Overexpression of BetS, a *Sinorhizobium meliloti* High-Affinity Betaine Transporter, in Bacteroids from *Medicago sativa* Nodules Sustains Nitrogen Fixation During Early Salt Stress Adaptation

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Submitted 7 December 2005. Accepted 20 March 2006.

Sinorhizobium meliloti possesses several betaine transporters to cope with salt stress, and BetS represents a crucial high-affinity glycine and proline betaine uptake system involved in the rapid acquisition of betaines by cells subjected to osmotic upshock. Using a transcriptional *lacZ* (β -galactosidase) fusion, we showed that *betS* is expressed during the establishment of the symbiosis and in mature nitrogen-fixing nodules. However, neither Nod nor Fix phenotypes were impaired in a *betS* mutant. BetS is functional in isolated bacteroids, and its activity is strongly activated by high osmolarity. In bacteroids from a *betS* mutant, glycine betaine and proline betaine uptake was reduced by 85 to 65%, indicating that BetS is a major component of the overall betaine uptake activity in bacteroids in response to osmotic stress. Upon *betS* overexpression (strain UNA349) in free-living cells, glycine betaine transport was 2.3-fold higher than in the wild-type strain. Interestingly, the accumulation of proline betaine, the endogenous betaine synthesized by alfalfa plants, was 41% higher in UNA349 bacteroids from alfalfa plants subjected to 1 week of salinization (0.3 M NaCl) than in wild-type bacteroids. In parallel, a much better maintenance of nitrogen fixation activity was observed in 7-day-salinized plants nodulated with the overexpressing strain than in wild-type nodulated plants. Taken altogether, these results are consistent with the major role of BetS as an emergency system involved in the rapid uptake of betaines in isolated and in planta osmotically stressed bacteroids of *S. meliloti*.

One of the most widely distributed strategies developed by bacteria, plants, and animals to respond to osmotic stress is the accumulation of a selected group of organic osmolytes, the so-called compatible solutes, that raise the intracellular osmotic pressure and protect some macromolecular structures against denaturation (Bourot et al. 2000; Le Rudulier et al. 1984; Timasheff 1992). Among the main osmolytes, betaines, particularly glycine betaine and proline betaine, also known as stachydrine, are probably the most efficient. *Sinorhizobium meliloti*, an alfalfa root-associated bacterium, possesses distinct

transport activities for betaine uptake, and two transporters, Hut and BetS, have been characterized fully. The Hut system is an ATP-binding cassette (ABC) histidine transporter also involved in proline and proline betaine uptake at high affinity and glycine betaine uptake at low affinity (Boncompagni et al. 2000). The BetS system, a single transmembrane protein which belongs to the betaine choline carnitine transporter (BCCT), is a high-affinity glycine betaine and proline betaine transporter (Boscari et al. 2002). BetS-mediated betaine uptake is the consequence of immediate activation of existing proteins by high osmolarity, most likely through posttranslational activation; thus, BetS appears as an emergency system involved in the rapid acquisition of betaines by cells subjected to osmotic upshock. Particularly relevant characteristics of BetS are briefly outlined below. i) It has a narrow specificity toward glycine betaine and proline betaine and fails to transport other trimethylammonium or related molecules such as choline, carnitine, and ectoine. ii) Its activation leads to protection by betaines after an osmotic upshock, and BetS represents a major component of the overall betaine uptake activities in response to salt stress. iii) *betS* is constitutively expressed in *S. meliloti* free-living cells. All these interesting features of BetS in a symbiotic species raise the question of its expression and role in bacteroids from nodules of alfalfa (*Medicago sativa* L.), a host plant of *S. meliloti*. It seems pertinent to point out that proline betaine is the major betaine found in alfalfa; however, other betaines, such as pipercolate betaine (homostachydrine), trigonelline, hydroxyproline betaine, and glycine betaine, also have been identified in various alfalfa genotypes (Wood et al. 1991). In addition, other *Medicago* spp., such as *M. truncatula*, *M. littoralis*, *M. rugosa*, and *M. polymorpha*, also produce proline betaine with significant genotypic variations (Naidu et al. 1992; Wood et al. 1991). Furthermore, we recently have demonstrated the ability of young nodulated alfalfa plants to accumulate proline betaine, through de novo synthesis, in shoots, roots, and nodules under salt stress treatment (Trinchant et al. 2004).

Obviously, betaines are available within alfalfa and, thus, the present study was initiated to gain an understanding of the role of BetS in alfalfa bacteroids. We have demonstrated the expression of *betS* during the early steps of the establishment of symbiosis and in mature nitrogen-fixing nodules. We also show that disruption of *betS* strongly reduced proline betaine transport in isolated bacteroids, whereas *betS* overexpression induced significant increase in proline betaine content within nodules produced by salt-stressed alfalfa. In addition, we pro-

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vided evidence that 1 week of salinization has a much more minor effect on nitrogen fixation in plants inoculated with the overexpressing strain than in plants nodulated with the wild-type strain.

