

Proline Betaine Uptake in *Sinorhizobium meliloti*: Characterization of Prb, an Opp-Like ABC Transporter Regulated by both Proline Betaine and Salinity Stress

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Sinorhizobium meliloti uses proline betaine (PB) as an osmoprotectant when osmotically stressed and as an energy source in low-osmolarity environments. To fulfill this dual function, two separate PB transporters, BetS and Hut, that contribute to PB uptake at high and low osmolarity, respectively, have been previously identified. Here, we characterized a novel transport system that mediates the uptake of PB at both high and low osmolarities. Sequence analysis of Tn5-*luxAB* chromosomal insertions from several PB-inducible mutants has revealed the presence of a four-gene locus encoding the components of an ABC transporter, Prb, which belongs to the oligopeptide permease (Opp) family. Surprisingly, *prb* mutants were impaired in their ability to transport PB, and oligopeptides were not shown to be competitors for PB uptake. Further analysis of Prb specificity has shown its ability to take up other quaternary ammonium compounds such as choline and, to a lesser extent, glycine betaine. Interestingly, salt stress and PB were found to control *prb* expression in a positive and synergistic way and to increase Prb transport activity. At low osmolarity, Prb is largely implicated in PB uptake by stationary-phase cells, likely to provide PB as a source of carbon and nitrogen. Furthermore, at high osmolarity, the analysis of *prb* and *betS* single and double mutants demonstrated that Prb, together with BetS, is a key system for protection by PB.

Bacteria have to cope with variations in their environment and particularly with changes in the external osmolarity. They protect themselves against high external osmolarity by accumulating osmoprotective organic compounds in their cytoplasm, the so-called compatible solutes (10). These usually nonionic, highly water-soluble molecules can be accumulated to very high levels in the cytoplasm of stressed cells without interfering with vital cellular functions. They correspond to a limited number of compounds including sugars (trehalose), free amino acids (e.g., glutamate and proline), and quaternary ammonium compounds (e.g., glycine betaine, proline betaine [PB], γ -butyrobetaine, and carnitine) that bacteria accumulate from de novo synthesis or from externally provided osmoprotectants such as choline, the precursor of glycine betaine (15, 36). Many bacteria are equipped with systems that facilitate the efficient transport of osmoprotectants under stressed conditions, and several of these osmoregulated systems have been identified previously (64). Among these systems, mainly ATP-binding cassette (ABC) transporters and secondary transporters have been characterized at the molecular level. All the ABC transporters reported until now in nonhalophilic bacteria are related to the ProU system from *Escherichia coli* (58), including OpuA, OpuB, and OpuC of *Bacillus subtilis* (29, 30), OusB of *Erwinia chrysanthemi* (14), and BusA of *Lactococcus lactis* (8).

In a few bacteria such as *Sinorhizobium meliloti*, the root

symbiont of *Medicago sativa* (alfalfa), quaternary ammonium transporters also have an important role at low osmolarity. In fact, this bacterium has the capacity to use compatible solutes such as glycine betaine and PB as carbon and nitrogen sources in addition to osmoprotection (5, 25). PB, also known as *N,N*-dimethylproline or stachydrine, is available in the rhizosphere, where it is secreted by germinating seedlings of many *Medicago* species nodulated by *S. meliloti* (47). The molecule is converted in proline after two steps of demethylation, with the first step of demethylation depending on *stc2* and *stc4* genes organized in a cluster on the megaplasmid pSyma and with the second step requiring the *stcD* gene located on the megaplasmid pSymb (11, 25, 46). PB catabolism is active at low osmolarity but reduced at high osmolarity, whereas PB uptake is increased. As a consequence, PB is accumulated in the cytoplasm, contributing to the reestablishment of turgor pressure (24). The expression of *stc2*, *stc4*, and *stcD* genes is enhanced in the presence of PB in the growth medium (25, 46), and PB has also been shown to induce the expression of nodulation (*nod*) genes via the activation of the NodD2 transcriptional regulator (45). Thus, more likely, the PB uptake activity also contributes to PB-mediated gene regulation in *S. meliloti*.

Two systems involved in the uptake of PB in *S. meliloti* have previously been characterized. Hut is a ProU-like ABC transporter with a high affinity for histidine, proline, and PB. Its activity is controlled at the transcriptional level by histidine but not by salt stress, and Hut contributes to the use of histidine as a nitrogen source (6). The BetS system is a Na⁺-coupled secondary transporter, with a high affinity for glycine betaine and PB. This system is activated posttranslationally by osmotic stress and plays a crucial role in the rapid response to osmotic upshock (7). However, a *betS* mutant is still able to use PB as

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TABLE 1. Bacterial strains and plasmids used in this study

Strain, plasmid, or transposon	Relevant characteristics ^a	Source or reference
Strains		
<i>S. meliloti</i>		
Rm1021	SU47 <i>str-21</i> Sm ^r	37
Rm5000	SU47 <i>rif-5</i> Rif ^r	20
Rm1021-S1 (S1)	Rm1021::Tn5 pRL1063a Sm ^r Neo ^r	46
Rm1021-S2 (S2)	Rm1021::Tn5 pRL1063a Sm ^r Neo ^r	46
Rm1021-S3 (S3)	Rm1021::Tn5 pRL1063a Sm ^r Neo ^r	46
Rm1021-S4 (S4)	Rm1021::Tn5 pRL1063a Sm ^r Neo ^r	46
Rm1021-S5 (S5)	Rm1021::Tn5 pRL1063a Sm ^r Neo ^r	46
Rm1021-S6 (S6)	Rm1021::Tn5 pRL1063a Sm ^r Neo ^r	46
Rm1021-S7 (S7)	Rm1021::Tn5 pRL1063a Sm ^r Neo ^r	46
Rm1021-S8 (S8)	Rm1021::Tn5 pRL1063a Sm ^r Neo ^r	46
RmUNA369	Rm1021 <i>betS</i> ::Ω Sm ^r Spc ^r	This work
RmUNA370	S7 <i>betS</i> ::Ω Sm ^r Spc ^r Neo ^r	This work
RmHY220	Rm5000 <i>hutX</i> ::Ω	6
<i>E. coli</i>		
DH5α	<i>supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	53
MT616	MT607 pRK600	21
Plasmids		
pRK600	ColE1 replicon with RK2 transfer region; Cm ^r	21
pH 45-Ω	pBR322 derivative with interposon Ω; Amp ^r Sm ^r Spc ^r	49
pSup 202	ColE1 replicon; Mob ⁺ Tc ^r Amp ^r Cm ^r	56
pSBI39	Derivative of pSup202 with an <i>S. meliloti</i> 3.4-kb insert containing the <i>betS</i> gene	7
pSM28	pSBI39 <i>betS</i> ::Ω (KpnI insertion)	This work
pSIF7	10-kb EcoRI fragment of Rm1021-S7 containing <i>prbB</i> ::Tn5 pR1063a; Km ^r	This work
pSIF5	4.3-kb EcoRI fragment of Rm1021-S5 containing <i>prbB</i> ::Tn5 pR1063a; Km ^r	This work
pSIF3	4.3-kb EcoRI fragment of Rm1021-S3 containing <i>prbC</i> ::Tn5 pR1063a; Km ^r	This work
Transposon		
Tn5pRL1063a	Tn5 derivative; promoterless <i>luxAB</i> ; <i>oriV</i> Km ^r	63

