Rhizobia are gram-negative soil bacteria that are able to establish nitrogen-fixing symbioses with legume plants under conditions of nitrogen deprivation. During this process, an exchange of molecular signals occurs between the two partners, leading to the formation of the root nodule, where biological nitrogen fixation takes place (12). It has been observed that rhizobium mutants affected in adaptation to high salinity present deficiencies in their symbiotic capacity (27, 7, 25). These results emphasize the importance of studying the adaptation mechanisms of rhizobia to osmotically unbalanced environmental conditions.

Response and adaptation to environmental stresses are probably complex phenomena involving many physiological and biochemical processes that likely reflect changes in gene expression and in the activity of enzymes and transport proteins (6, 43). Rhizobia may use distinct mechanisms in response to hyperosmotic conditions, such as stimulation of potassium uptake (7), changes in cell morphology and size, or modifications in the pattern of extracellular polysaccharides (18, 35, 42). Nevertheless, one of the most general responses for long-term adaptation to osmotic stress is the intracellular accumulation of osmoprotective compounds (24, 45). Trehalose synthesis pathways are present in the genome of S. meliloti 1021: OtsA, TreYZ, and TreS. Among these, OtsA has a major role in trehalose accumulation under all of the conditions tested and is the main system involved in osmoadaptation. Nevertheless, the other two systems are also important for growth in hyperosmotic medium. Genes for the three pathways are transcriptionally responsive to osmotic stress. The presence of at least one functional trehalose biosynthesis pathway is required for optimal competitiveness of S. meliloti to nodulate alfalfa roots.

The disaccharide trehalose is a well-known osmoprotectant, and trehalose accumulation through de novo biosynthesis is a common response of bacteria to abiotic stress. In this study, we have investigated the role of endogenous trehalose synthesis in the osmotolerance of S. meliloti. Genes coding for three possible trehalose synthesis pathways are present in the genome of S. meliloti 1021: OtsA, TreYZ, and TreS. Among these, OtsA has a major role in trehalose accumulation under all of the conditions tested and is the main system involved in osmoadaptation. Nevertheless, the other two systems are also important for growth in hyperosmotic medium. Genes for the three pathways are transcriptionally responsive to osmotic stress. The presence of at least one functional trehalose biosynthesis pathway is required for optimal competitiveness of S. meliloti to nodulate alfalfa roots.
### TABLE 1. Bacterial strains, plasmids, and primers used in this study

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<th>Strain, plasmid, or gene</th>
<th>Relevant characteristics or primer sequences&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>1000SS</td>
<td>1021 (∆otsA::Sm/Sp) Sm&lt;sup&gt;+&lt;/sup&gt; Sp&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>10OSS (treY::Km) Sm&lt;sup&gt;+&lt;/sup&gt; Sp&lt;sup&gt;+&lt;/sup&gt; Kmr&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>otsA (SMa0322)</td>
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<sup>a</sup> Sm<sup>+</sup>, streptomycin resistance; Km<sup>+</sup>, kanamycin resistance; Sp<sup>+</sup>, spectinomycin resistance; Gm<sup>+</sup>, gentamicin resistance; Tp<sup>+</sup>, trimethoprim resistance; Ap<sup>+</sup>, ampicillin resistance; Tc<sup>+</sup>, tetracycline resistance.

<sup>b</sup> Forward/reverse primer sequences (5′–3′) are shown. Underlined are the endonuclease XbaI action sites used to clone the mutated gene versions into the shuttle vector.

