti* genome (http://sequence.toulouse.inra.fr/meliloti.html) has shown that this nucleotide sequence is localized on the pSymb megaplasmid (smbY20333).

A search for related proteins revealed significant sequence identities with all BCCT transport systems involved in the uptake of trimethylammonium compounds. When considering BetS in its entire length, the highest identity scores were obtained with the BetT -like protein of *H. influenzae* (37%), BetT of *E. coli* (34%), OpuD of *B. subtilis* (30%), BetL of *L. monocytogenes* (28%), and BetP of *C. glutamicum* (25%). The hydrophobicity profile (DAS program; Stockholm University, Stockholm, Sweden) of BetS predicts 12 membrane-spanning segments (Fig. 1B), each with a length of approximately 20 amino acid residues and with both the N- and C-terminal extensions located in the cytoplasm. Considering the predicted structure and the identity scores mentioned previously, it is obvious that BetS of *S. meliloti* belongs to the BCCT transporter family.

It should be noticed that the smbY20333 sequence from the annotated genome of *S. meliloti* refers to a gene encoding a polypeptide of 621 amino acids, i.e., 85 residues shorter than BetS. Indeed, the ATG used to describe BetS is located 255 bp upstream from the ATG codon used for smbY20333. The putative protein encoded by smbY20333 lacks the first transmembrane helix and thus presents an N-terminal extension located in the periplasmic space, which is not a structure characteristic of BCCT transporters.

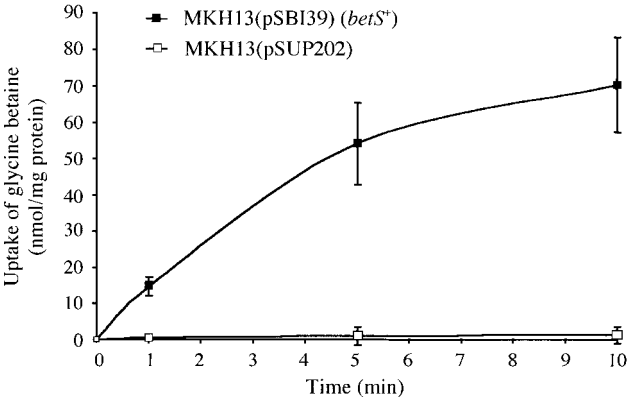

FIG. 2. BetS-mediated glycine betaine uptake in *E. coli* MKH13. Cells were grown in M63 medium containing 0.3 M NaCl to mid-log phase and assayed for the uptake of [^{14}C]glycine betaine at a final substrate concentration of 10 μM. Values are means from three independent experiments with standard errors as indicated.

Interestingly, the BetS protein possesses an important cytoplasmic C-terminal end (169 amino acid residues). Among members of the BCCT family, a similar C-terminal extension is present only in the BetT proteins of *E. coli* and *H. influenzae.* This C-terminal end is much shorter (about 50 amino acids) in the EctP (38) and BetP (36) transporters of *C. glutamicum*. *S. meliloti* BetS shows a cytoplasmic N-terminal domain of 52 amino acids, and such a long extension is found only in the BetP protein of *C. glutamicum*.

Transport activities and specificity of BetS expressed in *E. coli* MKH13. To investigate the function of BetS, the *betS* gene of *S. meliloti* was expressed in the MKH13 mutant strain of *E. coli*. In contrast to the *E. coli* parental strain, MC4100, the mutant MKH13, which cannot synthesize glycine betaine from its precursor, choline, and lacks the PutP, ProP, and ProU transport systems, is unable to grow on minimal medium of high osmolarity containing choline or glycine betaine as an osmoprotectant (18). Plasmid pSBI39, which contains the *betS* gene cloned in pSUP202, was transferred into MKH13. The presence of pSBI39, but not of pSUP202, restored the growth of MKH13 on high-osmolarity (0.5 M NaCl) minimal medium plates supplemented with 1 mM glycine betaine. As in MKH13, [^{14}C]glycine betaine transport at high affinity (10 μM) could not be detected in strains MKH13(pSUP202) and MKH13 (pSBI39) when grown in low-osmolarity minimal medium containing 1 mM glycine betaine or not (data not shown). However, in contrast to MKH13 and MKH13(pSUP202), [^{14}C]glycine betaine was taken up by MKH13(pSBI39) when the cells were grown in high-osmolarity medium (0.3 M NaCl), supplemented or not with glycine betaine (Fig. 2). Therefore, the functional complementation observed in MKH13(pSBI39) demonstrates that *betS* encodes a glycine betaine transporter.

Since the maximum uptake rate was observed when the bacteria were grown in the presence of 0.3 M NaCl without glycine betaine (data not shown), this condition was used to

TABLE 2. Effects of various compounds on glycine betaine uptake in *E. coli* MKH13(pSBI39)[a]

Competitor	% Inhibition of uptake with unlabeled competitor at:	
	100 μM	1 mM
Choline	−5	4
Carnitine	4	13
Ectoine	−2	3
Proline	1	2
Proline betaine	47	89
Histidine	−1	1

[a] Cells were grown in M63 medium containing 0.3 M NaCl. The results are expressed as percent inhibition of glycine betaine uptake and are means of three independent experiments with variations of less than 5%. Uptake was realized with 10 μM [^{14}C]glycine betaine. The control value without competitor was 6.4 nmol of glycine betaine transported per min per mg of protein.