^a Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Neo^r, neomycin resistance; Rif^r, rifampicin resistance; Spc^r, spectinomycin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance.

an osmoprotectant, suggesting the existence of another PB transport system(s). In this study, we described the characterization of a new ABC transporter, called Prb, involved in the uptake of PB. Prb is homologous to an oligopeptide permease and not to ProU, while it also transports choline and glycine betaine. We also demonstrated that Prb is active at low and high osmolarities and is required for a maximal adaptation to high-salinity conditions, since a *prb betS* mutant does not use PB as an osmoprotectant.

MATERIALS AND METHODS

Chemicals. Proline betaine was purchased from Extrasynthèse (Genay, France). Choline, glycine betaine, proline, Gly-Gly, Gly-His, Ala-Pro, Gly-Pro-Arg-Pro, Tyr-Gly-Gly-Phe-Leu (leucine enkephaline), and *N,N*-dimethylglycine were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). *N*-Methylproline was synthesized from the sodium salt of proline, as described previously (44). γ -Butyrobetaine and dimethylsulfonioacetate (DMSA) were prepared as described previously (3). Radioactive [U-¹⁴C]PB (4.6 GBq/mmol) was obtained from the Commissariat à l'Énergie Atomique (Gif-sur-Yvette, France), and [methyl-¹⁴C]choline (2.04 GBq/mmol) was purchased from Amersham Corp. (Little Chalfont, United Kingdom). [methyl-¹⁴C]glycine betaine was prepared from [methyl-¹⁴C]choline as previously described (44).

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. meliloti* strains were maintained at 30°C in Luria-Bertani (LB) medium supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂. For luciferase, transport, and osmoprotection assays, *S. meliloti* strains

were grown in MCAA semisynthetic medium containing 0.1% sodium malate as the carbon source and 0.1% Casamino Acids (technical) as the nitrogen source (57). For physiological analysis of the role of Prb and Hut, cells were grown in M9 minimal medium (37) supplemented with 0.2% mannitol or 0.2% PB as a carbon source. The osmolarity of the medium was increased by the addition of NaCl, as indicated. *E. coli* strains were grown in LB broth at 37°C. Antibiotics were used in *E. coli* (or *S. meliloti*) cultures at the following concentrations: kanamycin, 25 μg/ml; spectinomycin, 200 μg/ml; chloramphenicol, 20 μg/ml; streptomycin, 200 μg/ml; neomycin, 200 μg/ml. Bacterial growth was monitored spectrophotometrically at 600 nm, and protein concentrations were determined by the method of Bradford (9), with bovine serum albumin as a standard.

DNA manipulations and sequence analysis. Genomic and plasmidic DNA extractions, digestions by restriction enzymes, ligations, and transformations were performed by using standard methods (53). To recover *S. meliloti* genomic sequences contiguous with the inserted transposon, genomic DNA from each Rm1021::Tn5 pRL1063a mutant was digested by EcoRI, which does not cut within the transposon, ligated, and transformed into DH5α cells where *oriV* in pRL1063a maintained the DNA as a plasmid. For each mutant strain, plasmid DNA was purified, and the DNA sequence of the *S. meliloti* fragments was determined using unique outward primers corresponding to the left and right ends of Tn5 pRL1063a as described previously (38). Nucleotide sequence determination was performed by MWG-Biotech (Ebersberg, Germany) by using the fluorescent ABI dye-labeled deoxy terminator method. DNA and protein sequences were analyzed by using BLAST protocols (2).

Construction of *betS* mutants. *betS* mutants were constructed by using the Ω interposon encoding spectinomycin resistance. pSBI39, which carries the entire *betS* gene (7), was linearized by KpnI, which cuts inside the *betS* coding sequence, blunt ended, and ligated with an SmaI-digested Ω interposon. The resulting

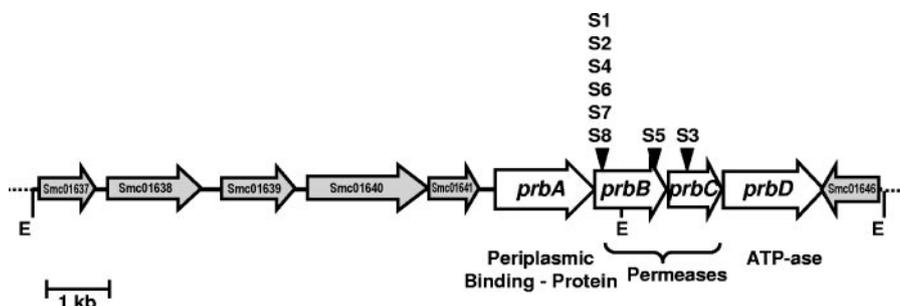


FIG. 1. Genetic organization of the chromosomal *prb* locus region of *S. meliloti* strain Rm1021. EcoRI sites used for subcloning are indicated (E), and locations of the Tn5-*luxAB* insertions are indicated by an arrowhead. Open reading frames are as follows: Smc01637, unknown function; Smc01638, hydroxyacyl-CoA dehydrogenase; Smc01639, acyl-CoA dehydrogenase; Smc01640, acyl-CoA synthetase; Smc01641, enoyl-CoA hydratase; Smc01646, unknown function.

plasmid was transferred by conjugation into strains Rm1021 and S7 by using triparental spot matings with *E. coli* MT616 as a helper strain (21). The Ω insertions were finally recombined into the *S. meliloti* genome by the plasmid incompatibility technique (52) as previously described (43). Genetic exchange of the wild-type *betS* gene by the *betS*- Ω insertion was confirmed by Southern hybridization analysis.