30°C in TY complex medium (1) or in minimal medium (MM) containing glutamate (7.5 mM), mannitol (55 mM), and mineral salts (K2HPO4 at 1.3 mM, KH2PO4 · 3H2O at 2.2 mM, MgSO4 · 7H2O at 0.6 mM, CaCl2 · 2H2O at 0.34 mM, FeCl3 · 6H2O at 0.022 mM, and NaCl at 0.86 mM) (30). When a specific pH was required, the MM was buffered with Tris/morpholineethanesulfonic acid (MES) at 5 mM. Escherichia coli strains were propagated in Luria-Bertani medium (31). When required, antibiotics were added at the following final concentrations: for E. coli, streptomycin (Sm) at 50 μg/ml, spectinomycin (Sp) at 50 μg/ml, kanamycin (Km) at 50 μg/ml, gentamicin (Gm) at 20 μg/ml, tetracycline (Tc) at 10 μg/ml, and ampicillin (Ap) at 200 μg/ml; for S. meliloti, Sm at 200 μg/ml, Sp at 100 μg/ml, Km at 200 μg/ml, Gm at 15 μg/ml, and Tc at 0.1 μg/ml for chromosomal insertions or 5 μg/ml for plasmid selection.

For the different experiments, MM was inoculated from cultures grown in TY with antibiotics to stationary phase. During the experiments, antibiotics were only added to avoid the possibility of plasmid loss in the case of transcriptional studies. When osmotic stress conditions were required, the osmolarity of the medium was increased by addition of appropriate amounts of NaCl from a 4 M stock solution (made in MM) or sucrose. These conditions were either imposed from the beginning of the experiment or, in the case of experiments involving an osmotic shock, when the culture reached mid-exponential phase. Growth was followed by measuring the optical density at 600 nm in cultures incubated in a rotary shaker or by comparative growth on solid medium.

**Genetic procedures and construction of S. meliloti mutants.** Standard techniques were used for genetic manipulations (31). To construct plasmids for the disruption of the different genes, we first amplified the corresponding regions of the S. meliloti genome by PCR with custom-synthesized oligonucleotides (Table 1). After cloning the amplicons into appropriate vectors, we deleted part of the gene sequence by endonuclease digestion and inserted antibiotic resistance cassettes to obtain OtsA and TreY mutant constructs in vitro. The constructs were then subcloned into the shuttle vectors, and the plasmids obtained were transferred by conjugation to S. meliloti 1021. In the case of the TreS system, an internal fragment from treS was directly cloned into the shuttle vector. Transconjugants were selected on medium containing the appropriate antibiotics. In the case of pK18mobSacB, the use of sucrose addition during selection as described by Schauer and associates (32) was avoided since its osmotic action could affect the growth of osmosensitive mutants. Double and triple mutants were obtained by transduction with bacteriophage φM12 as described by Finn and associates (10). Disruption of the genes of interest was confirmed by Southern hybridization with specific gene probes.
Determination of the trehalose contents of cells. For the analysis of trehalose content, four aliquots of the bacterial culture were collected by centrifugation. Cells were washed with water and then extracted in 75% (vol/vol) ethanol at room temperature for 24 h. The supernatants were collected, and the pellet was extracted once more. The resulting supernatants were combined and dried in a SpeedVac (SPD111V; ThermoSavant). The solids were dissolved in 250 μl of 135 mM citrate buffer, pH 5.7, and then preheated to 37°C, and three of the aliquots were treated with 0.008 U trehalase (Sigma). After 1 h at 37°C, the reaction was stopped by adding 250 μl of 500 mM Tris buffer, pH 7.5, and the glucose derived from the trehalose present in the sample was determined with the glucose (GO) assay kit (Sigma) using the non-trehalose-treated aliquot as blank. Mean values were calculated from the three replicates, and differences between samples were assessed by using a single-factor analysis of variance (ANOVA) test with five degrees of freedom (P < 0.01). The experiments presented were performed at least twice.