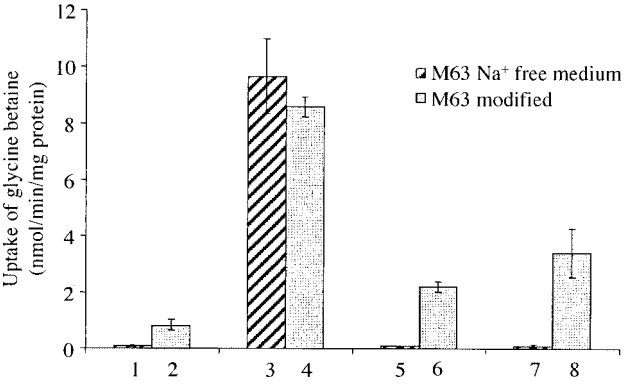

FIG. 3. Influence of medium composition on BetS activity in *E. coli* MKH13(pSBI39). [^{14}C]glycine betaine uptake was measured in cells grown in M63 medium with or without Na^+, at low osmolarity (1 and 2), or subjected to osmotic upshock with 0.3 M NaCl (3 and 4), 0.3 M KCl (5 and 6), or 0.48 M mannitol (7 and 8). Measurements were done in the presence of 10 μM substrate 10 min after solute addition. Values are means from duplicates of three independent cultures with standard errors as indicated.

determine the Michaelis-Menten parameters of BetS in the heterologous background strain MKH13(pSBI39). The apparent K_m value of glycine betaine uptake was found to be 16 ± 2 μM, with a V_{max} of 41 ± 5 nmol/min/mg of protein, indicating a high-affinity transport system. A similar K_m value, 10 μM, was previously reported for glycine betaine uptake in wild-type *S. meliloti* cells grown in high-osmolarity medium (2).

Since BCCT transporters are involved in the uptake of choline, ectoine, and carnitine in addition to glycine betaine, the substrate specificity of BetS was analyzed by competition assays using strain MKH13(pSBI39) grown in the presence of 0.3 M NaCl. The uptake of 10 μM [^{14}C]glycine betaine (Table 2) was greatly inhibited by the addition of unlabeled proline betaine, with 50 and 90% inhibition, with a 10- and 100-fold excess, respectively, of competitor. Measurements of [^{14}C]proline betaine uptake in strain MKH13(pSBI39) grown in the presence of 0.3 M NaCl showed a maximum uptake rate of 75 ± 18 nmol/min/mg of protein and a K_m of 56 ± 6 μM, whereas strain MKH13(pSUP202) did not show any transport. The addition of choline, ectoine, and carnitine had no effect on glycine betaine uptake activity despite a 100-fold excess of competitor. Proline and histidine, substrates of the Hut transporter which is also involved in glycine betaine uptake in *S. meliloti* (3), were not competitors. These results demonstrated that BetS is a high-affinity uptake system with a narrow specificity for glycine betaine and proline betaine.

Regulation of BetS activity in *E. coli* MKH13. Since the transport activities of glycine betaine and proline betaine mediated by BetS were detectable in the *E. coli* MKH13 mutant complemented by pSBI39 only when NaCl was added to the medium, we wanted to determine whether BetS activity is coupled to the transport of sodium and subject to immediate activation by the osmolarity of the medium. Thus, *E. coli* MKH13(pSBI39) cells grown in low-osmolarity medium were subjected to sudden osmotic upshift by the addition of NaCl, KCl, or mannitol (Fig. 3). When the cells were cultivated at low osmolarity in M63 Na^+-free medium, glycine betaine transport was barely detectable (less than 0.1 nmol/min/mg of protein), whereas addition of NaCl (0.3 M final concentration) strongly increased the uptake within 2 min following upshock (9.5 nmol/min/mg of protein). When the uptake was performed by the addition of KCl or mannitol used at concentrations osmotically equivalent to 0.3 M NaCl, no activation of uptake was observed (Fig. 3).

For further elucidation, the cells were grown at low osmolarity in M63 modified medium containing Na_2HPO_4 instead of K_2HPO_4 (40.8 mM Na^+). In this medium, glycine betaine uptake in unstressed cells was clearly increased but still remained quite low. As observed previously, BetS was again strongly activated after upshock performed with NaCl. Interestingly, in contrast to what was found for cells grown in Na^+-free medium, addition of KCl or mannitol also led to a strong enhancement in transport activity even though the stimulation was 4- and 2.6-fold less, respectively, than the increase observed in the presence of NaCl (Fig. 3). The difference may result from a specific effect of Na^+ at high concentration, but can also be due to a weaker stress because of significant uptake of K^+ or mannitol. Taken together, uptake measurements in cells grown in the presence or the absence of NaCl and upshock experiments carried out with NaCl, KCl, or mannitol indicate that Na^+ is most likely the coupling ion for BetS activity and suggest that BetS is subjected to posttranslational activation by high osmolarity, since such rapid regulation cannot be the consequence of activated transcription.