Measurement of luciferase activity. Cells from 500 μ l of bacterial culture were harvested, resuspended in fresh MCAA medium to obtain a final A_{600} of 1, mixed with 2 μ l of *n*-decyl aldehyde (Sigma, St. Louis, Mo.), and vortexed for 30 s. Light emission was measured for 60 s with a luminometer (CHEM-GLOW photometer model J4-7441; American Instrument Company, Md.). The assays were performed on two independent cultures in triplicates. Bioluminescence is expressed as arbitrary defined relative light units.

Transport assays. *S. meliloti* cells were harvested at an A_{600} of 0.4 to 1.2, washed twice in the medium used for the culture, and resuspended at a final A_{600} of 0.5. All assays were carried out at 30°C with 1 ml of the cell suspension. Uptake of radioactive substrate (100,000 dpm) was determined after a 2-min incubation period by rapid filtration through GF/F glass microfiber filters (Whatman), which were rinsed with 3 ml of the same medium, as previously described (19). The radioactivity remaining on the filters was determined with a liquid scintillation spectrometer (model LS6000SC; Beckman Instruments, Villepinte, France). For competition experiments, unlabeled compounds were added at a final concentration of 4 mM to a 40 μ M [14 C]PB solution.

Periplasmic protein extraction and binding assays. *S. meliloti* strain Rm1021 was grown to an A_{600} of 1.5 in MCAA medium supplemented with 0.3 M NaCl and 100 μ M PB. Cells were collected by centrifugation (10,000 \times *g* for 10 min at 20°C) and resuspended in 10 mM Tris-HCl (pH 7.5). Periplasmic proteins were released by cold osmotic shock according to a method described previously by Neu and Heppel (42). The activity of one periplasmic marker enzyme, cyclic phosphodiesterase, and the activity of one typical cytoplasmic marker, malate dehydrogenase, were measured to determine the amount of marker enzyme activities released, as described previously (35). Periplasmic proteins were concentrated by ultrafiltration according to a standard procedure (35). Binding assays with 150 μ g of periplasmic proteins were performed using ammonium sulfate precipitation, as described previously (19). Measurements were done in triplicates from two independent periplasmic preparations.

RESULTS

Identification of *prb*, a new PB-inducible locus of *S. meliloti*.

Twelve Tn5-*luxAB* insertions mutants (S1 to S12) containing PB-inducible genes have been isolated from *S. meliloti* Rm1021 (46). The only two mutants that could not use PB as a source of carbon and nitrogen were characterized further and were shown to be inactivated in the *putA* gene, encoding proline dehydrogenase (strain S11), and in the *stcD* gene, which is involved in PB catabolism (strain S10) (46). In order to identify other PB-inducible loci, we cloned the tagged genes in other mutants in *E. coli*, and their DNA sequences were determined and compared to the genomic sequence of *S. meliloti* strain

Rm1021 (<http://sequence.toulouse.inra.fr/meliloti/html>). Eight insertions were located in the same region of the chromosome that we called *prb* (*prb* for proline betaine-inducible transporter), which represents a cluster of four open reading frames (ORFs) oriented in the same direction, designated *prbA* (Smc01642), *prbB* (Smc01643), *prbC* (Smc01644), and *prbD* (Smc01645) (Fig. 1). Seven insertions (S1, S2, S4, S5, S6, S7, and S8) were localized in *prbB*, with strains S1, S2, S4, S6, S7, and S8 corresponding to a single site of insertion. The transposon insertion in strain S3 was located in the third gene of the locus, *prbC*. At the 5' extremity of the locus, a cluster of four genes involved in the metabolism of acyl coenzyme A (CoA) was found directly upstream of the *prb* locus, separated from *prbA* by a 317-bp-long sequence. At the 3' extremity, a 795-bp-long ORF (Smc01646) of unknown function was found on the reverse strand, 29 bp downstream of *prbD*. Inside the *prb* locus, a strong translational coupling probably exists between all the genes, as supported by (i) the identity of the last base of the TGA stop codon of *prbA* and the first base of the ATG initiation codon of *prbB*, (ii) the overlapping of four bases in the ATGA sequence of *prbB* and *prbC*, and (iii) the overlapping of eight bases in the ATGCCTGA sequence of *prbC* and *prbD*.

This locus resembles an ABC transporter operon, with a periplasmic binding protein (PrbA), two inner membrane components (PrbB and PrbC), and a peripheral ATPase (PrbD). The ORF corresponding to *prbA* (Smc01642) is reported to encode a hydrophilic protein of 538 amino acid residues according to the *S. meliloti* website. However, sequence analysis (<http://www.cbs.dtu.dk/services/SignalP/>) of the NH₂-terminal part of the potential encoded protein revealed the presence of a characteristic signal peptide for secretion in the periplasm if the first amino acid of the encoded protein is the Met residue located 75 bp downstream of the predicted one. Thus, we estimated that the *prbA*-encoded peptide is 513 amino acid residues long and corresponds to the unprocessed form of the periplasmic protein PrbA. PrbB and PrbC (319 and 275 amino acids, respectively) are highly hydrophobic and contain six and five putative transmembrane helices, respectively, typical of integral membrane proteins. The last gene, *prbD*, encodes a 543-amino-acid-residue protein, which is likely the ATPase of the transporter. This protein contains two fused ABC domains presenting 33% sequence identity, and each of them contains