Gene expression assays. Three different fusions to the β-glucoronidase gene uidA were constructed by cloning the promoter regions from otsA, treY, and treS in the appropriate direction into plasmid p53Gus. The promoter regions from otsA and treY were obtained by endonuclease digestion (XbaI-EcoRI for otsA and EcoRI-XhoI for treY) of the PCR fragments used for mutagenesis. For the treY fusion, primers PTreS-1 (5′-TTTTATCTAGACAGGACGAGATTAGAA-3′) and PTreS-2 (5′-TTTTAATCTAGATCGTCATGCATACTCCACCAGACG-3′) were used to amplify a 4,040-bp DNA fragment containing part of treS and the upstream genes and promoter region of the putative operon in which treS is included. The PCR fragment was cloned as an XbaI fragment into p53Gus (restriction enzyme sites underlined in the primer sequences), and the correct direction was selected to obtain the treS:uidA fusion. The p53Gus derivatives were then introduced into S. meliloti 1021 by conjugation. To quantify β-glucoronidase activity, three aliquots of the bacterial culture were collected by centrifugation, washed, and resuspended in assay buffer (dithiothreitol, 5 mM; EDTA, 1 mM; Na2HPO4·NaH2PO4, 50 mM). A 200-μl portion of each aliquot was used to determine the β-glucoronidase activity by mixing with 740 μl assay buffer, 50 μl 0.1% sodium dodecyl sulfate, and 100 μl chloroform and vortexing for 15 s twice. Samples were incubated at 37°C for 10 min, and 10 μl of 100 mM 4-nitrophenyl-β-D-glucoronide, preheated at 37°C, was then added. When the mixture turned yellow, the reactions were ended by adding 200 μl of 1 M Na2CO3. Samples were centrifuged for 5 min (12,000 rpm), and the absorbance of the upper phase was measured at 405 nm.

RESULTS

Genes for trehalose biosynthesis in S. meliloti 1021. An in silico analysis of the S. meliloti 1021 genome revealed the presence of putative genes coding for three trehalose biosynthesis systems: OtsA, TreYZ, and TreS. A gene annotated as otsA (SMa0233) is located in plasmid pSymA, and its product presents high sequence similarity to trehalose-6-phosphate synthases from other rhizobia (46% to 94% sequence identity). The OtsAB pathway involves a second step, which is catalyzed by the OtsB protein. We could not find any open reading frame in the S. meliloti 1021 genome encoding an OtsB-like protein. Nevertheless, the activity of this biosynthetic pathway has been previously reported (37).

The S. meliloti gene SMb20574, located in plasmid pSymB, is annotated as a putative maltoligosyl-trehalose synthase, and in fact its product presents 32 to 34% identity with TreY from Mycobacterium or Corynebacterium and up to 47% identity with TreY from R. leguminosarum bv. trifolii (4, 44, 22). Although no treZ gene has been annotated in the S. meliloti genome, a gene homologous to treZ could be identified. The product of the gene SMb21447, annotated as gbgB2 (encodes a putative 1,4-glucan branching enzyme), presents significant sequence similarity to TreZ from Arthrobacter sp. strain Q36 (38% identity) or Rhizobium sp. strain M11 (37% identity) (20, 21). Although both gbgB and treZ code for α-amylases, GlgB is well conserved among distantly related bacteria and the product of the S. meliloti open reading frame Smc03922, annotated as gbgB1, presents as much as 53% identity with E. coli GlgB, which in turn shows only 27% identity with the N-terminal part of the Smb21447 product. Due to its high homology to treZ genes and its low similarity to gbgB genes, we concluded that SMb21447 is misannotated in the S. meliloti 1021 genome and should be renamed treZ.

Finally, the gene SMb20099, located in the S. meliloti 1021 plasmid pSymB, is annotated as a maltose-α-d-glucosyltransferase, an alternative name for trehalose synthase. The product of the Smb20099 gene presents significant sequence similarity (35 to 40% identity) to known trehalose synthases from M. tuberculosis, C. glutamicum, or Pelmobacter sp. strain R48 (41, 4, 44). treS-like genes are commonly found in rhizobial genomes, and some may carry more than one copy. In S. meliloti, treS is the fifth gene of a putative operon of six genes (SMb20095 to SMb21000). None of the other five genes has a specific function assigned.