Glycine betaine and proline betaine transport activities in *S. meliloti*. To characterize the contribution of BetS to glycine betaine and proline betaine uptake in *S. meliloti*, a double recombinant GMI211 strain carrying a *lacZ*-Km^r cassette in the *betS* gene inserted in the opposite orientation (UNA316) was constructed as described in Materials and Methods and shown in Fig. 1A. Uptake of glycine betaine and proline betaine, used at a final concentration of 40 μM, was measured in both strains, wild type and mutant UNA316, grown overnight in MCAA medium in either the presence or the absence of 0.3 M NaCl. While the mutant strain was completely unaffected in glycine betaine uptake compared with the wild-type strain when the cells were grown at low osmolarity, at high osmolarity it showed a 60% decrease in uptake rate (Table 3). When glycine betaine was added to the growth medium, again a

TABLE 3. Glycine betaine and proline betaine uptake activities in *S. meliloti* wild-type (GMI211) and *betS* mutant (UNA316) strains[a]

Growth conditions	Uptake activity (nmol/min/mg of protein)			
	Glycine betaine		Proline betaine	
	GMI211	UNA316	GMI211	UNA316
Control	3.9 ± 0.1	3.6 ± 0.6	1.1 ± 0.2	1.0 ± 0.1
+ Glycine betaine (1 mM)	10.5 ± 0.9	10.6 ± 0.8	2.0 ± 0.3	1.9 ± 0.2
+ NaCl (0.3 M)	18.4 ± 1.4	7.0 ± 1.0	7.2 ± 0.4	3.0 ± 0.5
+ NaCl (0.3 M) + glycine betaine (1 mM)	20.0 ± 4.0	7.7 ± 0.6	7.8 ± 0.9	3.6 ± 0.5

[a] Cells were grown for 16 h in low-osmolarity MCAA medium (control) or in MCAA medium supplemented as indicated. Uptake measurements were done at a final substrate concentration of 40 μM. Values are means from duplicates of three independent cultures, and standard deviations are also shown.

strong reduction of uptake was observed only in the mutant salt-stressed cells. In addition, the presence of the substrate stimulated the uptake in cells grown at low osmolarity by two- to threefold, suggesting the presence of a glycine betaine-induced transporter(s) in both strains. Such stimulation was not observed in cells grown at high osmolarity.

When uptake of proline betaine (40 μM) was measured with cells grown as previously, the results obtained with the wild-type and the mutant strains were in good agreement with the corresponding results obtained with glycine betaine (Table 3). If proline betaine uptake activity appeared about three- to fourfold lower than glycine betaine uptake activity, depending on the growth, it is important to note that 40 μM substrate represents 2.5-fold the K_m value for glycine betaine but less than the K_m value for proline betaine. Given that the strong reduction of betaine uptake in the mutant strain was observed only in cells grown at high osmolarity, we assume that the BetS transporter represents an important component in the overall glycine betaine and proline betaine uptake activities in response to salt stress in *S. meliloti*.

Osmotic regulation of BetS in *S. meliloti*. To analyze the expression of *betS* in *S. meliloti,* a transcriptional *betS-lacZ* fusion was constructed, resulting in strain UNA315 (see Materials and Methods). The β-galactosidase activity of UNA315 was measured in cells grown in LAS minimal medium and compared to the basal level of β-galactosidase activity of UNA316. In strain UNA315, the *betS-lacZ* fusion was strongly expressed in cells grown in minimal medium (Table 4). Addition of NaCl (0.3 M), glycine betaine (1 mM), or a combination of both did not significantly induce *betS* expression. These results indicate that the *betS* gene is constitutively expressed and exclude significant transcriptional induction by either salt stress or glycine betaine. Consequently, in *S. meliloti*, the activation of BetS-mediated glycine betaine transport by osmotic stress is most likely due to a posttranscriptional regulation, as previously observed in the complemented *E. coli* MKH13 strain.

TABLE 4. β-Galactosidase activities of *S. meliloti* GMI211 carrying a *betS-lacZ* fusion integrated into the chromosome (strain UNA315)[a]

Growth conditions	β-Galactosidase activity (Miller units)
Control	1,155 ± 76
+ NaCl (0.3 M)	1,425 ± 51
+ Glycine betaine (1 mM)	1,180 ± 7
+ NaCl (0.3 M) + glycine betaine (1 mM)	1,472 ± 23

[a] Cells were grown overnight in low-osmolarity LAS minimal medium (control). Values are means from duplicates of two independent cultures, and standard deviations are also shown.

Since *betS* is not regulated at the expression level, it was important to establish whether BetS activity, which is observed only at high osmolarity, depends on osmotic activation of existing BetS proteins. Thus, glycine betaine uptake was measured in exponentially growing wild-type GMI211 and *betS* mutant UNA316 cells, treated or not with chloramphenicol, and subjected to a sudden osmotic upshock by adding NaCl (final concentration, 0.3 M). As shown in Fig. 4, glycine betaine uptake by GMI211 was immediately stimulated upon osmotic upshock (from 4 to 15 nmol/min/mg of protein). A strong stimulation was observed in less than 2 min, but maximal activation required approximately 8 to 10 min. Then, the rate of uptake continued to rise slowly over a period of 180 min to reach approximately 20 nmol/min/mg of protein. Chloramphenicol-treated cells showed a similar response to the upshock. However, the slow further increase observed previously could no longer be detected, and the rate of transport remained constant during 180 min. In contrast to what was observed with the wild type, glycine betaine uptake in the *betS* mutant was slightly decreased for about 10 min after the osmotic upshock and then slowly increased as in the wild type. In chloramphenicol-treated cells, this increase was completely abolished. Almost similar results were obtained with proline betaine (80 μM) as the substrate (data not shown).