RESULTS

betS expression during symbiosis.

We previously have shown that *betS* is constitutively expressed in *S. meliloti* under free-living conditions (Boscari et al. 2002). However, considering the low growth rate of this bacterium within the infection threads during the development of the symbiotic relationship with its host plant alfalfa (Gage et al. 1996), and the switch off of numerous cell structural genes during bacteroid differentiation (Becker et al. 2004), it was of interest to determine whether the expression of *betS* could occur during symbiosis. Thus, using a transcriptional *betS-lacZ* fusion (strain UNA315, *betS*⁺, kanamycin resistant [Km^r]), the expression of *betS* was monitored in situ during the infection process and in mature nitrogen-fixing nodules. The histochemical detection of β -galactosidase activity was performed using X-gal as substrate (Fig. 1). Seven days after infection, the indigo coloration resulting from the expression of the *betS-lacZ* fusion was observed inside root hair infection threads (Fig. 1A and B) and in the infection zone of 7-day-old developing nodules (Fig. 1C). As expected, analysis of mature 4-week-old nitrogen-fixing nodules revealed no X-gal staining in the apical meristematic zone formed of uninfected cells (Fig. 1D). In contrast, a strong expression of the *betS-lacZ* fusion was detected in the infection prefixing zone II and within the nitrogen-fixing zone III (Fig. 1D), as referred to the

specific histological zone of alfalfa nodules (Vasse et al. 1990). Observations of nodules from plants infected by the *betS* mutant strain UNA316, which contains the *lacZ* cartridge in the opposite orientation (*betS*⁻), indicated no expression of the *betS-lacZ* fusion inside the infection threads, and only a weak staining background in 7-day- and 4-week-old nodules (Fig. 1E and F). Hence, *betS* clearly is expressed during all successive symbiotic steps, from the infection thread formation to the differentiated bacteroids in mature nodules.

BetS is a major betaine transporter in bacteroids.

It previously has been established that bacteroids isolated from alfalfa nodules can taken up both glycine betaine and proline betaine (Fougère and Le Rudulier 1990a; Trinchant et al. 2004). Because BetS represents a major component of the overall betaine uptake activities in response to salt stress in free-living *S. meliloti*, we were interested in determining whether BetS could play such an important role in bacteroids, and also in testing to what extent BetS activity in bacteroids could be modulated by the osmolarity. For this purpose, alfalfa plantlets were infected with the *betS* mutant strain UNA316, and the GMI211 strain as control. Bacteroids were prepared from 5-week-old root nodules and the rates of transport were investigated by incubating purified free bacteroids with 40 μ M [¹⁴C]glycine betaine or [¹⁴C]proline betaine, at various osmolarities.

With [¹⁴C]glycine betaine as a substrate (Fig. 2), uptake by GMI211 bacteroids was very limited (approximately 0.40 nmol/15 min/mg of protein) at low osmolarity (100 mOsm/liter), whereas a strong stimulation (up to 10-fold) was observed upon osmotic upshock. The maximal transport activity was obtained at 500 to 600 mOsm/liter. In contrast, uptake by

Fig. 1. Histochemical localization of *betS* gene expression during *Sinorhizobium meliloti*–*Medicago sativa* nodule development. β -Galactosidase activity was detected using X-gal as substrate. *betS::lacZ* fusion (strain UNA315) in hair roots **A** and **B**, 4 days after infection, **C**, in 7-day-old nodule, and **D**, in 4-week-old nodule. β -Galactosidase staining in **E**, 7-day-old nodule and **F**, 4-week-old nodule infected with *betS* mutant (strain UNA316). Arrows indicate infection threads. Specific histological zones (I, II, III, and IV) are indicated on mature nodule cross sections. Scale bars: 25 μ m (A and B), 100 μ m (C and E), and 400 μ m (D and F).

UNA316 bacteroids was not osmotically activated, and maximal rate remained almost constant at approximately 1 nmol/15 min/mg of protein. Clearly, BetS-mediated glycine betaine transport in bacteroids is functional, and osmotically-dependent, as in free-living *S. meliloti* cells (Boscari et al. 2002), with a maximal activation obtained at an osmolarity of 600 mOsm/liter, equivalent to 0.3 M NaCl. To investigate the consequence of salt stress application to the host plants on glycine betaine uptake by bacteroids, nodulated alfalfa plants (3-week-old nodules) were subjected to 0.3 M NaCl during 2 weeks before bacteroid preparation. Salinization of the host plant tended to stimulate the uptake rate in GMI211 bacteroids: a 30% increase was observed at high osmolarity (600 mOsm/liter), which gave the highest rate (Fig. 2). However, salinization had no effect on uptake in UNA316 bacteroids.