To study the involvement of each of the three possible trehalose biosynthesis systems identified in the S. meliloti 1021 genome, we constructed strains lacking each of them by allelic exchange with interrupted versions of the genes otsA, treY, and treS (Fig. 1). We also obtained double mutants retaining only one of the systems described and a triple mutant devoid of all three systems.

Role of trehalose biosynthesis systems in S. meliloti osmotolerance. To check whether any mutation could cause growth defects in S. meliloti, the different mutants were tested in TY complex medium and in defined MM. In both media, all mutants displayed wild-type growth (data not shown). To determine the involvement of each trehalose biosynthesis system in osmotolerance, growth curves of all of the mutants in liquid MM containing different concentrations of NaCl (0.3 M, 0.4 M, or 0.5 M) or sucrose (0.5 M or 0.7 M) were obtained. Among all of the mutants tested, only 10trOt (TreS+OtsA−) and 10SYOt (TreS−TreY−OtsA−), lacking both the OtsA and TreS systems, presented a significant growth delay in medium containing NaCl or sucrose. For both mutants, the growth delay was only detectable at the highest concentrations of osmolytes tested and was more
The expression of the \( otsA \), \( treY \), and \( treS \) genes in \( S. meliloti \) was assayed throughout growth in MM. All of the genes presented low levels of expression during exponential growth on MM, but induction of \( treS \) transcription could be detected upon entry into stationary phase, whereas \( otsA \) and \( treY \) expression remained virtually unchanged throughout the growth curve (Fig. 5). However, the expression of \( otsA \) and \( treY \) was significantly increased during growth on MM with NaCl added (Fig. 5). Induction of \( treS \) expression could also be detected under these conditions but to a lower extent than that of \( otsA \) and \( treY \), and the effect was masked during early stationary phase due to the induction of the gene at this stage. Gene expression at different NaCl concentrations (0.4 M and 0.5 M) was also studied, and dose-dependent induction could be assessed (data not shown). For instance, the expression of \( otsA \) at mid-exponential phase in MM was around 20 Miller units, 40 in MM with 0.4 M NaCl added, and 130 when the NaCl concentration was 0.5 M. These observations were corroborated by studying gene expression after the addition of different concentrations of NaCl (0.5 M and 0.6 M) to exponentially growing cultures (NaCl shock). The addition of the highest salt concentration also resulted in the highest expression levels for all three genes (data not shown).

**Trehalose accumulation.** The accumulation of trehalose in *R. leguminosarum* bv. trifolii has been reported to reach a peak at the early stationary phase, declining afterwards (22). To establish how the disaccharide accumulates in *S. meliloti*, the levels of trehalose were determined in the wild-type strain throughout the growth curve. We observed that in *S. meliloti* 1021, trehalose accumulated during exponential growth in MM and its levels continued to increase in the stationary phase, reaching a maximum at the end of the growth curve (Fig. 6a). To determine the involvement of the different biosynthetic systems in trehalose accumulation, the amounts of trehalose...
present in different mutants were measured at the late stationary phase of cultures. We observed that the mutants lacking OtsA or TreY (10OtSS and 10MOTK) presented reduced levels of trehalose with respect to the parental strain, whereas mutants lacking both systems (10OtM and 10SYOt) showed a further reduction of the accumulated trehalose, suggesting that accumulation of trehalose in the stationary phase is mediated by the OtsA and TreY systems (Fig. 6b).

FIG. 2. Growth curves of S. meliloti 1021-derived strains in MM (a), MM supplemented with 0.7 M sucrose (b), and MM supplemented with 0.5 M NaCl (c). Only the growth curves of mutants showing significant differences with the wild-type strain are shown. Wild-type strain 1021, squares; TreS⁻OtsA⁺ mutant 10trOt, triangles; TreS⁻ TreY⁻ OtsA⁺ mutant 10SYOt, circles. The data shown are representative of at least two independent experiments. OD600, optical density at 600 nm.