TABLE 2. Sequence identities and similarities^a between components of Prb and selected transporters of the oligopeptide transport system family in gram-negative bacteria

Transporter (bacterium)	% Identity (% similarity)				Substrate(s)	Reference
	PrbA	PrbB	PrbC	PrbD ^b		
Dpp (<i>E. coli</i>)	22 (46)	27 (44)	28 (53)	33 (56)/26 (49) 34 (59)/39 (62)	Dipeptides, 5-aminolevulinic acid	1, 61 61
Opp (<i>Salmonella enterica</i> serovar Typhimurium)	17 (41)	23 (44)	25 (48)	32 (57)/32 (54) 37 (56)/40 (63)	Oligopeptides	27
Dpp (<i>Rhizobium leguminosarum</i>)	21 (41)	28 (49)	31 (55)	32 (54)/30 (48) 36 (58)/38 (63)	Dipeptides, 5-aminolevulinic acid	13
Agp (<i>S. meliloti</i>)	20 (38)	23 (47)	28 (53)	36 (60)	α -Galactosides	23
Nik (<i>E. coli</i>)	19 (41)	22 (43)	28 (53)	31 (50)/26 (49) 31 (49)/36 (60)	Nickel	41
Acc (<i>Agrobacterium tumefaciens</i>)	20 (40)	28 (50)	35 (55)	28 (49)/34 (52) 33 (55)/35 (56)	Agrocinopine	26

^a The percentages of identity and similarity (in parentheses) were determined with Clustal W.

^b Each half containing the ABC domain of PrbD (amino-terminal domain/carboxy-terminal domain) was compared to the first encoded (first line) and the second encoded (second line) ATPase of the transporter. For AgpD, which is the unique ATPase encoded by Agp and which contains two ABC cassette domains, comparison with PrbD concerns the entire proteins.

the Walker A and Walker B sites, the Q loop, and the switch domain, forming the nucleotide-binding site (54, 55). The conserved signature motif (LSGGQ) unique to the ABC transporters (54, 55) is present in the carboxyl-terminal ABC domain, while a very close sequence (VSGGQ) is present in the amino-terminal ABC domain.

Comparison of the Prb protein sequences with protein sequences in the data libraries revealed that the highest amino acid similarity was obtained with the components from systems of the peptide ABC transporter superfamily (Table 2). These transporters are known for their broad substrate specificities and have been shown to be involved in the uptake of peptides and nonpeptide molecules such as nickel or sugars (16).

PB and NaCl inducibility of *prb* gene expression. As a first step to characterize Prb function, the potential induction of *prb* gene expression was analyzed under different growth conditions. The PB inducibility of Tn5-*luxAB* mutants, and particularly of the *prb* mutants, was initially detected in cells grown at low osmolarity (46). Since PB has an important role as an osmoprotectant in *S. meliloti*, PB inducibility of *prb* gene expression was determined in cells grown at high osmolarity and was compared to that of cells grown at low osmolarity. The *prb* expression level was measured by monitoring the luciferase activity in strain S7, a *prbB::luxAB* insertion mutant. This strain was grown in MCAA semisynthetic medium with or without PB (100 μ M) in the presence or absence of 0.3 M NaCl, a salt concentration that is not too restrictive for growth (Fig. 2A). In the absence of NaCl, PB induction of the *luxAB* reporter gene was significant only at stationary phase, and the activity was eightfold higher in induced cultures than in uninduced cultures after 14 h of growth (Fig. 2B). The addition of NaCl led to a much earlier and stronger PB induction, since after only 6 h of growth in MCAA medium containing NaCl and PB, the cell luciferase activity was more than 30-fold higher than the activity measured in MCAA medium (Fig. 2C); activity was even higher in cells entering stationary phase, after 14 h. The pos-

itive effect of NaCl without PB in the growth medium on *prbB* expression was also measurable from 6 h to 14 h, with about a twofold increase compared to the level in MCAA medium (Fig. 2B and C). These results showed that *prb* gene expression is controlled by both PB and NaCl, with a synergistic effect between the two compounds. Since the higher level of expression was observed at the beginning of the stationary phase under all tested conditions, the growth phase also plays a role in the control of *prb* gene expression. Moreover, the PB inducing effect could not be attributed to catabolite derivatives of PB, as neither *N*-methylproline nor proline induced the expression of *prb* (data not shown). Similarly, no induction by choline or glycine betaine could be detected, whereas these compounds are also transported by Prb (see below). Furthermore, despite the homology between Prb and oligopeptide transporters, no induction was observed after growth in the presence of a dipeptide (Gly-Gly) and oligopeptides of various sizes (Gly-Pro-Arg-Pro and Tyr-Gly-Gly-Phe-Leu), strengthening the specificity of the PB inducing effect on *prb* gene expression.

TABLE 3. A *prb* mutant is affected in the uptake of proline betaine at low osmolarity^a

Strain	Growth with PB	PB uptake activity ^b (nmol/min/mg of protein) \pm SD	
		Exponential phase	Stationary phase
Rm1021	No	0.56 \pm 0.10	0.70 \pm 0.10
	Yes	0.53 \pm 0.05	1.82 \pm 0.12
S7	No	0.51 \pm 0.05	0.69 \pm 0.03
	Yes	0.39 \pm 0.05	0.78 \pm 0.03

^a Cells were grown in MCAA medium with or without 100 μ M PB and harvested at mid-log phase (A_{600} of 0.4) or early stationary phase (A_{600} of 1.5).

^b Uptake measurements of PB were done at a final substrate concentration of 40 μ M. Values are means from duplicates of three independent cultures \pm standard deviations.

