Functional Role of *Bradyrhizobium japonicum* Trehalose Biosynthesis and Metabolism Genes during Physiological Stress and Nodulation^{▽†}

Masayuki Sugawara,¹ Eddie J. Cytryn,^{1‡} and Michael J. Sadowsky^{1,2,3*}

Department of Soil, Water, and Climate,¹ Microbial and Plant Genomics Institute,² and
BioTechnology Institute,³ University of Minnesota, St. Paul, Minnesota 55108

Received 13 October 2009/Accepted 10 December 2009

Trehalose, a disaccharide accumulated by many microorganisms, acts as a protectant during periods of physiological stress, such as salinity and desiccation. Previous studies reported that the trehalose biosynthetic genes (*otsA*, *treS*, and *treY*) in *Bradyrhizobium japonicum* were induced by salinity and desiccation stresses. Functional mutational analyses indicated that disruption of *otsA* decreased trehalose accumulation in cells and that an *otsA treY* double mutant accumulated an extremely low level of trehalose. In contrast, trehalose accumulated to a greater extent in a *treS* mutant, and maltose levels decreased relative to that seen with the wild-type strain. Mutant strains lacking the OtsA pathway, including the single, double, and triple Δ *otsA*, Δ *otsA* Δ *treS* and Δ *otsA* Δ *treY*, and Δ *otsA* Δ *treS* Δ *treY* mutants, were inhibited for growth on 60 mM NaCl. While mutants lacking functional OtsAB and TreYZ pathways failed to grow on complex medium containing 60 mM NaCl, there was no difference in the viability of the double mutant strain when cells were grown under conditions of desiccation stress. In contrast, mutants lacking a functional TreS pathway were less tolerant of desiccation stress than the wild-type strain. Soybean plants inoculated with mutants lacking the OtsAB and TreYZ pathways produced fewer mature nodules and a greater number of immature nodules relative to those produced by the wild-type strain. Taken together, results of these studies indicate that stress-induced trehalose biosynthesis in *B. japonicum* is due mainly to the OtsAB pathway and that the TreS pathway is likely involved in the degradation of trehalose to maltose. Trehalose accumulation in *B. japonicum* enhances survival under conditions of salinity stress and plays a role in the development of symbiotic nitrogen-fixing root nodules on soybean plants.

Rhizobia induce the formation of nodules on the roots of legume plants, in which atmospheric nitrogen is fixed and supplied to the host plant, thereby enhancing growth under nitrogen-limiting conditions. The symbiotic interaction between rhizobia and their cognate leguminous plants is important for agricultural productivity, especially in less developed countries. However, physiological stresses, such as desiccation and salinity, negatively affect these symbiotic interactions by limiting nitrogen fixation (44). The osmotic environment within the rhizosphere may affect root colonization, infection thread development, nodule development, and the formation of effective N₂-fixing nodules (21). Moreover, when legume seeds are inoculated with appropriate rhizobial strains prior to planting in the field, the vast majority of nodules produced are often not formed by the inoculant bacteria but rather by indigenous strains in the soil (36). This is in part due to the death of inoculant strains from rapid seed coat-mediated desiccation. Therefore, improvement of the survival of rhizobia under conditions of physiological stresses may promote biological nitrogen fixation and enhance plant growth.

Rhizobia synthesize and accumulate compatible solutes, including trehalose, in response to desiccation and solute-mediated physiological stresses (5, 21, 42). Trehalose, a nonreducing disaccharide with an α , α -1,1 linkage between the two glucose molecules, has been shown to protect cell membranes and proteins from stress-induced inactivation and denaturation (8, 23, 24). The relationship between trehalose accumulation and symbiotic phenotype is dependent on rhizobial species and host genotype. Suarez et al. (39) reported an increase in root nodule number and nitrogen fixation by *Phaseolus vulgaris* inoculated with a trehalose-6-phosphate synthase-overexpressing strain of *Rhizobium etli*. In contrast, trehalose accumulation in *Rhizobium leguminosarum* and *Sinorhizobium meliloti* cells did not result in an increase in nitrogen-fixing nodules but led to enhancement of competitiveness on clover and on certain alfalfa genotypes, respectively (1, 16, 20).