From all of these experiments, we conclude the following: (i) upon osmotic upshock, the strong increase in glycine betaine and proline betaine uptake via the BetS transporter is the consequence of the activation of existing BetS proteins, probably through conformational changes; (ii) besides BetS, at least one other uptake system for both betaines is operative, and its expression is most likely transcriptionally induced by osmotic stress. For further elucidation of the role of osmolarity on BetS activation, glycine betaine transport was measured in GMI211 and UNA316 following osmotic upshock obtained by the addition of increased NaCl concentrations (Fig. 5). Clearly, the maximal activation of BetS-mediated glycine betaine transport was obtained with 0.3 M NaCl. At higher concentrations, the rate of transport decreased, probably because of inhibitory effects of the high ion concentration.

Contribution of BetS to osmoprotection by glycine betaine and proline betaine in *S. meliloti*. To assess the contribution of the BetS transporter to osmoprotection by glycine betaine and proline betaine, GMI211 and UNA316 were grown in MCAA medium under high osmolarity (0.5 M NaCl) in the absence or

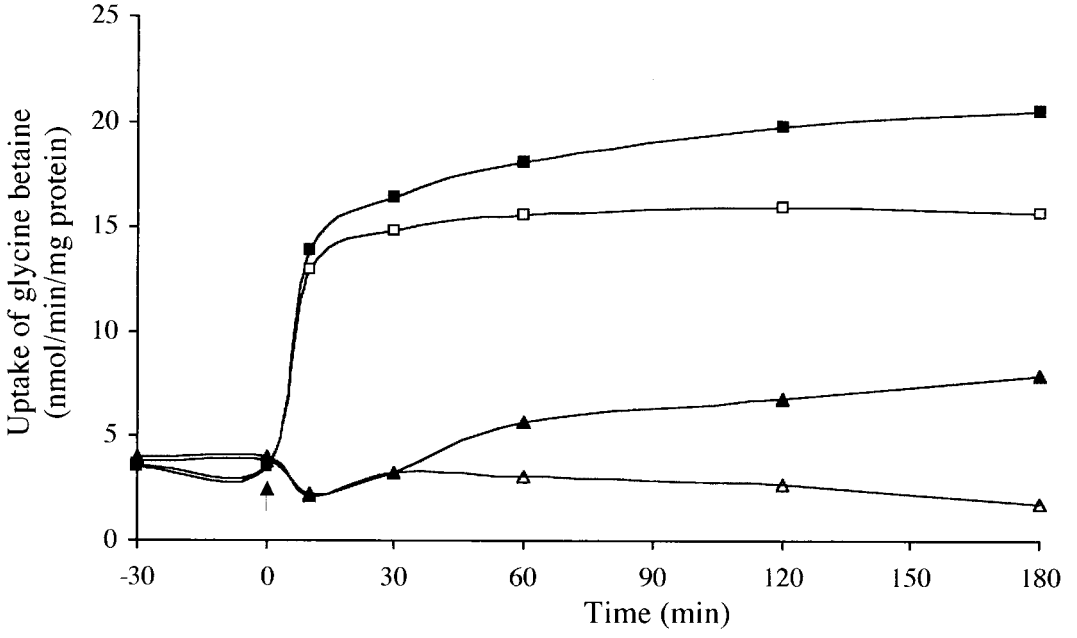

FIG. 4. Effect of NaCl upshock on glycine betaine uptake activity in *S. meliloti* GMI211 (■, □) and *betS* mutant (▲, △) strains. Cells were grown in MCAA low-osmolarity medium to mid-log phase and subjected to a sudden upshock (indicated by the arrow) by adding NaCl at a final concentration of 0.3 M. Uptake measurements of [^{14}C]glycine betaine were done at a final concentration of 40 μM using cells treated (□, △) or not (■, ▲) with chloramphenicol (100 μg/ml). Values are means from duplicates of three independent cultures, with standard errors of less than 5%.

presence of betaine (1 mM). As already known, (i) growth of both strains was strongly impaired in the absence of osmoprotectant, (ii) addition of 1 mM glycine betaine or 1 mM proline betaine strongly alleviated growth inhibition in GMI211, and (iii) glycine betaine was more efficient than proline betaine in protecting *S. meliloti* against osmotic stress (Fig. 6). During the exponential growth phase, the generation time obtained for salt-stressed cells grown in the presence of betaines was not significantly different between GMI211 and UNA316. However, UNA316 showed a much longer delay in its response to osmotic stress: for example, in the presence of glycine betaine, the lag phase for GMI211 was about 3 h, compared to 12 h for UNA316 (Fig. 6A). Similar results were obtained when proline betaine replaced glycine betaine (Fig. 6B). Hence, the BetS transporter of *S. meliloti* is absolutely required for a rapid adaptation to osmotic stress, through uptake of glycine betaine or proline betaine.

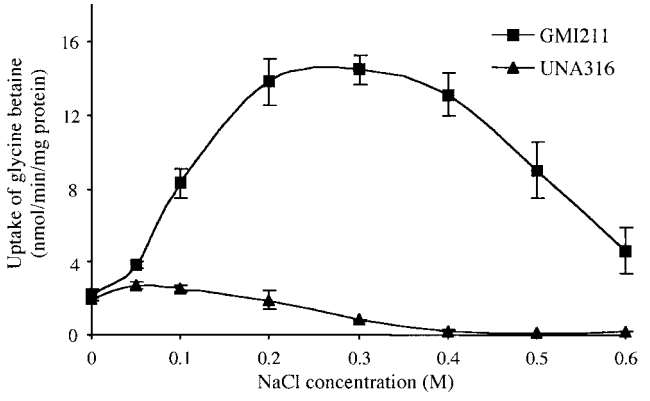

FIG. 5. Dependence of glycine betaine uptake in *S. meliloti* wild-type and *betS* mutant strains on increases in medium osmolarity imposed by NaCl. Cells were grown in low-osmolarity MCAA medium and then subjected to osmotic upshock by addition of NaCl. The initial uptake rates were determined at 40 μM [^{14}C]glycine betaine, 10 min after upshock, and plotted against the NaCl concentration added to the medium. Data are means from duplicates of three separate experiments; standard deviations are also shown.