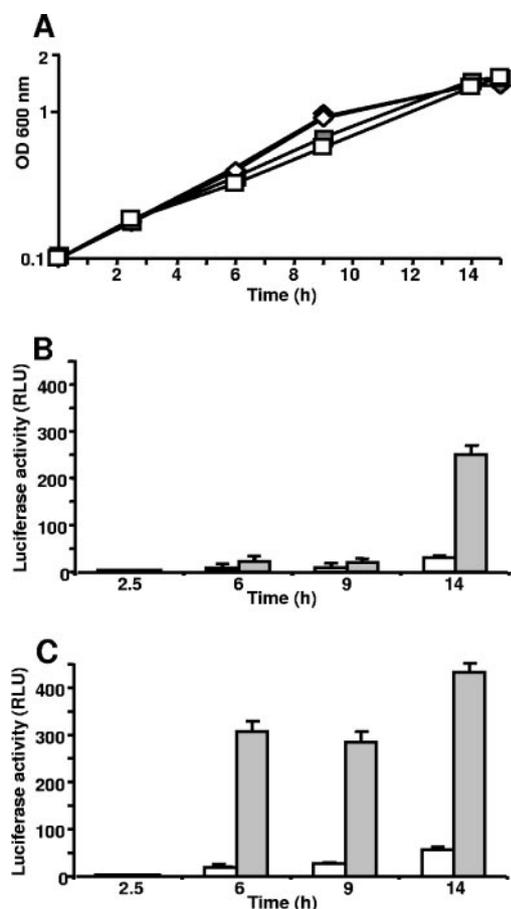


FIG. 2. *prb* gene expression is induced by both PB and NaCl. Cells of strain S7 (*prbB::Tn5-luxAB* fusion) were grown overnight in MCAA medium and then diluted in the same medium or in MCAA medium with 0.3 M NaCl in the presence (gray symbols and gray bars) or absence (white symbols and white bars) of PB. (A) Growth curves in MCAA medium (\diamond and \blacklozenge) and MCAA medium with NaCl (\square and \blacksquare). (B and C) Luciferase activity at various times during the growth cycle in MCAA medium and MCAA with NaCl. Results are means of duplicates from three independent experiments. The error bars represent the standard deviations from the means. OD, optical density; RLU, relative light units.

Prb contribution to PB uptake at low osmolarity. Assuming that PB inducibility of *prb* genes indicates that PB is a potential substrate for the Prb transport system, the contribution of Prb to PB uptake was investigated. Transport experiments using radiolabeled PB at 40 μ M were performed with wild-type and S7 cells grown in low-osmolarity MCAA medium in the presence or absence of PB (100 μ M). PB uptake was measured using cells grown to mid-exponential phase and cells grown to stationary phase. As shown in Table 3, wild-type and mutant strains collected at the exponential phase presented similar PB uptake activities, which were not enhanced by the presence of PB. This result is in full agreement with the low level of *prb* gene induction by PB at this growth stage, as shown previously. On the contrary, in stationary-phase cells, the addition of PB was followed by a 2.6-fold increase in PB uptake activity in the wild-type strain, but such stimulation was not observed in the mutant strain. These results showed that PB uptake activity can

be stimulated by the presence of PB in a low-osmolarity growth medium and that such increased activity is Prb dependent.

To investigate the physiological role of Prb in cells maintained at low osmolarity, growth experiments were performed using minimal medium containing PB as the unique carbon and nitrogen source. No difference in the growth kinetics between the wild-type and the *prb* mutant could be observed, which probably indicates that another PB transport system(s) compensates for the Prb deficiency. Hut was a good candidate, but the growth of *hut* mutant strains could not be distinguished from that of the wild-type strain, unless histidine (100 μ M) was present as a *hut* inducer in the growth medium. Thus, in addition to Prb, it is more likely that another transporter is involved in the utilization of PB as a carbon and nitrogen source.

Prb contribution to PB uptake at high osmolarity. PB transport assays were also performed using cells that were exposed to NaCl in MCAA medium with 0.3 M NaCl during 1.5 h and 15 h in the presence (100 μ M) or absence of PB (Fig. 3). In the wild-type strain, the PB uptake activity increased in response to the time of exposure to NaCl. Interestingly, this NaCl stimulation was enhanced by the presence of PB in the growth medium: after 15 h, the activity increased from 3.5 to 5.3 nmol/min/mg of protein when PB was added. This higher level corresponds to about a 10-fold increase in PB uptake activity compared to the activity in cells grown in MCAA medium (0.6 nmol/min/mg of protein) (Table 2). In the mutant strain, stimulation of uptake by NaCl was much lower, with a maximal activity of 1.6 nmol/min/mg of protein. Consequently, after 15 h of growth in the presence of both NaCl and PB, PB uptake activity in the *prb* mutant represented only one-third of the activity in wild-type cells grown under the same conditions. These results demonstrated that Prb is the major PB uptake system functioning under prolonged conditions of high salinity

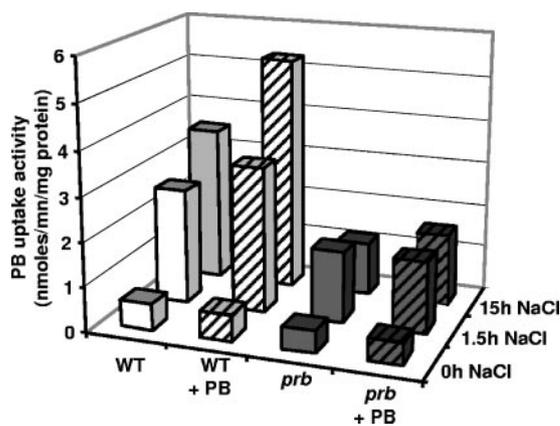


FIG. 3. A *prb* mutant is affected in the uptake of PB at high osmolarity. Cells of strains Rm1021 (wild type [WT]) and S7 (*prb*) grown to mid-log phase in MCAA medium were subjected to a 1.5-h NaCl treatment (final concentration of 0.3 M) in the presence or absence of 100 μ M PB or diluted in MCAA medium with 0.3 M NaCl supplemented or not supplemented with 100 μ M PB and grown for 15 h to mid-log phase. Uptake measurements of PB were done at a final concentration of 40 μ M. Results are means of duplicates from three independent experiments, and standard deviations did not exceed 15%.