Four trehalose biosynthetic pathways, mediated by OtsAB, TreS, TreYZ, and TreT, have been reported thus far for prokaryotes (8, 25). The OtsAB pathway results in the condensation of glucose-6-phosphate with UDP-glucose by trehalose-6-phosphate synthase (OtsA) to form trehalose-6-phosphate. Trehalose is subsequently formed from trehalose-6-phosphate by the action of trehalose-6-phosphate phosphatase (OtsB). The TreS pathway involves a reversible transglycosylation reaction in which trehalose synthase (TreS) converts maltose, a disaccharide with α , α -1,4 linkage between the two glucose molecules, to trehalose. The third pathway, mediated by TreYZ, involves the conversion of maltodextrins into trehalose. The terminal α -1,1-glycosidic bond at the end of the maltodextrin polymer is hydrolyzed by malto-

* Corresponding author. Mailing address: University of Minnesota, Department of Soil, Water, and Climate, 1991 Upper Buford Circle, 439 Borlaug Hall, St. Paul, MN 55108. Phone: (612) 624-2706. Fax: (612) 625-2208. E-mail: sadowsky@umn.edu.

‡ Present address: Institute for Soil, Water, and Environmental Sciences, ARO, Volcani Research Center, Bet Dagan 50-250, Israel.

† Supplemental material for this article may be found at <http://aem.asm.org/>.

[▽] Published ahead of print on 18 December 2009.

oligosyltrehalose synthase (TreY), and trehalose is subsequently released from the end of the polymer via hydrolysis by maltoligosyltrehalose trehalohydrolase (TreZ). More recently, a trehalose glycosyltransferring synthase (TreT) was shown to catalyze the reversible formation of trehalose from ADP-glucose and glucose (25).

In addition to biosynthesis, Gram-negative bacteria have also been reported to have trehalose degradation systems. Typically, trehalose is hydrolyzed into two glucose moieties by periplasmic and cytoplasmic trehalase enzymes, TreA and TreF, respectively (13, 15). However, *Sinorhizobium meliloti* also uses ThuA and ThuB for trehalose utilization (16).

Bradyrhizobium japonicum, the root nodule symbiont of soybeans, accumulates trehalose in cultured cells and bacteroids (34, 35). Biochemical studies indicated that *B. japonicum* has three independent trehalose biosynthetic pathways involving trehalose synthase (TreS), maltoligosyltrehalose synthase (TreYZ), and trehalose-6-phosphate synthetase (OtsAB) (38). Sequence analysis of the *B. japonicum* USDA 110 genome identified the genes that encode these biosynthetic pathways: *otsAB* (bll0322 to bll0323), two homologs of *treS* (blr6767 and bll0902), and *treYZ* (blr6770 to blr6771), but not *treT* (17). Orthologous gene sequences to the trehalose degradation genes *treA*, *treF*, and *thuAB* have not been found in the genome of *B. japonicum* USDA 110. Cytryn et al. (6) reported that expression of *otsA*, *treS* (blr6767), and *treY* genes were highly induced by desiccation stress. Moreover, the concentrations of these three enzymes increased when *B. japonicum* was cultured in the presence of salt (38). Trehalose concentration in *B. japonicum* has been reported to increase due to desiccation stress (6), and this sugar is purported to act as an osmoprotectant. The addition of exogenously supplied trehalose has been reported to enhance the survival of *B. japonicum* in response to desiccation and salinity stresses (9, 37). Despite this information, little is known about how the various trehalose biosynthetic pathways modulate stress tolerance and symbiotic performance in *B. japonicum*.