DISCUSSION

Glycine betaine and proline betaine accumulation in salt-stressed *S. meliloti* allows the cells to grow at high osmolarity, both betaines being very potent osmoprotectants (17, 25). These betaines are mainly plant-derived compounds which may reach soil bacteria through root exudation or plant decomposition (41). In addition, because proline betaine occurs widely in *Medicago* species (42) and not in other genera of the Leguminosae (54), this betaine, which is also released from germinating *Medicago* seeds (42), may be ecologically important for root colonization of alfalfa by *S. meliloti*.

In this study, we report the identification and characterization of BetS, a major *S. meliloti* transporter for glycine betaine and proline betaine, whose inactivation results in loss of protection by both betaines after an osmotic upshock. BetS shows significant identity with various BCCT transporters (BetT, OpuD, BetL, and BetP) and, from a computer-assisted analysis, possesses 12 transmembrane domains, with both the N- and C-terminal ends located in the cytoplasm, a structural feature common in secondary transporters (46). In addition, a highly

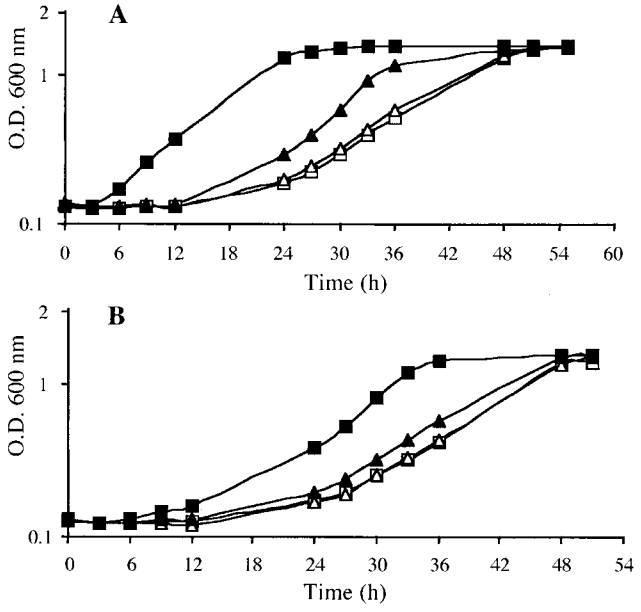

FIG. 6. Growth of *S. meliloti* GMI211 wild-type (■, □) and *betS* mutant (▲, △) strains. Cells were grown in MCAA medium with 0.5 M NaCl in the presence (solid symbols) or not (open symbols) of 1 mM glycine betaine (A) or 1 mM proline betaine (B). Values are means from duplicates of three independent cultures, with variations of less than 3%.

conserved amino acid sequence within helix 8 contains, at residue 357, the BCCT signature, (GSDN)-WT-(LIVM)-X-(FY)-WXWW. With respect to betaine uptake in *S. meliloti*, only an ATP-binding cassette histidine transporter (Hut system), also involved in glycine betaine and proline betaine, has been characterized previously (3). However, this system, which is induced by histidine and not by high osmolarity, most likely has a catabolic role (nitrogen assimilation) rather than being involved in osmoprotection. Hence, the BetS system characterized here is the first osmoregulated BCCT identified in *S. meliloti* and furthermore the only bacterial BCCT showing high affinity for proline betaine. The uptake of this betaine is mediated through the ABC system ProU in *E. coli* (18) and through the ProU homologues OpuA and OpuC in *B. subtilis* (4), even though their affinities for proline betaine have not been determined so far.

Upon expression in a betaine uptake-deficient *E. coli* mutant grown at elevated osmolarity, BetS displays high affinity towards glycine betaine (K_m value of 16 μM) and proline betaine (K_m value of 56 μM), allowing *S. meliloti* to scavenge both betaines from the rhizosphere or the soil. BetS appeared to be driven via cotransport with Na^+, another feature common to this subclass of symporters. In addition, BetS failed to transport other trimethylammonium or related molecules, such as choline, carnitine, and ectoine, which proves a narrow specificity of BetS towards glycine betaine and proline betaine. A restricted specificity towards one or two molecules is also relevant to the BCCT transporters: OpuD, BetL, and BetP are highly specific for glycine betaine, BetT is a choline carrier, and EctT, a new BCCT recently characterized in *Virgibacillus pantothenticus,* is mainly involved in ectoine and hydroxyectoine uptake (A. Kuhlmann and E. Bremer, personal communication). Given the competition among soil bacteria for osmoprotectants, it is tempting to argue that *S. meliloti* is particularly well adapted for using proline betaine exuded in the rhizosphere of alfalfa.