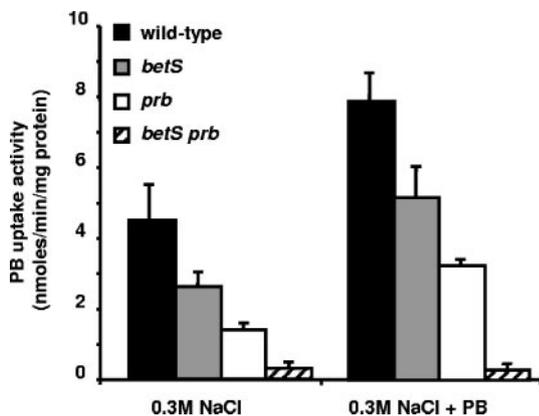


FIG. 4. PB uptake activity is severely impaired in a *prb betS* double mutant. Cells were grown for 18 h in MCAA medium containing 0.3 M NaCl or 0.3 M NaCl and 100 μM PB. Assays for the uptake of PB were done at a final concentration of 40 μM using cells from strains Rm1021 (wild type), RmUNA369 (*betS*), S7 (*prb*), and RmUNA370 (*prb betS*). The results are means of duplicates from three independent cultures; standard deviations from the means are also shown.

and that the enhancement of PB uptake activity by the presence of PB is essentially due to Prb stimulation.

Since both Prb and BetS systems are involved in the uptake of PB at high osmolarity (7), a *betS prb* double mutant should be severely impaired in PB uptake. Thus, a *betS* inactivated allele was introduced into strains Rm1021 and S7, resulting in strains UNA369 and UNA370 (see Materials and Methods). PB uptake activities of *prb* and *betS* single and double mutants (strains S7, RmUNA369, and RmUNA370, respectively) in cells grown at high osmolarity during 18 h in the presence or the absence of PB were then determined. Figure 4 shows that PB uptake activity was nearly abolished in the *betS prb* double mutant, indicating that only Prb and BetS significantly contributed to PB uptake at a high osmolarity. Moreover, PB uptake activity in *betS* and *prb* single mutants increased following the addition of PB to MCAA medium, suggesting that BetS activity, in addition to Prb activity, was stimulated by PB.

Evidence for PB-binding activity in periplasmic fractions. In gram-negative bacteria, high-affinity ABC transporters are dependent upon at least one periplasmic substrate binding protein, and Prb-mediated uptake of PB more likely depends on the existence of such a component. However, no PB-binding activity has previously been detected in periplasmic fractions of cells grown at low or high osmolarity (24). Since the results presented above have shown that Prb activity is strongly increased in the presence of both NaCl and PB, binding assays were performed with periplasmic fractions extracted from cells grown in MCAA medium containing NaCl (0.3 M) and PB (100 μM). In such fractions, a PB-binding activity of 35 pmol/mg of protein was detected in the presence of a free-ligand concentration of 80 μM. In extracts from cells grown at low osmolarity or in the presence of 0.3 M NaCl without PB, binding activity was barely measurable. These results demonstrated the existence of a PB binding protein(s) among periplasmic fractions from *S. meliloti* but with a level of activity that was rather low.

Prb is involved in the uptake of choline and glycine betaine. In *E. coli* and *B. subtilis*, the different systems involved in PB

TABLE 4. Various compounds inhibit proline betaine uptake in a *betS* mutant^a

Competitor	% Inhibition of PB uptake ^b with unlabeled competitor
Glycine betaine	94
Trigonelline.....	9
γ-Butyrobetaine.....	53
Choline	70
N,N-Dimethylglycine	40
N-Methylproline.....	55
DMSA	84

^a Cells of RmUNA369 (*betS::Ω*) were grown to mid-log phase in MCAA medium with 0.3 M NaCl.

^b Uptake assays were performed with 40 μM PB and different competitors added at a 100-fold molar excess. The results are expressed as the percentage of inhibition of PB uptake and are means of duplicates with standard deviations of less than 5%. The control value without competitor was 2.2 nmol of PB transported per min per mg of protein.

uptake also transport other betaines or related compounds (31). Similarly, in *S. meliloti*, BetS is a high-affinity transporter for PB and also for glycine betaine (7). In addition, in the wild-type strain, uptake of PB is inhibited by other quaternary ammonium compounds (24). In a first approach to determine potential substrates for Prb, different molecules were used in competition experiments conducted with strain UNA369 (*betS* mutant). Uptake of 40 μM PB in the presence of a 100-fold molar excess of unlabeled competitors was measured in cells grown in MCAA medium containing 0.3 M NaCl (Table 4). Trigonelline, which strongly inhibited PB uptake in the wild-type strain (24), poorly competed in the *betS* mutant, suggesting that it is a substrate for BetS and not for Prb. In contrast, several other compounds (glycine betaine, γ-butyrobetaine, choline, N,N-dimethylglycine, N-methylproline, and DMSA) effectively inhibited the uptake of PB in the *betS* mutant. Among these compounds, only choline and glycine betaine are available in a radioactive form. Thus, in order to precisely define the substrate of Prb, transport assays with radiolabeled choline and glycine betaine were performed using *prb*⁺ strains and *prb* mutants grown at high osmolarity (0.3 M NaCl). Choline (40 μM) uptake activity was significantly affected in the *prb* mutant, and the presence of PB in the growth medium strongly increased the uptake activity in the wild-type strain (Table 5). This suggested that stimulation of Prb by PB likewise plays an important role in the uptake of choline at high osmolarity. Conversely, the transport of glycine betaine (40 μM) was nei-

TABLE 5. A *prb* mutant is affected in the uptake of choline^a

Strain	Uptake activity (nmol/min/mg of protein) ^b ± SD			
	Choline		Glycine betaine	
	A	B	A	B
Rm1021 (wild type)	0.8 ± 0.1	6.8 ± 0.4	6.7 ± 0.4	7.3 ± 1.0
S7 (<i>prb</i>)	0.5 ± 0.1	0.3 ± 0.1	7.7 ± 0.5	6.3 ± 0.4
UNA369 (<i>betS</i>)	ND	ND	2.5 ± 0.1	3.9 ± 0.8
UNA370 (<i>prb betS</i>)	ND	ND	1.5 ± 0.1	1.6 ± 0.2

^a Cells grown in MCAA medium with 0.3 M NaCl in the absence (A) or the presence (B) of 100 mM PB were harvested at mid-log phase.