The purpose of this study was to examine the functional role(s) of the *B. japonicum* trehalose biosynthetic pathways on stress survival by constructing single, double, and triple mutants and by producing strains that overexpress the trehalose biosynthesis enzymes. Here we report on the relationship between trehalose accumulation and physiological responses to salinity and desiccation stresses in mutant and overexpression strains and that mutations in the trehalose biosynthesis pathways altered the symbiotic performance of *B. japonicum* USDA 110 on soybeans. Results of these studies indicate that trehalose accumulation in *B. japonicum* plays a prominent role in the saprophytic and symbiotic competence of this agriculturally important soil bacterium.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are shown in Table 1. The *B. japonicum* strains were grown at 30°C in arabinose-gluconate (AG) medium (26), and the *Escherichia coli* strains were grown at 37°C in LB medium (29). Media were supplemented with antibiotics, as required, at the following concentrations (per ml): for *B. japonicum*, kanamycin at 100 µg, streptomycin at 100 µg, spectinomycin at 100 µg, tetracycline at 100 µg, and polymyxin B at 50 µg; and for *E. coli*, ampicillin at 50 µg, kanamycin at 50 µg, tetracycline at 12.5 µg, streptomycin at 25 µg, and spectinomycin at 25 µg.

DNA manipulations. The isolation of plasmid DNA, restriction enzyme digestions, DNA ligations, and the transformation of *E. coli* were done as described by Sambrook and Russell (29).

Construction of *B. japonicum* USDA 110 mutants in trehalose synthesis genes. A mobilizable *otsA* inactivation plasmid was constructed as follows: the 1.0-kb *otsA* (bll0322) coding region from *B. japonicum* USDA 110 was amplified by PCR using genomic DNA as the template and the oligonucleotide primers tre6PSFN and tre6PR (see Table S1 in the supplemental material). The PCR product was ligated into the pGEM-T Easy vector (Promega, Madison, WI), to create pGEM-*otsA*. The Ω cassette, which encodes resistance to spectinomycin and streptomycin (via *aadA*), was digested from pH45 Ω (10) and inserted into the NruI site of pGEM-*otsA*, resulting in pGEM-*otsA*:: Ω . Plasmid pGEM-*otsA*:: Ω was digested with EcoRI, and the 3.0-kb fragment containing *otsA*:: Ω was ligated into suicide vector pK18mob (30) to create pK18mob-*otsA*:: Ω (Fig. 1). For inactivation of the *treS* gene, the 1.7-kb *treS* (blr6767) coding region from *B. japonicum* USDA 110 was amplified by PCR using genomic DNA as the template and the oligonucleotide primers treSF and treS2R (see Table S1). The PCR product was ligated into the pGEM-T Easy vector, yielding pGEM-*treS*. The Ω cassette, or the aminoglycoside 3-phosphotransferase (*aph*) cassette from pUC4-K (41), was inserted into the BamHI site of pGEM-*treS*, to create pGEM-*treS*:: Ω and pGEM-*treS*::*aph*, respectively. Plasmid pGEM-*treS*:: Ω was digested with EcoRI, and the resulting 3.7-kb fragment containing *treS*:: Ω was ligated into suicide vector pK18mob to create pK18mob-*treS*:: Ω (Fig. 1). Plasmid pGEM-*treS*::*aph* was digested with BamHI, and the 2.9-kb fragment containing *treS*::*aph* was ligated into suicide vector pSUP202 (31), resulting in pSUP-*treS*::*aph* (Fig. 1). For inactivation of the *treY* gene, the 1.5-kb *treY* (blr6771) coding region from *B. japonicum* USDA 110 was amplified by PCR using genomic DNA as the template and the oligonucleotide primers treY-Fbam and treY-Rbam (see Table S1) and ligated into pGEM-T vector (Promega, Madison, WI), yielding pGEM-*treY*. The *tetA* gene, which confers tetracycline resistance, was amplified by PCR from plasmid pALTER-1 (Promega, Madison, WI) by using the oligonucleotide primers tetA-Feco and tetA-Reco (see Table S1). The *tetA* fragment was subsequently digested with EcoRI and inserted into the EcoRI site of pGEM-*treY*, resulting in pGEM-*treY*::*tetA*. Plasmid pGEM-*treY*::*tetA* was digested with BamHI, and the 3.1-kb fragment containing *treY*::*tetA* was ligated into suicide vector pK18mob to create pK18mob-*treY*::*tetA* (Fig. 1). Plasmids containing pK18mob-*otsA*:: Ω (for the *otsA* mutation), pK18mob-*treS*:: Ω (for the *treS* mutation), pSUP-*treS*::*aph* (for the *treS* mutation), and pK18mob-*treY*::*tetA* (for the *treY* mutation) were introduced into *B. japonicum* USDA 110 by triparental mating, using pRK2013 as a helper plasmid (11). Mutated strains were selected on AG agar plates containing 50 µg of polymyxin B per ml (to eliminate *E. coli*) and other appropriate antibiotics as required. Gene replacement, double crossover mutants were verified by antibiotic resistance phenotypes and by PCR using primers for the inserted gene and primers that spanned the insertion sites.