Uptake experiments also were conducted with [14 C]proline betaine as a substrate (Fig. 3). Bacteroids were purified from nodules produced on alfalfa plants subjected or not to 0.3 M NaCl during 2 weeks, as in the previous experiment. They were resuspended in medium of low (100 mOsm/liter) or high (600 mOsm/liter) osmolarity, before [14 C]proline betaine uptake measurements. As observed in the experiment with [14 C]glycine betaine, salinization of the host plant induced a 25% increase in proline betaine uptake by GMI211 bacteroids maintained at 600 mOsm/liter. Other similarities can be noticed: i) at 100 mOsm/liter, uptake was rather low (1 nmol/15 min/mg of protein), and even lower in UNA316 bacteroids; and ii) at 600 mOsm/liter, a 4.2-fold stimulation was observed in GMI211 bacteroids compared with only a 2.5-fold in UNA316 bacteroids, and again the uptake activity was much lower in mutant bacteroids than in wild-type bacteroids (2.8-fold). However, it should be noticed that the maximal uptake rate of proline betaine was significantly lower than glycine betaine uptake (1.8-fold), and this could be due to a lower affinity of *S. meliloti* BetS for proline betaine (K_m of 56 μ M) than for glycine betaine (K_m of 16 μ M). In addition, the residual proline betaine uptake activity in UNA316 bacteroids maintained at 600 mOsm/liter still represented 36% of the activity in GMI211, compared with only 13% with glycine betaine.

Fig. 2. Dependence of glycine betaine uptake in bacteroids isolated from *Medicago sativa* nodules on increases in incubation medium osmolarity. Nodulated plants were grown on nitrogen-free nutrient solution during 3 weeks, and maintained on this control medium (○ and □) or subjected to salt stress (NaCl, 0.3 M) during 2 weeks (● and ■) before bacteroid preparation. Bacteroids were isolated from nodules produced on alfalfa roots by strains GMI211 (● and ○) or UNA316 (■ and □) and resuspended in media of increased osmolarity imposed by addition of NaCl; 600 mOsm/liter is equivalent to 0.3 M NaCl. At 10 min after upshock, the uptake was determined at 40 μ M [methyl- 14 C] glycine betaine. Data are means from duplicates of three separate experiments; standard deviations also are shown.

In planta, the osmolarity of infected cells has never been determined accurately. However, it is well established that symbiosomes which contain bacteroids behave as osmometers (Ou Yang and Day 1992). Consequently, the buffers used during symbiosome preparations need to be isotonic with the peribacteroid space in order to avoid bursting of these nitrogen-fixing units. In the case of symbiosomes from alfalfa nodules, we have demonstrated recently that the osmolarities of such buffers have to be adjusted at 450 and 600 mOsm/liter for symbiosomes from unstressed and salt-stressed plants, respectively (Trinchant et al. 2004). Thus, it is more likely that in planta bacteroids are subjected to osmolarities of the same range and, under such conditions, our results indicated that BetS is the main efficient betaine transporter; its activity would represent approximately 65 to 85% of the overall proline betaine and glycine betaine uptake activities, respectively. However, it should be noticed that betaine uptake activity is much lower (30-fold) in isolated bacteroids than in free-living cells. A similar observation already has been reported in the case of succinate and malate transport by bacteroids and free-living cells of *Bradyrhizobium japonicum* USDA3278 (San Francisco and Jacobson 1985), and might be related to lower physiological activities of bacteroids compared with free-living cells.

Overexpression of *betS* in free-living *S. meliloti*.

To better assess the contribution of the BetS transporter to cell osmoprotection and betaine accumulation, an *S. meliloti* strain overexpressing *betS* (named UNA349) was constructed (discussed below). The wild-type and UNA349 strains were grown overnight in mannitol and casamino acids (MCAA) medium, harvested by centrifugation, and resuspended in high osmolarity medium (MCAA + 0.5 M NaCl). The rate of [14 C]glycine betaine was measured at a final concentration of 40 μ M 10 min after upshock. In the UNA349 strain, the transport rate was 2.3-fold higher than in the control strain (Table 1), indicating significant overexpression of *betS*. For further investigation, and to study the consequence of an increased uptake rate on intracellular glycine betaine accumulation, the cells were grown in high-osmolarity (0.5 M NaCl) MCAA medium supplemented with 1 mM glycine betaine. Cells were

Fig. 3. Proline betaine uptake in bacteroids isolated from *Medicago sativa* nodules. Nodulated plants were grown on nitrogen-free nutrient solution during 3 weeks and then subjected to salt stress (NaCl, 0.3 M) during 2 weeks before bacteroid preparation. Bacteroids were isolated from nodules produced on alfalfa roots by strains GMI211 or UNA316, and resuspended in medium of low osmolarity without NaCl (-) or in medium of high osmolarity containing 0.3 M NaCl (+). At 10 min after upshock, the uptake was determined at 40 μ M [14 C] proline betaine. Data are means from duplicates of three separate experiments; standard deviations also are shown.