FIG. 3. Ability of 1021-derived mutants to grow in MM with 0.5 M NaCl added at pH 7 (a) and pH 6.5 (b). In each row, drops contain approximately the number of CFU indicated at the left. A representative example of at least two experiments is shown. Pictures were taken after 6 to 8 days of incubation under the indicated conditions.
The ability of the parental strain *S. meliloti* 1021 to accumulate trehalose upon NaCl addition to exponentially growing cultures was also tested by using different concentrations of the stressing compound. We found that trehalose accumulation was part of the response to osmotic stress, but in contrast to the dose-dependent induction of the biosynthetic genes, the amount of trehalose accumulated was lower with the highest concentration of NaCl tested (Fig. 7a).

We also determined the trehalose accumulated by each of the mutants after a 0.3 M NaCl osmotic shock. The results indicated that the main system involved in trehalose accumulation under these conditions was OtsA, although in the absence of OtsA, the involvement of TreY became apparent, so that the lack of both systems caused a further reduction in trehalose accumulated in 100trOt (OtsA<sup>−</sup> TreY<sup>−</sup> OtsA<sup>−</sup> mutant) and 10SYOt (TreS<sup>−</sup> TreY<sup>−</sup> OtsA<sup>−</sup>) (Fig. 7b). Trehalose accumulation by the triple mutant 10SYOt (TreS<sup>−</sup> TreY<sup>−</sup> OtsA<sup>−</sup>) recovered to the levels of wild-type strain 1021 upon complementation with the *otsA* gene alone (pJB3otsA), while complementation with the *treY* gene (pJB3treY) restored trehalose accumulation in the triple mutant to the levels of the double mutant 10trOt (TreS<sup>−</sup> OtsA<sup>−</sup>), still showing the effect of the missing OtsA pathway (data not shown). The triple mutant 10SYOt (TreS<sup>−</sup> TreY<sup>−</sup> OtsA<sup>−</sup>), the triple mutant complemented with the *treS* gene (pJB3treS), and the double mutant 100trOt (OtsA<sup>−</sup> TreY<sup>−</sup>) accumulated comparably low amounts of trehalose under these conditions, showing the lack of involvement of the TreS system in the accumulation process (data not shown).

**Importance of trehalose biosynthesis systems for symbiosis.** All of the mutants obtained were able to induce nitrogen-fixing root nodules on alfalfa plants and displayed nodulation kinetics comparable to that of wild-type strain 1021 upon complementation with the *otrA* gene (pJB3otrA), and the double mutant 10OtM (OtsA<sup>−</sup> TreY<sup>−</sup> OtsA<sup>−</sup>) recovered to the levels of wild-type strain 1021 to accumulate trehalose upon NaCl addition to exponentially growing cultures. The results indicated that the main system involved in trehalose accumulation under these conditions was OtsA, although in the absence of OtsA, the involvement of TreY became apparent, so that the lack of both systems caused a further reduction in trehalose accumulated in 100trOt (OtsA<sup>−</sup> TreY<sup>−</sup> OtsA<sup>−</sup> mutant) and 10SYOt (TreS<sup>−</sup> TreY<sup>−</sup> OtsA<sup>−</sup>) (Fig. 7b). Trehalose accumulation by the triple mutant 10SYOt (TreS<sup>−</sup> TreY<sup>−</sup> OtsA<sup>−</sup>) recovered to the levels of wild-type strain 1021 upon complementation with the *otsA* gene alone (pJB3otsA), while complementation with the *treY* gene (pJB3treY) restored trehalose accumulation in the triple mutant to the levels of the double mutant 10trOt (TreS<sup>−</sup> OtsA<sup>−</sup>), still showing the effect of the missing OtsA pathway (data not shown). The triple mutant 10SYOt (TreS<sup>−</sup> TreY<sup>−</sup> OtsA<sup>−</sup>), the triple mutant complemented with the *treS* gene (pJB3treS), and the double mutant 100trOt (OtsA<sup>−</sup> TreY<sup>−</sup>) accumulated comparably low amounts of trehalose under these conditions, showing the lack of involvement of the TreS system in the accumulation process (data not shown).