Construction of strains overexpressing trehalose synthesis genes. To overexpress *OtsA*, the 1.5-kb full-length *otsA* gene, including its Shine-Dalgarno (SD) sequence, from *B. japonicum* USDA 110 was amplified by PCR using genomic DNA as the template and the oligonucleotide primers otsAFpst and otsARbam (see Table S1 in the supplemental material). The PCR product was ligated into the pGEM-T Easy vector to create pGEM-*otsA*EX. The pGEM-*otsA*EX was subsequently digested with PstI and BamHI, and the 1.5-kb fragment containing *otsA* was inserted into the PstI and BamHI sites, downstream of the *trp* promoter, of the broad-host-range vector pTE3 (7), yielding plasmid pTE3-*otsA*. Expression of genes inserted into this vector are constitutively expressed and under the control of the *trp* promoter. For overexpression and complementation of *treS*, the 3.3-kb full-length *treS* gene, including its SD sequence, was amplified by PCR using genomic DNA as the template and the oligonucleotide primers treSF-pst3 and treSR-pst3 (see Table S1). The PCR product was digested with PstI and ligated into pBluescript II SK(+) vector (Invitrogen Co., Carlsbad, CA) to create pBS-*treSEX*. The pBS-*treSEX* was digested with PstI to obtain the 3.3-kb *treS*-containing fragment and ligated into the PstI site of pTE3, resulting in pTE3-*treS*. For overexpression of *treY*, the 3.0-kb full-length *treY* gene, including its SD sequence, from *B. japonicum* USDA 110 was amplified by PCR using genomic DNA as the template and the oligonucleotide primers treY-FpstEX2 and treY-RbamEX (see Table S1). The PCR product was digested with PstI and BamHI and ligated into the pBluescript II SK(+) vector to create pBS-*treYEX*. The pBS-*treYEX* vector was digested with PstI and BamHI, and the 3.0-kb fragment containing *treY* was inserted into the PstI site of pTE3, resulting in pTE3-*treY*.