A prominent finding of this work is the regulation of BetS. Upon *betS* expression in *E. coli*, whereas the carrier was completely inactive in cells grown at low osmolarity, its activity was strongly increased in cells subjected to salt stress. In *S. meliloti*, a transcriptional *betS-lacZ* fusion is constitutively expressed, but osmotic stress was required for BetS activity. More conclusively, a sudden osmotic upshock strongly activated BetS within minutes following stress application in the presence or the absence of a protein synthesis inhibitor. Hence, BetS is positively regulated by osmotic stress at the posttranslational level, most probably by conformational changes, as observed for BetP in *C. glutamicum* (36). The level of BetS activation was dependent on the intensity of the stress, and maximal induction was found at an NaCl concentration of 0.3 M (Fig. 5). The pattern of BetS activation is similar to that observed for BetP in *C. glutamicum*, which, however, presents optimal activity at 0.6 M NaCl (36), probably reflecting the higher turgor pressure in gram-positive bacteria compared to gram-negative bacteria.

Some osmoprotectant carriers, including both ABC transporters and ion symporters, have been shown to be upregulated at the posttranslational level by elevation of medium osmolarity (16, 37, 44). Because osmotic shifts elicit dramatic changes in bacterial cell structure, a model of direct sensing of the osmolarity of the environment by monitoring alteration in membrane tensions has been proposed to account for the activation of various osmoregulated transporters. Among them, the activation mechanisms of the H^+-symporter ProP of *E. coli*, the Na^+-dependent BCCT transporter BetP of *C. glutamicum*, and the ABC OpuA transporter of *Lactococcus lactis* have been analyzed in great details (37, 44, 45, 52). All three proteins have been purified, and the osmoregulated responses of the activities have been examined in proteoliposomes, indicating that osmoregulation of these transporters is mediated by changes in membrane properties, with the lipids playing an essential role. These carriers act as both osmoregulators and osmosensors for the cells.

In both ProP and BetP transporters, the positively charged C-terminal extension seems to be involved in sensing and/or transducing osmotic changes to the domain of the protein responsible for the translocation of the substrate (37). Interestingly, BetS contains a much longer stretch of internal hydrophilic residues (169 amino acids) in its C-terminal part than ProP and BetP. This extension is identical in length to that of the choline BetT transporter of *E. coli*. The BetS and BetT C-terminal regions show 23% identity but do not show similarity to the C-terminal domain of BetP. In addition, they do not contain any coiled-coil sequence, as in ProP (9, 32). Further studies are necessary to fully elucidate the role of the C-terminal end of BetS in the activation and the stability of the active conformation of the protein.

In view of the osmotic upshock results and growth experiments, *S. meliloti* BetS is clearly a major betaine transporter used for rapid adaptation to sudden increases in osmolarity. However, BetS can be distinguished from other osmopro-

tectant emergency transporters, such as the EctP system from *C. glutamicum*, by its narrow specificity. In contrast to EctP, which accepts glycine betaine, ectoine, proline, and probably all known compatible solutes, in *C. glutamicum* (38), BetS can only transport glycine betaine and proline betaine.