harvested after 2 or 6 h, washed, and extracted with 70% ethanol as described earlier (Fougère et al. 1991), and the glycine betaine content was analyzed. After 2 h of incubation, glycine betaine content in UNA349 cells was already maximal, and 2.2-fold higher than in GMI211 cells. Even after 6 h of incubation, the accumulation in GMI211 cells remained 20% lower than in UNA349 cells. These results demonstrate that overexpression of *betS* strongly induced a very rapid and consequent accumulation of glycine betaine after upshock, and confirm the role of BetS as an emergency system. Also, the overexpression rapidly alleviated growth inhibition at 0.6 M NaCl after addition of glycine betaine: the lag phase for UNA349 strain was 30 min, compared with 1 h for GMI211 and 20 h for the UNA316 *betS* mutant (data not shown). These results are perfectly in agreement with the transport rate of glycine betaine by the three strains, because this activity was very low in the *betS* mutant (Boscari et al. 2002).

Consequence of *betS* overexpression on proline betaine accumulation in bacteroids.

Because proline betaine accumulation in wild-type alfalfa bacteroids was observed previously in salt-stressed plants (Trinchant et al. 2004), the effects of *betS* overexpression and *betS* disruption on bacteroid proline betaine content were examined. Alfalfa seedlings were inoculated with strains GMI211, UNA349, or UNA316 and, 3 weeks after inoculation, the plants were subjected or not to salt stress (0.3 M NaCl) during 7 or 14 days. Bacteroids were purified and instantaneously extracted with 70% ethanol, and proline betaine contents were determined. In bacteroids from unstressed plants, proline betaine content was not dependent on the strain used as inoculant (Table 2), and remained almost constant during the 2 weeks of the experiment. Whatever the strain used for inoculation, 1 week of salinization of the host plants already enhanced bacteroid proline betaine content, resulting in a 1.4-fold increase in GMI211 and UNA316 bacteroids and a 2.2-fold increase in UNA349 bacteroids. These data indicate that *betS* overexpression induced a higher proline betaine content (41% more in UNA349 than in GMI211), whereas *betS* disruption had no significant effect. During the second week of salt stress, increases in proline betaine contents still persisted and, at the end of the salt treatment, the highest content was observed in UNA349 bacteroids, whereas the lowest appeared in UNA316 bacteroids. However, if the difference in proline betaine content between UNA349 and UNA316 bacteroids was significant, the level of proline betaine in UNA349 bacteroids was not signifi-

cantly higher than in GMI211 bacteroids. Based on the intracellular aqueous volume of alfalfa bacteroids previously determined (Fougère et al. 1991; Trinchant et al. 2004), proline betaine concentration in bacteroids from salt-stressed plants was estimated at approximately 8 mM in GMI211 and UNA349 bacteroids, and slightly lower (6.7 mM) in UNA316 bacteroids. These results supported the view that BetS plays a role in proline betaine accumulation in bacteroids, but mainly during the initial phase of adaptation to salt stress. On a longer term, other proline betaine transporters present in *S. meliloti*, as demonstrated with the *betS* mutant (Fig. 3), probably are activated. In conjunction with the existence of a catabolism of this compound (Trinchant et al. 2004), the activity of such transporters can modulate the intracellular proline betaine level in bacteroids.

Symbiotic phenotype of the *betS* overexpressing and mutant strains.

Because a significant increase in proline betaine level in UNA349 bacteroids was observed, the effects of *betS* overexpression on the nodulation and fixation capacities of the bacteria subsequently was investigated. Alfalfa plantlets were infected either with strains UNA349 or UNA316, or the GMI211 as a control, and the number of nodules was determined every week. The kinetics of nodulation was identical with the three strains, and the numbers of nodules obtained 5 weeks after infection were comparable with an average of 10 nodules per plant.

The acetylene reduction activity (ARA) was assayed 3 weeks after infection and during 2 additional weeks with plants subjected or not to salinization (0.3 M NaCl). Whatever the strain used for inoculation, the plantlets maintained at low osmolarity showed equivalent ARA over the 3 weeks of measurements. In each case, an average ARA of 41 ± 5 μ mol of ethylene produced per gram of fresh weight nodules per hour was obtained 3 weeks after inoculation, and a slight decrease of 18% was noticed 5 weeks after infection (Fig. 4). Obviously, in the absence of salt stress, the overexpression of a functional BetS system or its absence in *S. meliloti* is not crucial for the establishment and functioning of symbiosis. After 1 week of salt stress, if a 60 and 45% reduction in ARA was observed in plants inoculated with the UNA316 and the GMI211 strains, respectively, the plants nodulated with the UNA349 overexpressing strain exhibited only a 4% ARA reduction compared with the activity of nonsalinized plants of the same age. Even with non-negligible standard deviations, the results indicated a much better resistance of nitrogen fixation to salinization in UNA349-nodulated plants than in plants nodulated with the other strains. However, at the end of the 2-week salt treatment, the ARA in plants inoculated with the UNA349 strain was altered as in the other plants (approximately 50% reduction). Thus, the higher ARA in plants nodulated with the overexpressing strain is time limited, and