The pTE3-*otsA*, pTE3-*treS*, or pTE3-*treY* plasmid construct was introduced into wild-type *B. japonicum* USDA 110 by triparental mating as described above. Transconjugant strains were selected on AG agar plates containing 50 µg of polymyxin B per ml and 100 µg of tetracycline per ml. For comple-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype/relevant characteristics ^a	Source or reference
<i>Bradyrhizobium japonicum</i> strains		
USDA 110	Wild-type strain	USDA, Beltsville, MD
Δ otsA mutant	otsA:: Ω mutant of USDA 110; Sm ^r , Sp ^r	This study
Δ treS mutant	treS:: Ω mutant of USDA 110; Sm ^r , Sp ^r	This study
Δ treY mutant	treY::tetA mutant of USDA 110; Tc ^r	This study
Δ otsA Δ treS mutant	otsA:: Ω , treS::aph double mutant of USDA 110; Km ^r , Sm ^r , Sp ^r	This study
Δ otsA Δ treY mutant	otsA:: Ω , treY::tetA double mutant of USDA 110; Sm ^r , Sp ^r , Tc ^r	This study
Δ treS Δ treY mutant	treS:: Ω , treY::tetA double mutant of USDA 110; Sm ^r , Sp ^r , Tc ^r	This study
Δ otsA Δ treS Δ treY mutant	otsA:: Ω , treS::aph, treY::tetA triple mutant of USDA 110; Km ^r , Sm ^r , Sp ^r , Tc ^r	This study
<i>Escherichia coli</i> strains		
DH5 α	recA; cloning strain	Invitrogen
S17-1	RK2 tra regulon	31
Plasmids		
pGEM-T	Cloning vector for TA cloning; Ap ^r	Promega
pGEM-T Easy	Cloning vector for TA cloning; Ap ^r	Promega
pBluescript II SK(+)	Cloning vector; Ap ^r	Invitrogen
pK18mob	Mobilizable suicide vector; Km ^r	30
pSUP202	Mobilizable suicide vector; Ap ^r , Cm ^r , Tc ^r	31
pHP45 Ω	Plasmid carrying Ω cassette; Sm ^r , Sp ^r	10
pUC4-K	Plasmid carrying aph cassette; Km ^r	41
pALTER-1	Plasmid carrying tetA; Ap ^r , Tc ^r	Promega
pTE3	Broad-host-range plasmid pLAFR1 carrying trp promoter; Tc ^r	7
pRK2013	ColE1 replicon carrying RK2 transfer genes; Km ^r	11
pGEM-otsAEX	pGEM-T Easy carrying 1.5-kb PCR product of full length of otsA from <i>B. japonicum</i> USDA 110	This study
pBS-treSEX	pBluescript II SK(+) carrying 3.3-kb PCR product of full length of treS from <i>B. japonicum</i> USDA 110	This study
pBS-treYEX	pBluescript II SK(+) carrying 3.0-kb PCR product of full-length of treY from <i>B. japonicum</i> USDA 110	This study
pTE3-otsA	pTE3 carrying 1.5-kb PstI-BamHI fragment from pGEM-otsAEX	This study
pTE3-treS	pTE3 carrying 3.3-kb PstI fragment from pBS-treSEX	This study
pTE3-treY	pTE3 carrying 3.0-kb PstI-BamHI fragment from pBS-treYEX	This study
pGEM-otsA	pGEM-T Easy carrying 1.0-kb PCR product of otsA fragment from <i>B. japonicum</i> USDA 110	This study
pGEM-otsA:: Ω	pGEM-otsA carrying 2.0-kb Ω cassette at NruI site	This study
pK18mob-otsA:: Ω	pK18mob carrying 3.0-kb EcoRI fragment from pGEM-otsA:: Ω	This study
pGEM-treS	pGEM-T Easy carrying 1.7-kb PCR product of treS fragment from <i>B. japonicum</i> USDA 110	This study
pGEM-treS:: Ω	pGEM-treS carrying 2.0-kb Ω cassette at BamHI site	This study
pK18mob-treS:: Ω	pK18mob carrying 3.7-kb EcoRI fragment from pGEM-treS:: Ω	This study
pGEM-treS::aph	pGEM-treS carrying 1.2-kb aph cassette at BamHI site	This study
pSUP-treS::aph	pSUP202 carrying 2.9-kb BamHI fragment from pGEM-treS::aph	This study
pGEM-treY	pGEM-T vector carrying 1.5-kb PCR product of treY fragment from <i>B. japonicum</i> USDA 110	This study
pGEM-treY::tetA	pGEM-treY carrying 1.6-kb tetA PCR fragment from pALTER-1 at EcoRI site	This study
pK18mob-treY::tetA	pK18mob carrying 3.1-kb BamHI fragment from pGEM-treY::tetA	This study

^a Resistant to (i) ampicillin, Ap^r; (ii) chloramphenicol, Cm^r; (iii) kanamycin, Km^r; (iv) streptomycin, Sm^r; (v) spectinomycin, Sp^r; and (vi) tetracycline, Tc^r. aph is the aminoglycosidase-3-O-phosphotransferase gene, and tetA is the tetracycline efflux protein gene.

mentation analyses, pTE3-treS was introduced into the treS mutant by triparental mating, and transconjugants were selected on AG agar plates containing 50 μ g of polymyxin B per ml and 100 μ g of tetracycline, spectinomycin, and streptomycin per ml.