Since the *betS* mutant retains 40% of the betaine transport capacity of the wild type under high osmolarity, *S. meliloti* uses at least one other uptake system, most probably transcriptionally induced and involved in long-term adaptation to high salt concentration. Furthermore, the measurements of glycine betaine uptake by cells grown at low osmolarity (Table 3) indicate that an additional betaine uptake activity inducible by the substrate is present in *S. meliloti*. Thus, in addition to BetS and to the Hut system, this bacterium may contain at least two other betaine carriers. A multiplicity of betaine uptake systems has been observed in various bacteria, such as *B. subtilis* (20). It is important to note that *S. meliloti* is unusually well equipped with transporters, particularly on the megaplasmid pSymb, as evidenced by the genome annotation (13), and this perfectly correlates with the extremely large spectrum of osmoprotectants and energy sources, including betaines, used by this bacterium.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment research tool. J. Mol. Biol. **215:**403–410.
2. **Bernard, T., J.-A. Pocard, B. Perroud, and D. Le Rudulier.** 1986. Variations in the response of salt-stressed *Rhizobium* strains to betaines. Arch. Microbiol. **29:**189–198.
3. **Boncompagni, E., L. Dupont, T. Mignot, M. Østerås, A. Lambert, M.-C. Poggi, and D. Le Rudulier.** 2000. Characterization of a *Sinorhizobium meliloti* ATP-binding cassette histidine transporter also involved in betaines and proline uptake. J. Bacteriol. **182:**3717–3725.
4. **Bremer, E., and R. Krämer.** 2000. Coping with osmotic challenges: osmoregulation through accumulation and release of compatible solutes in bacteria, p. 79–97. *In* G. Storz and R. Hengge-Aronis (ed.), Bacterial stress responses. American Society for Microbiology, Washington, D.C.
5. **Cairney, J., I. R. Booth, and C. F. Higgins.** 1985. *Salmonella typhimurium proP* gene encodes a transport system for the osmoprotectant betaine. J. Bacteriol. **164:**1218–1223.
6. **Cairney, J., I. R. Booth, and C. F. Higgins.** 1985. Osmoregulation of gene expression in *Salmonella typhimurium*: *proU* encodes an osmotically induced betaine transport system. J. Bacteriol. **164:**1224–1232.
7. **Cohen, G. N., and H. V. Rickenberg.** 1956. Concentration spécifique réversible des aminoacides chez *E. coli*. Ann. Inst. Pasteur **91:**693–720.
8. **Csonka, L. N., and W. Epstein.** 1996. Osmoregulation, p. 1210–1223. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella:* cellular and molecular biology, 2nd ed. American Society for Microbiology, Washington, D.C.
9. **Culham, D. E., B. Tripet, K. I. Racher, R. T. Voegele, R. S. Hodges, and J. M. Wood.** 2000. The role of the carboxyl terminal α-helical coiled-coil domain in osmosensing by transporter ProP of *Escherichia coli*. J. Mol. Recognit. **13:** 309–322.
10. **Farwick, M., R. M. Siewe, and R. Krämer.** 1995. Glycine betaine uptake after hyperosmotic shift in *Corynebacterium glutamicum*. J. Bacteriol. **177:**4690–4695.
11. **Finan, T. M., E. K. Hartwieg, K. LeMieux, K. Bergman, G. C. Walker, and E. R. Signer.** 1984. General transduction in *Rhizobium meliloti*. J. Bacteriol. **159:**120–124.
12. **Finan, T. M., B. Kunkel, G. F. DeVos, and E. R. Signer.** 1986. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. J. Bacteriol. **167:**66–72.
13. **Finan, T. M., S. Weidner, K. Wong, J. Buhrmester, P. Chain, F. J. Vorhölter, I. Hernandez-Lucas, A. Becker, A. Cowie, J. Gouzy, B. Golding, and A. Pühler.** 2001. The complete sequence of the 1,683-kb pSymB megaplasmid from the N_2-fixing endosymbiont *Sinorhizobium meliloti*. Proc. Natl. Acad. Sci. USA **98:**9889–9894.
14. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, K. McKennie, G. Sutton, W. FitzHugh, C. Fields, J. D. Gocayne, J. Scott, R. Shirley, L.-I. Liu, A. Glodek, M. Kelley, J. F. Weidmann, T. Phillipps, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritchmann, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter.** 1995. Whole random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
15. **Friedman, A. M., S. R. Long, W. S. E. Brown, W. J. Buikema, and F. M. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene **18:**289–296.
16. **Glaasker, E., E. H. M. L. Heuberger, W. N. Konings, and B. Poolman.** 1998. Mechanism of osmotic activation of the quaternary ammonium compound transporter (QacT) of *Lactobacillus plantarum.* J. Bacteriol. **180:**5540–5546.
17. **Gloux, K., and D. Le Rudulier.** 1989. Transport et catabolism of proline betaine in salt-stressed *Rhizobium meliloti.* Arch. Microbiol. **151:**143–148.
18. **Haardt, M., B. Kempf, E. Faatz, and E. Bremer.** 1995. The osmoprotectant proline betaine is a major substrate for binding-protein-dependent transport system ProU of *Escherichia coli* K-12. Mol. Gen. Genet. **246:**783–786.
19. **Kappes, R. M., and E. Bremer.** 1998. Responses of *Bacillus subtilis* to high osmolarity: uptake of carnitine, crotonobetaine, and γ-butyrobetaine via the ABC transport system OpuC. Microbiology **144:**83–90.
20. **Kappes, R. M., B. Kempf, and E. Bremer.** 1996. Three transport systems for the osmoprotectant glycine betaine operate in *Bacillus subtilis*: characterization of *opuD*. J. Bacteriol. **178:**5071–5079.
21. **Kempf, B., and E. Bremer.** 1995. OpuA, an osmotically regulated binding protein-dependent transport system for the osmoprotectant glycine betaine in *Bacillus subtilis*. J. Biol. Chem. **270:**16701–16713.
22. **Kempf, B., J. Gade, and E. Bremer.** 1997. Lipoprotein from the osmoregulated ABC transport system OpuA of *Bacillus subtilis*: purification of the glycine betaine binding protein and characterization of a functional lipidless mutant. J. Bacteriol. **179:**6213–6220.
23. **Kokotek, W., and W. Lotz.** 1989. Construction of a *lacZ*-kanamycin-resistance cassette, useful for site directed mutagenesis and as a promoter probe. Gene **84:**467–471.
24. **Lamark, T., I. Kaasen, M. W. Eshoo, P. Falkenberg, J. McDougall, and A. R. Strøm.** 1991. DNA sequence and analysis of the *bet* genes encoding the osmoregulatory choline-glycine betaine pathway of *Escherichia coli*. Mol. Microbiol. **5:**1049–1064.
25. **Le Rudulier, D., and T. Bernard.** 1986. Salt tolerance in *Rhizobium*: a possible role for betaines. FEMS Microbiol. Rev. **39:**67–72.
26. **Le Rudulier, D., K. Gloux, and N. Riou.** 1991. Identification of an osmotically induced periplasmic glycine betaine-binding protein from *Rhizobium meliloti.* Biochim. Biophys. Acta **1061:**199–205.
27. **Le Rudulier, D., A. R. Strøm, A. M. Dandekar, L. T. Smith, and R. C. Valentine.** 1984. Molecular biology of osmoregulation. Science **224:**1064–1068.
28. **Lucht, J. M., and E. Bremer.** 1994. Adaptation of *Escherichia coli* to high osmolarity environments: osmoregulation of the high-affinity glycine betaine transport system ProU. FEMS Microbiol. Rev. **14:**3–20.
29. **Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149:**114–122.
30. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. **Milner, J. L., S. Grothe, and J. M. Wood.** 1988. Proline porter II is activated by a hyperosmotic shift in both whole cells and membrane vesicules of *Escherichia coli* K12. J. Biol. Chem. **263:**14900–14905.
32. **Milner, J. L., and J. M. Wood.** 1989. Insertion *proQ220*::Tn*5* alters regulation of proline porter II, a transporter of proline and glycine betaine in *Escherichia coli*. J. Bacteriol. **171:**947–951.
33. **Niel, C., J. B. Guillaume, and M. Bechet.** 1977. Mise en évidence de deux enzymes présentant une activité β-galactosidase chez *Rhizobium meliloti*. Can. J. Microbiol. **23:**1178–1181.
34. **Østerås, M., E. Boncompagni, N. Vincent, M. C. Poggi, and D. Le Rudulier.** 1998. Presence of a gene encoding choline sulfatase in *Sinorhizobium meliloti bet* operon: choline-*O*-sulfate is metabolized into glycine betaine. Proc. Natl. Acad. Sci. USA **95:**11394–11399.
35. **Perroud, B., and D. Le Rudulier.** 1985. Glycine betaine transport in *Escherichia coli*: osmotic regulation. J. Bacteriol. **161:**393–401.
36. **Peter, H., A. Burkovski, and R. Krämer.** 1996. Isolation, characterisation, and expression of the *Corynebacterium glutamicum betP* gene, encoding the