Table 1. Glycine betaine uptake activity and accumulation in *Sinorhizobium meliloti* GMI211 wild-type cells and *betS* overexpressing (UNA349) cells^a

Strains	Uptake (nmol/min/mg) ^c	Accumulation (nmol/mg) ^b	
		2 h	6 h
GMI211	8.7 $\pm$ 0.7	998 $\pm$ 104	1,700 $\pm$ 360
UNA349	20.3 $\pm$ 0.9	2,136 $\pm$ 275	2,126 $\pm$ 330

^a Cells were grown overnight at low osmolarity in mannitol and casamino acids (MCAA) medium, harvested by centrifugation, and resuspended in high osmolarity (NaCl 0.5 M) MCAA medium at an optical density at 600 nm of 1.0.

^b Glycine betaine accumulation (nmol/mg of protein) was determined in cells maintained during 2 or 6 h in MCAA medium containing 0.5 M NaCl and 1 mM glycine betaine. Cells were extracted with ethanol and betaine content was determined by high performance liquid chromatography. Values are as above.

^c Uptake of [methyl-¹⁴C] glycine betaine (nmol/min/mg of protein) was assayed at a final concentration of 40 μ M, 10 min after osmotic upshock. Values are the mean from duplicates of three independent cultures $\pm$ standard deviation.

Table 2. Effect of salt stress on the proline betaine content (nmol/g of dry weight of nodules) of bacteroids isolated from alfalfa nodules^a

Strains ^c	Salinization of host plants (0.3 M NaCl) ^b		
	Control	7 days	14 days
GMI211	710 $\pm$ 70	1,030 $\pm$ 10	1,560 $\pm$ 200
UNA349	670 $\pm$ 10	1,450 $\pm$ 14	1,710 $\pm$ 30
UNA316	710 $\pm$ 80	1,050 $\pm$ 10	1,360 $\pm$ 380

^a Proline betaine was extracted from isolated bacteroids and analyzed by high performance liquid chromatography.

^b Salt stress was applied 3 weeks after inoculation. Values are the mean from duplicates of three independent plant growth experiments $\pm$ standard deviation.

^c Alfalfa plants were inoculated with strains GMI211 (wild-type), UNA349 (*betS* overexpression), or UNA316 (*betS* mutant).

could be correlated with the higher increase in proline betaine level in UNA349 bacteroids than in GMI211 bacteroids during the initial period of stress.

DISCUSSION

Proline betaine occurs widely in *Medicago* spp. (Wood et al. 1991) and is accumulated largely in alfalfa nodules, particularly in the cytosol, but also in bacteroids, from plants submitted to salt stress (Trinchant et al. 2004). It also was established that proline betaine was taken up by purified symbiosomes and free bacteroids. Because BetS is a major betaine uptake system in *S. meliloti* (Boscari et al. 2002), the aim of the present study was to determine its contribution in the accumulation of proline betaine by bacteroids. The data presented in this communication clearly indicate that the BetS system is expressed during nodule formation and in differentiated bacteroids. Glycine betaine and proline betaine uptake activities are reduced by 85 and 65%, respectively, in bacteroids from a *betS* mutant in response to osmotic stress (Figs. 2 and 3); therefore, BetS appears to be a major betaine transporter in purified alfalfa bacteroids. BetS-mediated activity is stimulated in an osmotically dependent manner, as in *S. meliloti* free-living cells (Boscari et al. 2002).

Disruption of the *betS* gene had no significant effect on the symbiotic nodulation and fixation phenotype, even when the host plants were submitted to salt treatment. This finding raised the questions of i) whether bacteroids can perceive osmotic modulation within nodules from salt-stressed plants, and ii) whether compensatory changes can be made by bacteroids for the purpose of osmotic adjustment. Important ultrastructural modification of symbiosomes and major biochemical changes within bacteroids from salt-stressed alfalfa nodules already have been reported (Fougère et al. 1991; Trinchant et al. 2004). Indeed, in such nodules, the peribacteroid space is consistently enlarged, suggesting an increased turgor pressure inside the symbiosome which might be due partially to an elevated concentration in compatible solutes (proline and proline betaine). Furthermore, salinization of the plants strongly enhances the concentration of some other metabolites within bacteroids, such as asparagine, lactate, and pinitol. Collectively, these data are in full agreement with the proposal that bacteroids in planta can sense osmotic variations and can adapt their internal

turgor pressure through modifications of transport activities or de novo biosynthesis of compatible solutes using mechanisms that could be distinct from that used by free-living cells. This is specifically the case for proline which is accumulated by bacteroids, whereas this imino acid is not used as an osmoprotectant by *S. meliloti* (Gloux and Le Rudulier 1989). It also is interesting to note that the remaining proline betaine uptake activity in the *betS*-deficient mutant (35% of total activity) indicated that there are other transporter for this betaine. The activity of such transporters could explain why disruption of the BetS system had no significant consequence on the symbiotic phenotype. In support of this hypothesis, G. Alloing, I. Travers, B. Sagot, and D. Le Rudulier (*personal communication*) recently have characterized one other osmotically inducible ABC proline betaine transporter in *S. meliloti*, a system which more likely is functioning in bacteroids. Furthermore, because salinization increased the proline betaine content of the nodule cytosol up to several millimolars (Trinchant et al. 2004), low-affinity proline betaine transporters also can be operational in bacteroids.