Determination of trehalose and maltose contents in *Bradyrhizobium* cells. Cell extracts of wild-type and mutant strains were prepared by using a hot ethanol extraction method. The *B. japonicum* cells were cultured in 25 ml AG medium or AG medium supplemented with 60 mM NaCl, until an optical density at 600 nm (OD₆₀₀) of 0.5 to 0.6 was reached. Cells were collected by centrifugation at 10,000 $\times$ g for 10 min, resuspended in 500 μ l 80% ethanol, and incubated at 85°C for 15 min. The cell suspension was centrifuged at 15,000 $\times$ g for 2 min, and the supernatant was transferred to a 1.5-ml microcentrifuge tube. This procedure was repeated, and the approximately 1.0 ml of cell extract was evaporated by using a Spin-Vap centrifugal concentrator. The dried sample was suspended in

100 μ l distilled H₂O and stored at –20°C until used for trehalose and maltose quantification analyses.

Trehalose and maltose in cell extracts were detected following enzymatic conversion to glucose by reaction with trehalase (Sigma, St. Louis, MO) and maltase (Sigma), respectively. Reaction mixtures (50 μ l) using trehalase contained 30 μ l of 135 mM citric acid buffer, pH 5.7; 10 μ l of cell extract; and 10 μ l of trehalase enzyme solution, which was prepared by dissolving 0.2 units of enzyme per ml in cold 135 mM citric acid buffer, pH 5.7. Reactions were incubated for 1 h at 37°C and terminated by addition of 50 μ l of 500 mM Tris-HCl, pH 7.5. Reaction mixtures using maltase contained 20 μ l of 50 mM potassium phosphate buffer, pH 6.0; 25 μ l of cell extract; and 5 μ l of maltase enzyme solution, which was prepared by dissolving 1.0 unit of enzyme per ml in cold 50 mM potassium phosphate buffer, pH 6.0. Reactions were incubated for 1 h at 25°C. The amount of glucose produced was measured by using a glucose assay kit (Sigma), according to the manufacturer's

FIG. 1. Physical maps of the *otsA*, *treS*, and *treY* genes (and their flanking regions) used in the construction of *B. japonicum* USDA 110 mutants. (a) *otsA* (bl0322) and its flanking region. The internal NruI fragment in *otsA* was removed and replaced with the Ω cassette to create pK18mob-*otsA*:: Ω . Arrows denote open reading frames. (b) *treS* (blr6767) and its flanking region. The Ω or *aph* cassette was inserted into the internal BamHI site of *treS* to create pK18mob-*treS*:: Ω or pSUP-*treS*::*aph*, respectively. (c) *treY* (blr6771) and its flanking region. The *tetA* gene fragment was inserted into the internal EcoRI site of *treY* to create pK18mob-*treY*::*tetA*. B, BamHI; E, EcoRI; N, NruI.

instruction. The concentration of trehalose or maltose in samples was calculated from the difference in the concentration of glucose in samples with and without the addition of trehalase or maltase, respectively.