^b Uptake assays of choline and glycine betaine were done at a final substrate concentration of 40 μM. Values are means from duplicates of three independent cultures ± standard deviations. ND, not determined.

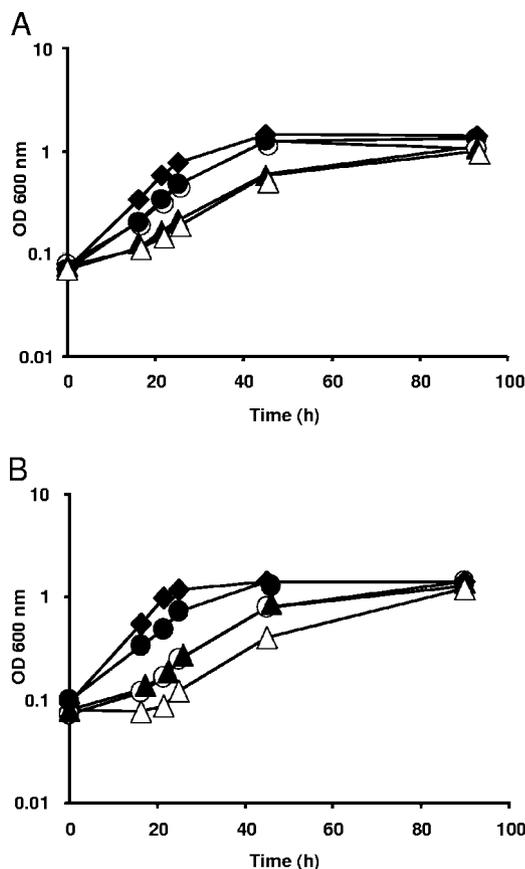


FIG. 5. Inactivation of *prb* has a strong negative effect on osmoprotection by PB and glycine betaine. Cells were grown in MCAA medium with 0.6 M NaCl in the presence of 1 mM PB (A) or 1 mM glycine betaine (GB) (B). Strains used were Rm1021 (wild-type) (◆), S7 (*prb*) (●), RmUNA369 (*betS*) (○), and RmUNA370 (*prbB betS*) (▲). Growth of RmUNA370 in MCAA medium with 0.6 M NaCl (without osmoprotectant) is also shown (△). The results are means of three independent cultures, and standard deviations did not exceed 5%. OD, optical density.

ther significantly stimulated by the addition of PB nor affected in the single *prb* mutant. However, it was more affected in the *prb betS* mutant than in the single *betS* mutant, showing that Prb also contributes to glycine betaine uptake but at a lower affinity than BetS. Finally, because of the rather high level of homology between Prb and oligopeptide transporters, peptides of different sizes (Ala-Pro, Gly-His, Gly-Pro-Arg-Pro, and Tyr-Gly-Gly-Phe-Leu) were used in competition experiments using the wild-type strain. None of them significantly inhibited PB uptake in cells grown in the presence or absence of NaCl (data not shown), suggesting that oligopeptide uptake is not mediated by Prb.

Prb plays a significant role in osmoprotection by PB and glycine betaine. Since Prb is involved in the transport of PB, choline, and glycine betaine in cells grown at high osmolarity, it could significantly contribute to osmoprotection. Therefore, the effect of *prb* inactivation was investigated by growing the cells at high osmolarity (0.6 M NaCl) in the absence or presence of PB, choline, or glycine betaine. No difference in osmoprotection by choline (7 mM) could be observed in a *prb*

mutant (strain S7) compared to the wild-type strain, indicating that another choline uptake activity compensated for the *prb* deficiency (data not shown). This result corroborates the previous identification of a choline uptake activity that is inducible by choline and fully active at high osmolarity (48). When PB (1 mM) was added, growth inhibition was much more efficiently alleviated in the wild-type strain than in *betS* and *prb* mutants (Fig. 5A). It is noteworthy that PB was no longer used as an osmoprotectant by the *betS prb* double mutant. These results offer evidence that Prb is necessary for maximal osmoprotection by PB and that beside Prb and BetS, no other transport system is required for adaptation to salinity stress through PB utilization. Osmoprotection by glycine betaine was also less efficient in the *prb* mutant than in the wild-type strain but was clearly much more efficient than that in the *betS* mutant (Fig. 5B). This result is consistent with the moderate contribution of Prb to glycine betaine uptake, as indicated by the results of transport assays. The remaining capacity of the *betS prb* double mutant to use glycine betaine for osmoprotection also indicates the existence of another system or other systems for glycine betaine transport in *S. meliloti*.

DISCUSSION

Proline betaine, a quaternary ammonium compound released in the rhizosphere by germinating alfalfa seeds (47), functions as an osmoprotectant, an energy substrate, and an inducer of nodulation genes in *S. meliloti*. For the first physiological processes, this bacterium has evolved several types of PB uptake systems, two of which are the Hut (6) and the BetS (7) transporter systems. In this study, we have localized Tn5-*luxAB* insertions in genes encoding components of a novel ABC transporter, called Prb, also involved in the uptake of PB, which differs from Hut and BetS by its inducibility by both PB and salt stress. The general organization of the *prb* locus is similar to that of ABC transporter operons, and each component of the Prb system shows significant similarity with components of the peptide ABC transporters (Opp-like transporters) of various bacteria. These systems are known to transport a large variety of substrates, such as peptides of various lengths, used as carbon and nitrogen sources or as pheromones (32, 34) but also heme precursor (13, 61), nickel (41), and sugar and modified sugar molecules (23, 26). In *S. meliloti*, 27 of the 176 predicted ABC transporters in the whole genome belong to the peptide transport system subfamily (4, 12, 22). However, their substrate specificities have not been characterized so far, except in the case of Agp system, which transports α -galactosides (23). It has been suggested that some Opp-like transporters from *S. meliloti* could be involved in osmotic adaptation, since several genes from *opp*-like operons are among the salt-induced genes identified using a microarray approach (51). The presence of *prbB* and *prbD* among these genes and our present data demonstrating the contribution of Prb in PB, choline, and glycine betaine uptake at high osmolarity strongly support this suggestion. It is noteworthy that in spite of the homology between Prb and Opp proteins, Prb is unlikely to be involved in the uptake of peptides, since peptides of 2 to 5 amino acids failed to compete with PB. Interestingly, PrbA has an arginine residue instead of the aspartate residue, which is present in all OppA-like proteins involved in peptide binding

and which forms a salt bridge with the N-terminal α -amino group of the bound peptide (18).