Because of the availability of a large cytosolic pool of proline betaine in salt-stressed nodules, we have considered the possibility of enhancing proline betaine transport and accumulation as a way of improving nitrogen fixation activity of alfalfa plants submitted to salt stress. We investigated this issue by constructing a mutant strain that overexpressed *betS* (strain UNA349, *betS*⁺). Both in free-living bacteria and in bacteroids, enhanced expression of *betS* led to a clear increase in betaine transport capacity. The glycine betaine content of the wild-type cells remained 20% lower than in the overexpressing cells, even after a long period of incubation in the presence of the substrate (Table 1). It should be emphasized that *S. meliloti*, unlike many other bacteria, has the capacity to catabolize betaines (Bernard et al. 1986; Gloux and Le Rudulier 1989) and, thus, the steady-state intracellular betaine pool is controlled not only by transport but also by degradation activities. Although the overall betaine degradation in *S. meliloti* is inhibited under high osmolarity (Gloux and Le Rudulier 1989; Smith et al. 1988), the demethylation activities might be higher in the *betS*⁺ cells than in control cells, because the intracellular osmotic pressure was restored more quickly in the first category than in the second. In bacteroids, proline betaine catabolism also has been observed (Fougère and Le Rudulier

Fig. 4. Effect of salt stress on nitrogen fixation activity (acetylene reduction activity) of nodulated *Medicago sativa* plants. Plants were grown on nitrogen-free nutrient solution during 3 weeks and then subjected to salt stress with 0.3 M NaCl (black bars) or maintained in control medium (open bars) during 1 or 2 weeks. Alfalfa plants were inoculated by *Sinorhizobium meliloti* strains GMI211, UNA316, or UNA349. The control value of acetylene reduction activity measured 3 weeks after inoculation and before salinization of the plants (100%) was 41 $\mu\text{mol C}_2\text{H}_4$ produced/h/g of fresh weight nodule. Data are means from six replicates of three independent plant growth cultures; standard errors also are shown.

1990b), and two *S. meliloti* genes involved in such catabolism have been characterized: the *stc2* gene encodes a monooxygenase catalyzing the production of N-methyl proline (Burnet et al. 2000), and *stcD* encodes a demethylase that converts N-methyl proline to proline (Philipps et al. 1998). The existence of such a catabolic pathway may explain the limited capacity of bacteroids from the overexpressing strain to accumulate a much larger proline betaine pool than wild-type bacteroids, particularly after a 2-week salinization treatment (Table 2). In fact, after 2 weeks of salt treatment, the turgor pressure in bacteroids might be restored, leading to a strong reduction in BetS activity. Despite that, after 1 week of salt stress, the accumulation of proline betaine in bacteroids from the *betS⁺⁺* strain was 41% higher than in wild-type bacteroids. Simultaneously, the plantlets inoculated with the *betS⁺⁺* strain retained 96% of their nitrogen-fixation capacity, whereas the plants nodulated with the wild-type strain were much more affected, with a remaining nitrogenase activity of only 55% of the initial value. These data lead us to propose that, during the early step of stress adaptation, the rapid accumulation of proline betaine in the *betS⁺⁺* bacteroids favors the maintenance of the nitrogen fixation activity. On that line, it also is appropriate to underscore previous physiological studies demonstrating a beneficial effect of an exogenous supply of proline betaine on nitrogen fixation activity from plantlets grown in vitro and submitted to 0.2 M NaCl (Pocard et al. 1984). Therefore, we consider that the availability of a substantial amount of proline betaine within the bacteroids, either from exogenous or endogenous origin, can significantly alleviate, directly or indirectly, the negative effect of salt stress on nitrogen fixation. Nevertheless, the beneficial effect of proline betaine accumulation on nitrogen fixation is time limited. Obviously, a prolongation of the salinization treatment strongly affects many physiological properties of both the host plant and the bacteroids which cannot be restored only by proline betaine accumulation. For example, in addition to an inhibition of photosynthesis, increased osmolarity strengthens the oxygen diffusion barrier of the inner cortex of nodule and limits the oxygen availability to bacteroids (Diaz del Castillo et al. 1994), with a strong negative impact on the energy supply to the bacteroids. Furthermore, because the BetS system is an Na⁺/betaine symporter (Boscari et al. 2002), we cannot rule out the possibility that the reduction of nitrogen fixation activity observed after 2 weeks of salt treatment in plants inoculated with the *betS⁺⁺* strain might be the consequence of a concomitant substantial toxic Na⁺ accumulation. Indeed, a sizable increase in Na⁺ level (2.2-fold) already has been observed within wild-type bacteroids from 2-week-salinized nodules (Trinchant et al. 2004).