**DISCUSSION**

Many rhizobia accumulate trehalose in response to different stresses (26, 3). The biosynthesis of this disaccharide occurs in most organisms through the OtsAB pathway, although the coexistence of up to three different trehalose biosynthetic systems in some bacteria has been previously reported (4, 44, 19). The present study demonstrates that *S. meliloti* 1021 carries genes for three likely trehalose biosynthetic pathways (OtsA, TreYZ, and TreS) and that all are involved in the osmoadaptive response.

The relevance of trehalose biosynthesis for osmotic tolerance could only be revealed at very high osmolyte concentrations. Trehalose has been previously proposed to act in the osmotic adaptation of *S. meliloti* only under conditions of harsh stress, since the accumulation of other compounds like glutamate or N-acetyl glutaminyl glutamine amide during the exponential growth phase could protect the cell at lower stress levels (34). We assessed differences in the relative importance of each of the three systems for osmoregulation, the OtsA system being the most relevant one in this process. The OtsAB pathway has been reported to be active in *S. meliloti* 1021 under free-living conditions and is known to be involved in the osmotic tolerance of other rhizobia (37, 38). Nevertheless, we could also observe a role for *treS* in the osmotolerance of *S. meliloti* 1021, since the absence of both *otsA* and *treS* caused a reduction of the growth ability of the strain at high solute concentrations compared to *otsA* single mutants. The possibility of a polar effect of the *treS* mutation on the downstream gene (*smb20100*) cannot be ruled out, and in fact, the complete operon (*smb20095* to *smb20100*) could be involved in osmoregulation since all of the genes are upregulated after an NaCl shock (9). On the other hand, this is the first report of the possible involvement of TreS in the osmoregulation ability of rhizobia, although this system is known to be active in *Bradyrhizobium japonicum* and its activity increases during symbiosis.

**FIG. 4.** Growth curves of strains 1021 (wild type; squares) and 10SYOt (TreS<sup>−</sup> TreY<sup>−</sup> OtsA<sup>−</sup> mutant; circles) in MM with NaCl added at the times indicated by the arrows to a final concentration of 0 M (a) or 0.6 M (b). The data shown are representative of at least two independent experiments. OD600, optical density at 600 nm.
to become the dominant trehalose biosynthetic pathway in the bacteroids of this species (37). The minor relevance of the TreYZ system during the osmotic adaptation of *S. meliloti* 1021 observed in our studies contrasts with the reported involvement of both TreYZ and OtsAB in the desiccation tolerance of *R. leguminosarum* bv. trifolii (22). Nevertheless, we observed that the TreYZ pathway is involved in trehalose accumulation under various growth conditions, although its importance during osmotic adaptation was clearly lower than that of the OtsA system.

It has been reported that the accumulation of trehalose in *S. meliloti* in response to osmotic stress is maximal at the end of the exponential growth phase and the beginning of the stationary growth phase (39). A similar result was obtained for *R. leguminosarum* bv. trifolii (22). However, our studies show that *S. meliloti* 1021 accumulates trehalose during growth, reaching maximum amounts at late stationary phase. Furthermore, this accumulation depends mainly on the activity of both the OtsA and TreYZ systems. On the other hand, gene expression studies showed that, as observed in *R. leguminosarum* bv. trifolii (22), the *S. meliloti* 1021 *otsA* and *treY* genes display low constitutive expression levels during growth in osmotically balanced medium. Only *treS* expression was enhanced upon entry into stationary phase; however, TreS was apparently not involved in trehalose accumulation. These results suggest that trehalose accumulation during the stationary phase could be controlled at the posttranscriptional level or through control of trehalose breakdown rates.