Disruption of the *prbB* gene significantly reduces PB transport in cells grown at low or high osmolarity. In stationary-phase cells grown at low osmolarity, Prb contributes to PB uptake and could thus enhance PB acquisition when the main exogenous carbon and nitrogen sources become limited for the cells and when their capacity for amino acid biosynthesis becomes diminished. In addition, under high-salinity conditions, Prb uptake activity has a major role in osmoprotection, since a strain inactivated in both Prb and BetS systems is no longer able to use exogenously provided PB as an osmoprotectant. Moreover, the demonstration that Prb is also involved in the uptake of choline and, to a lesser extent, of glycine betaine highlights the importance of Prb in the adaptation to salinity stress.

In terms of the uptake of osmoprotective compounds, *S. meliloti* shows important differences compared to other bacterial models. In most bacteria, osmoprotectants are metabolically inert molecules and are not actively transported at a low osmolarity. The uptake of the few compounds that play a double role in energy supply and osmoprotection is mediated through separate transport systems. For example, the acquisition of proline as a carbon and nitrogen source is mediated in *E. coli* by the PutP permease for which expression is proline induced (39, 40) and is mediated in *B. subtilis* by an uncharacterized system, while the utilization of proline for osmoprotective purposes requires the osmotically regulated ProP and ProU transporters in *E. coli* and the OpuE system in *B. subtilis* (15, 62). Similarly, in *S. meliloti*, the uptake of PB at low and high osmolarities involves distinct systems, Hut for energy supply and BetS for immediate adaptation to an osmotic upshock. Interestingly, Prb is, up to now, the first uptake system active under both conditions. It has been shown previously that the uptake of ectoine, a nonaccumulated osmoprotectant, is also fulfilled by a unique system (Ehu) in *S. meliloti* cells grown either at a low or high osmolarity. However, the expression of *ehu* genes is induced only by ectoine (28), whose uptake is not increased in cells grown at high osmolarity (59).

A prominent finding of this work concerns the dual regulation of Prb. Data regarding the expression of a *prbB::Tn5-luxAB* fusion have led to the proposal that this regulation occurs, at least partly, at the transcriptional level and is responsive to both increases in salinity and the presence of PB in the growth medium. A modest increase (twofold) in *prb* gene expression was observed in cells maintained in the presence of 0.3 M NaCl, whereas a strong synergistic enhancement was obtained in the presence of PB (Fig. 2), causing massive *prb* transcription when PB was available. Such PB and NaCl transcriptional control, combined with the osmotic posttranslational activation of BetS (7), allows the rapid and long-term uptake of PB in salt-stressed cells with a very beneficial effect on the adaptation to salinity stress. Salt stimulation by the activation of preexisting Prb proteins could additionally occur, as observed for other osmotically induced osmoprotectant transporters (31).

The capacity to accumulate PB at high osmolarity is necessarily controlled to prevent an excessive accumulation of the osmoprotectant and to maintain the cell turgor within certain limits. In many bacteria, the presence of osmoprotectants in the growth medium frequently reduces the transcription of

osmotically induced genes encoding components for the uptake of these solutes (50). An opposite situation is observed in *S. meliloti*, and that original *prb* regulation could come from the capacity of the cells to catabolize PB, restraining the accumulated betaine to a certain threshold. Whereas the induction of *stc2* gene expression by PB is strongly reduced in cells subjected to increased osmolarities, substantial PB degradation is still likely to occur at a lower salt concentration, such as 0.3 M NaCl (G. Alloing, unpublished results).

The affinity and specificity of ABC transporters usually depend on at least one periplasmic binding protein. In *S. meliloti*, periplasmic binding activities for glycine betaine and choline have already been observed in cells grown at high osmolarity (35), and our present results demonstrate the existence of a PB-binding activity. The measured binding activity is rather low and might indicate an unstable PB-protein complex, which is nevertheless associated with effective uptake. Similarly, in *L. lactis* (17), poor binding activity of short peptides to OppA has previously been observed, whereas these compounds are efficiently transported by the Opp system. In that case, the dissociation rate constant is an important determinant of the transport rate, and even poorly bound peptides, associated with high dissociation rate constant values, are relatively well transported (17, 33). However, the poor PB-binding activity might also be the consequence of the preloading of periplasmic proteins with PB present in the growth medium. Obviously, in *S. meliloti*, additional analyses of PrbA-binding activity are needed, and its potential role in PB, glycine betaine, and choline binding and uptake remains to be clarified.

Collectively, the remaining PB uptake activity observed in a *prb* mutant, as well as our inability to detect any difference between the wild-type, *prb*, and *hut* mutant strains in the kinetics of growth in minimal low-osmolarity medium containing PB as the only carbon or nitrogen source, indicates the existence of as-yet-uncharacterized PB transport systems. The existence of several systems dedicated to PB transport is particularly helpful for the adaptation of *S. meliloti* to various environments, in the soil as a heterotrophic bacterium and in planta in an endosymbiotic form. In both situations, PB synthesized by alfalfa, a host plant of *S. meliloti*, is present as a root exudate in the rhizosphere and in the nodule, cytosol, and bacteroids (45, 60). Finally, how *prb* expression is regulated in planta and whether Prb activity is crucial for the endosymbiotic form of *S. meliloti* when the host plant is subjected or not subjected to salt stress are interesting questions that require additional investigation.

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