RNA extraction, cDNA synthesis, and quantitative RT-PCR. A 25-ml culture of *B. japonicum* was grown in AG medium or AG medium supplemented with 60 mM NaCl to an optical density at 600 nm of 0.5 to 0.7. Cultures were immediately transferred to centrifuge tubes containing 2.5 ml of cold stop solution (5% H₂O-saturated phenol, pH 4.3, in 95% ethanol). The suspension was centrifuged at 4°C for 10 min at 10,000 × g, and cell pellets were frozen immediately in liquid nitrogen and stored at -80°C until used for RNA isolation. Desiccated cell samples for RNA extraction were prepared as described by Cytryn et al. (6). RNA isolation from *B. japonicum* cells and DNA digestions were done as described by Chang et al. (4). The cDNA was synthesized from 5 µg of total RNA template. Reaction mixtures contained 250 ng of random hexamers (Invitrogen), 1 µl of 10 mM dNTP mix, and 11 µl of RNA solution. The mixture was heated for 5 min at 65°C and chilled on ice for 1 min, and 4 µl of 5× first-strand buffer, 1 µl of 0.1 M dithiothreitol, 1 µl of RNaseOUT (Invitrogen), and 200 units of Superscript III reverse transcriptase (RT) (Invitrogen) were added to the solution. The solution was incubated at 50°C for 1 h and placed in a 70°C heating

block for 15 min. The resulting cDNA sample was kept at -20°C until used in quantitative RT-PCR (qRT-PCR) experiments.

quantitative RT-PCR (qRT-PCR) experiments. For qRT-PCR, each 25- μ l reaction mixture contained the cDNA synthesized from 0.25 μ g RNA, 12.5 μ l iTaq SYBR green Supermix with ROX (Bio-Rad, Hercules, CA), 11.25 μ l nuclease-free water, and 0.5 μ M (each) forward and reverse gene-specific primers (see Table S1 in the supplemental material). The primer sets used in the PCR to amplify products from the transcript were as follows: bll0322-F2 and bll0322-R2 (for *otsA*), bllr6767-F2 and bllr6767-R2 (for *treS*), bll0902-F and bll0902-R (for bll0902), bllr6771-F and bllr6771-R (for *treY*), and bll0631-F and bll0631-R (for *parA*). The qRT-PCRs were run on an Applied Biosystems (Foster City, CA) 7500 real-time PCR system using Sequence Detection System software, version 1.3. The PCR program consisted of an initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. Expression values for three biological replicates for each treatment were normalized to the expression level of *parA* (bll0631), which is a housekeeping gene in the *B. japonicum* USDA 110 genome.

Measurement of viable cell numbers after desiccation stress. Desiccation stress assays were done as described by Cytryn et al. (6). The relative humidity (RH) in

FIG. 2. qRT-PCR analysis of differentially expressed trehalose synthesis genes (*otsA*, *treS*, and *treY*) in salinity- and desiccation-stressed *B. japonicum* USDA 110 cells. Values are normalized to those of the housekeeping gene *parA*. (A) Absolute gene expression values of trehalose synthesis genes in salinity-stressed cells. Black bars, nonstressed cells; gray bars, salt-stressed cells. (B) Absolute gene expression values of trehalose synthesis genes in desiccated cells. Black bars, nonstressed cells; gray bars, cells after 24 h incubation under desiccation stress; white bars, cells after 72 h incubation under desiccation stress. Error bars indicate standard deviations of data for three technical replicates.

the desiccators was adjusted to 50% by using a saturated solution of potassium acetate. The viability of desiccated cells was determined by using plate count assays.

Nodulation studies. Wild-type *B. japonicum* USDA 110 and the Δ *otsA* Δ *treY*, Δ *otsA* Δ *treS* Δ *treY*, and Δ *treS* mutant strains were grown in AG medium supplemented with the appropriate antibiotics, collected by centrifugation at $8,000 \times g$ for 10 min, and washed twice with sterile distilled water. These resultant cultures were diluted to 10^7 cells per ml, and 1-ml aliquots of these dilutions were inoculated onto surface-sterilized *Glycine max* cv. Lambert seeds. Plant assays were done in sterile Leonard jar assemblies containing a 3:1 mixture of vermiculite and perlite, as previously described (26). Seeds of *G. max* cv. Lambert were surface sterilized by immersion in 100% ethanol for 5 s and treated with 1.0% sodium hypochlorite for 5 min. Seeds were washed 10 times with sterile distilled water prior to planting. After inoculation, seeds were planted in a 3:1 mixture of vermiculite and perlite and covered with a 1-cm layer of sterilized paraffin-coated sand. Plants were watered with nitrogen-free plant nutrient solution (18) and incubated in a plant growth chamber at 25°C with a photoperiod of 16 h. The number of mature and immature nodules and nodule and plant dry weights (wt) were determined 31 days after inoculation.