Our studies also revealed that in *S. meliloti* 1021, the response to osmotic stress includes induction of the *otsA*, *treY*, and *treS* genes, in a dose-dependent manner, both after an osmotic shock and in response to prolonged growth on saline medium. These results confirm our previous microarray data showing upregulation of the three genes after an osmotic upshift (8).

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**FIG. 5.** Expression of the transcriptional fusions *otsA::uidA* (a), *treY::uidA* (b), and *treS::uidA* (c) during growth in MM (left) or MM plus 0.5 M NaCl (right). The data shown are representative of two independent experiments. OD600, optical density at 600 nm; Miller U, β-glucuronidase activity expressed as Miller units.
It was unexpected to observe that the osmolyte concentration yielding the highest gene expression levels in *S. meliloti* 1021 did not cause maximal trehalose accumulation. While the expression levels of the three genes positively correlated with increasing NaCl concentrations, the amount of accumulated trehalose decreased at very high NaCl concentrations (Fig. 7). Such a paradoxical situation is not without precedents. In *C. glutamicum*, *otsA* and *treS* expression increase significantly in response to osmotic stress, whereas *treY* transcription remains unchanged despite TreY being the main system involved in the osmoregulated synthesis of trehalose in this bacterium (44). Likewise, the transcriptional profile of the trehalose biosynthetic genes in *S. meliloti* may not necessarily correlate with their relative importance during osmotic adaptation, which would again suggest the existence of other control mechanisms in addition to transcriptional regulation. Nevertheless, the lower amounts of trehalose accumulated at the highest osmolarities could reflect a metabolic compromise caused by the severe stress conditions imposed rather than differential regulation. Thus, the inability of the metabolic systems to keep high trehalose biosynthesis rates under conditions of harsh stress would cause the observed reduction in disaccharide accumulation.

None of the trehalose biosynthesis systems studied in *S. meliloti* 1021 was essential for nodulation or nitrogen fixation, even under osmotic stress conditions. Nevertheless, the lack of the corresponding genes caused a significant reduction in the competitive ability of the triple mutant, which is in accordance with the observation by McIntyre and associates (22) that an *R. leguminosarum* bv. trifolii mutant unable to accumulate trehalose was less competitive than the parental strain for nodule occupancy but capable of nodulation and nitrogen fixation. It has also been reported that *S. meliloti* 1021 mutants defective in trehalose catabolism present higher rates of nodule occupancy than their parental strain but a similar nitrogen fixation ability (15). As suggested by these authors, the ability to accumulate trehalose (either by increasing biosynthesis or by reducing catabolism) could help bacteria to tolerate infection-related stresses during early stages of the interaction.

The situation in *S. meliloti* and *R. leguminosarum* seems different from that in *R. etli*, where an *otsA* mutant still able to accumulate trehalose to a certain extent displayed reduced
nodule and lower nitrogenase activity in symbiosis with Phaseolus vulgaris and consequently reduced plant biomass (38). In contrast, S. meliloti (this study) or R. leguminosarum (22) strains virtually unable to accumulate trehalose seem to display a more discrete symbiotic phenotype. Suárez and associates (38) suggested that the reduction in symbiotic effectiveness could be related to a putative role for trehalose as a signal molecule coordinating nitrogen and carbon metabolism and responses to stress. Taken together, these results suggest that the activity of rhizobial trehalose as a protectant compound and/or as a signal molecule may depend on the nature of the symbiotic partners. Our results show that in S. meliloti 1021, trehalose biosynthesis becomes important during the response to harsh stress conditions. The presence within the plant of molecules such as proline betaine, able to act as osmoprotectants for S. meliloti, has been reported in alfalfa (40). Such osmoprotectants would make trehalose biosynthesis unnecessary for stress adaptation during nodule development, although its production could still be an advantage during the early stages of the symbiotic interaction, as proposed by Jensen and associates (15).

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