transport system for the compatible solute glycine betaine. J. Bacteriol. **178:**5229–5234.
37. **Peter, H., A. Burkovski, and R. Krämer.** 1998. Osmo-sensing by N- and C-terminal extensions of the glycine betaine uptake system BetP of *Corynebacterium glutamicum*. J. Biol. Chem. **273:**2567–2574.
38. **Peter, H., B. Weil, A. Burkovski, R. Krämer, and S. Morbach.** 1998. *Corynebacterium glutamicum* is equipped with four secondary carriers for compatible solutes: identification, sequencing, and characterization of the proline/ectoine uptake system, ProP, and the ectoine/proline/glycine betaine carrier, EctP. J. Bacteriol. **180:**6005–6012.
39. **Philipp, W. J., S. Poulet, K. Eiglmeier, L. Pascopella, V. Balasubramanian, B. Heym, S. Bergh, B. R. Bloom, W. R. Jacobs, and S. T. Cole.** 1996. An integral map of the genome of the bacillus *Mycobacterium tuberculosis* H37Rv, and comparison with *Mycobacterium leprae*. Proc. Natl. Acad. Sci. USA **93:**3132–3137.
40. **Phillips, D. A., C. M. Joseph, and C. A. Maxwell.** 1992. Trigonelline and stachydrine released from alfalfa seeds activate NodD2 protein in *Rhizobium meliloti.* Plant Physiol. **99:**1526–1531.
41. **Phillips, D. A., and W. R. Streit.** 1997. Applying plant-microbe signalling concepts to alfalfa: roles for secondary metabolites, p. 319–342. *In* B. D. McKersie and D. C. W. Brown (ed.), Biotechnology and the improvement of forage legumes. CAB International, Wallingford, England.
42. **Phillips, D. A., J. Wery, C. M. Joseph, A. D. Jones, and L. R. Teuber.** 1995. Release of flavonoids and betaines from seeds of seven *Medicago* species. Crop Sci. **35:**805–808.
43. **Pocard, J.-A., N. Vincent, E. Boncompagni, L. T. Smith, M. C. Poggi, and D. Le Rudulier.** 1997. Molecular characterization of the *bet* genes encoding glycine betaine synthesis in *Sinorhizobium meliloti* 102F34. Microbiology **143:**1369–1379.
44. **Racher, K. I., R. T. Voegele, E. V. Marshall, D. E. Culham, J. M. Wood, H. Jung, M. Bacon, M. T. Cairns, S. M. Ferguson, W. J. Liang, P. J. F. Henderson, G. White, and F. R. Hallett.** 1999. Purification and reconstitution of an osmosensor: transporter ProP of *Escherichia coli* senses and responds to osmotic shifts. Biochemistry **38:**1676–1684.
45. **Rübenhagen, R., H. Rönsch, H. Jung, R. Krämer, and S. Morbach.** 2000. Osmosensor and osmoregulator properties of the betaine carrier BetP from *Corynebacterium glutamicum* in proteoliposomes. J. Biol. Chem. **275:**735–741.
46. **Saier, M. H., Jr.** 1994. Computer-aided analysis of transport protein sequences: gleaning evidence concerning function, structure, biogenesis, and evolution. Microbiol. Rev. **58:**71–93.
47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
48. **Simon, R., U. Priefer, and A. Pülher.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology **1:**784–791.
49. **Sleator, R. D., C. G. M. Gahan, T. Abee, and C. Hill.** 1999. Identification and disruption of BetL, a secondary glycine betaine transport system linked to the salt tolerance of *Listeria monocytogenes* LO28. Appl. Environ. Microbiol. **65:**2078–2083.
50. **Smith, L. T., J.-A. Pocard, T. Bernard, and D. Le Rudulier.** 1988. Osmotic control of glycine betaine biosynthesis and degradation in *Rhizobium meliloti*. J. Bacteriol. **170:**3142–3149.
51. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
52. **Van der Heide, T., and B. Poolman.** 2000. Osmoregulated ABC-transport system of *Lactococcus lactis* senses water stress via changes in the physical state of the membrane. Proc. Natl. Acad. Sci. USA **97:**7102–7106.
53. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153:**3–34.
54. **Wyn Jones, R. G., and R. Storey.** 1981. Betaines, p. 171–204. *In* L. P. Paleg and D. Aspinall (ed.), The physiology and biochemistry of drought resistance in plants. Academic Press, Ltd., Sydney, Australia.