Finally, if overexpression of *betS* contributes to accelerate proline betaine accumulation in *S. meliloti*, in free-living cells as in planta, and plays a positive role in the immediate adaptation to osmotic stress, a long-term resistance of nitrogen fixation is more likely the result of stimulation of a pyramid of complex interacting mechanisms.

MATERIALS AND METHODS

Chemicals.

Glycine betaine and proline betaine were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France), and from Extrasynthèse (Genay, France), respectively. [methyl-¹⁴C] glycine betaine was prepared as described previously (Perroud and Le Rudulier 1985) from [methyl-¹⁴C] choline (2.04 GBq/mmol) purchased from Amersham Corp. (Little Chalfont, U.K.). [U-¹⁴C] proline betaine (4.6 GBq/mmol) was obtained from the Commissariat à l'Énergie Atomique (Gif-sur-Yvette, France).

Bacterial strains and culture conditions.

S. meliloti GMI211 (derivative of wild-type RCR2011, streptomycin resistant, *lac*) (Niel et al. 1977) and derivatives UNA316 (*betS* mutant, a double recombinant GMI211 strain carrying a *lacZ*-Km^r cartridge inserted in the opposite orientation of the *betS* gene) (Boscari et al. 2002), UNA315 (a simple recombinant containing a transcriptional *betS::lacZ* fusion, *betS⁺*, Km^r) (Boscari et al. 2002), and UNA349 (*betS* overexpressed; this study), were grown aerobically at 30°C in Luria-Bertani (LB) (Sambrook et al. 1989), supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂ (LBmc). When required, antibiotics were added at the final concentration described previously (Finan et al. 1984). For physiological experiments, *S. meliloti* strains were grown overnight in LBmc medium and transferred in MCAA medium containing 0.1% sodium malate and 0.1% casamino acids (Smith et al. 1988). The osmotic strength of the media was increased by the addition of increased concentrations of NaCl, as indicated. The osmolalities of the different media were determined by freezing point depression with a microosmometer (model H. Roebeling; Bioblock Scientific, Illkirch, France). When necessary, betaines were added at a concentration of 1 mM. Bacterial growth was monitored spectrophotometrically by measuring the optical density at 600 nm (OD₆₀₀).

S. meliloti strain constructions.

The *betS* overexpressing strain (UNA349) was constructed by replacing the wild-type promoter of *betS* by the *trp* promoter of *Salmonella enterica* serovar Typhimurium. This *trp* promoter fragment is constitutively active in *Sinorhizobium meliloti* and results in high levels of expression of genes placed under its control (Gage et al. 1996). To construct strain UNA349, the *Bam*HI/*Xho*I fragment from the plasmid pTB93F (Gage et al. 1996) containing the *pTrp* promoter and the green fluorescent protein (GFP) cassette (850 bp) was extracted and cloned into the suicide vector pSUP202 (Simon et al. 1983). The GFP cartridge then was exchanged with the 2.8-kb *Xho*I/*Sal*I fragment containing the *betS* gene from the plasmid pBT58 (Boscari et al. 2002), resulting in the plasmid pSTS313. Finally, the *Pst*I-*Pst*I fragment, which contains the transcriptional fusion *pTrp-betS*, was removed and cloned in pSUP202 vector to yield pSTG7. This resulting plasmid, pSSTG7, was transferred into *S. meliloti* GMI211 by triparental mating, using *Escherichia coli* MT616 (pRK600) (Finan et al. 1986) as a helper. A simple recombinant clone (strain UNA349) was selected as streptomycin and tetracycline resistant, and the correct location of the recombinant event was verified by Southern hybridization.

Plant material, growth conditions, nodulation, and nitrogen fixation assays.

The nodulation efficiency of *S. meliloti* strains was assayed on alfalfa (*M. sativa* cv. Europe) surface-sterilized germinating seedlings. Plants were grown in sterile test tubes (three plantlets per tube) containing 15 ml of N-free nutrient medium (Boncompagni et al. 2000) with 1.5% agarose prepared as a slope. The plantlets were inoculated twice (2×10^7 cells/tube), 5 and 7 days after germination, with the appropriate *S. meliloti* strain. The number of nodules was determined on 72 plants every week during 5 weeks after the second inoculation, and phenotypes of bacteria recovered from nodules were checked on the appropriate media.

In order to produce a large amount of nodules for bacteroid preparation, alfalfa seedlings were grown on a sterilized vermiculite/sand (10:1, vol/vol) mixture in filled pots (30 by 30 cm, 6 liters) moistened with a nitrogen-free nutrient solution (Sirois and Petersen 1982) as described previously (Mandon et

al. 2003). The plantlets were inoculated 7 days after sowing with a bacterial suspension containing 7×10^8 cells/ml (200 ml of suspension per container). They were maintained in a growth chamber at $70\% \pm 5\%$ air humidity under a 16-h-light and 8-h-dark cycle at 24 ± 1 and $19 \pm 1^\circ\text{C}$, respectively, and a photosynthetically active photon flux density of $360 \mu\text{M m}^{-2} \text{s}^{-1}$. Every 3 days, each pot was weighted and, in order to compensate for the consumption of medium, the plants were watered alternatively with the nitrogen-free nutrient solution or with distilled water. When needed, osmotic stress was applied 3 weeks after inoculation by adding NaCl to the growth medium (final concentration 0.3 M) and maintained for 1 or 2 weeks. During the salinization period, in order to keep the salt concentration in the pot as constant as possible, the plants were watered as above, using alternatively 0.3 M NaCl medium or distilled water. Nitrogen fixation capacity was determined by C_2H_2 reduction, using a gas chromatograph (model 610; TI-Unicam, Cambridge, U.K.) equipped with a Porapak T column (Trinchant et al. 1994). Incubations, in six replicates for each condition, were made at 25°C in a 60-ml rubber cap vial containing five plants, oxygen at 20 kPa, and acetylene at 10 kPa. Measurements were made with three independent plant growth cultures.

Preparation of bacteroids.

Rapid isolation of bacteroids was essentially as described by Trinchant and associates (2004). Preparations were conducted aerobically from freshly harvested nodules (2 to 5 g, fresh weight) gently crushed in ice-cold buffer (50 mM KH_2PO_4 , pH 7.4) containing 10% (wt/vol) polyvinylpyrrolidone, and adjusted with mannitol to 450 or 600 mosmol per liter for control and salt-stressed nodules, respectively. The homogenate was filtered through nylon and immediately centrifuged ($1,500 \times g$ for 2 min). The supernatant was recovered and centrifuged at $10,000 \times g$ for 5 min. The pellets were used instantaneously for uptake measurements or extracted for proline betaine determinations.

Intracellular glycine betaine and proline betaine determinations.

Intracellular betaine pools were determined in free-living cells and in isolated bacteroids. The pellets were extracted at 60°C with 95% ethanol under stirring (20 min). The ethanol extracts were evaporated to dryness under vacuum at 37°C , and solids were dissolved in 1 ml of ultrapure water. Glycine betaine and proline betaine contents were determined by high-performance liquid chromatography (Waters Associates, Milford, MA, U.S.A.), as recommended by Gorham (1984). Separations were performed on a 250-by-5-mm Whatman column packed with Partisil 10-SCX (Waters Associates). Elution and detection were as described previously (Trinchant et al. 2004).

Transport assays.

The cells were grown in MCAA medium, harvested at an OD_{600} of 0.8 to 1.0, washed twice in the medium used for the culture, and resuspended at the same OD. All assays were carried out at 30°C with 1 ml of cell suspension and radioactive glycine betaine or proline betaine (100,000 dpm) at $40 \mu\text{M}$ for 1 to 5 min. Reactions were terminated by rapid filtration through glass microfiber filters (Whatman) as described previously (Boncompagni et al. 2000). Filters were rinsed with 3 ml of the corresponding cold assay medium and the radioactivity remaining on the filters was determined with a liquid scintillation spectrometer (model LS6000SC; Beckman Instruments, Villepinte, France).

Uptake by isolated bacteroids was measured under aerobic conditions at 25°C as described above for *S. meliloti* free-

living cells. The bacteroids (0.1 mg of protein) were resuspended at the requested osmolarity and uptake was initiated 10 min later by injecting the radioactive substrate into the incubation mixture, then stopped after 15 min by rapid filtration. For both free-living cells and isolated bacteroids, uptake was linear with time during the incubation period. Reactions were conducted in duplicate, with materials from three independent extracts. Protein contents were estimated according to Lowry and associates (1951) using bovine serum albumin as standard.

Histochemical detection of β -galactosidase activity nodules.

M. sativa plantlets were grown in sterile test tubes (three plantlets per tube) with nitrogen-free medium and inoculated with the appropriate strain as for nodulation assays. At 1 or 4 weeks after inoculation, histochemical detection of β -galactosidase activity in entire roots and cross sections was performed according to Boivin and associates (1990) and Jiménez-Zurdo and associates (1997). Roots and mature nodules were fixed with 1.25% glutaraldehyde in 0.2 M cacodylate, pH 7.2. Sections of 4-week-old nodules ($100 \mu\text{m}$) were obtained with a vibratome (VT 1000 Plus; Labo Nord, Templemars, France) and fixed again with 1.25% glutaraldehyde. Samples were transferred to the staining solution and mounted on slides for observation and photography with an Olympus microscope.

ACKNOWLEDGMENTS

This work was supported by the Centre National de la Recherche Scientifique. A. Boscarì received a doctoral fellowship from the Ministère de l'Enseignement Supérieur et de la Recherche. We gratefully acknowledge D. Tepfer for the generous gift of [^{14}C] proline betaine.

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