RESULTS

Expression of trehalose biosynthetic genes under stress conditions. *otsAB* (bll0322 to bll0323), two copies of *treS* (blr6767 and bll0902), and *treYZ* (blr6770 to bll6771) were previously annotated as trehalose biosynthetic genes in *B. japonicum* strain USDA 110 (17). In this study, real-time qRT-PCR analyses were done to examine expression of these genes under conditions of salinity and desiccation stress, relative to that seen with nonstressed cells. Results depicted in Fig. 2 show that the expression of *otsA* (bll0322), the two homologs of *treS* (blr6767 and bll0902), and *treY* (blr6771) were induced by stress imparted by supplementation of the growth medium with 60 mM NaCl and by growth under desiccating conditions. The absolute gene expression value of *otsA* was greater than those seen for the other trehalose synthesis genes. In contrast, expression of bll0902, coding for the TreS homolog, was the lowest of all the tested genes. These results suggested that the OtsAB pathway may play a major role in stress-induced trehalose biosynthesis in *B. japonicum*.

Construction of trehalose biosynthesis mutants and overexpressing strains. In order to investigate the roles that the different trehalose biosynthesis pathways play in stress survival of *B. japonicum* USDA 110, antibiotic resistance cassettes were used to create insertions in genes coding for OtsA, TreS

(blr6767), and TreY (Fig. 1). Single, double, and triple mutations in these three genes were constructed by homologous recombination. The *treS* (bll0902) gene was eliminated as a target in this study, since the expression of this gene under stress conditions was lower than that of other genes (Fig. 2).

Trehalose accumulation in wild-type cells increased about fourfold in response to salinity stress (4.7 ± 0.21 μ mol/g fresh wt) compared to cells grown under nonstressed conditions (1.2 ± 0.02 μ mol/g fresh wt). This result correlated well with gene expression data (Fig. 2A). In contrast, strains growing under salt stress conditions and carrying the *otsA* mutation, the Δ *otsA*, Δ *otsA* Δ *treS*, Δ *otsA* Δ *treY*, and Δ *otsA* Δ *treS* Δ *treY* mutants, accumulated smaller amounts of trehalose, relative to the wild-type strain (Fig. 3A). In particular, the two strains lacking the OtsAB and TreYZ pathways, the Δ *otsA* Δ *treY* and Δ *otsA* Δ *treS* Δ *treY* mutants, accumulated extremely low levels of trehalose. Surprisingly, the *treS* mutant (Δ *treS*) strain accumulated a larger amount of trehalose than that seen with salt-grown wild-type cells. The strain containing the *treY* single mutation and the wild-type strain accumulated approximately the same amount of trehalose, suggesting that the maltotriose-trehalose synthase does not play a major role in trehalose synthesis in *B. japonicum* under the tested growth conditions.

Wild-type USDA 110 strains overexpressing OtsA or TreY accumulated slightly greater amounts of trehalose than those of the wild-type strain carrying the empty plasmid vector pTE3. In contrast, trehalose accumulation in wild-type cells overexpressing TreS was less than that found with wild-type USDA 110(pTE3) cells. This result suggested that TreS was involved in trehalose degradation in *B. japonicum* USDA 110. In addition, the Δ *treS* mutant strain complemented with the *treS* gene, Δ *treS* (pTE3-*treS*), accumulated less trehalose than the parental control Δ *treS* (pTE3) strain. Taken together, these results suggested that trehalose biosynthesis in *B. japonicum* is mediated mainly by the OtsAB pathway and marginally by TreYZ pathways and that the TreS protein encoded by blr6767 is likely involved in trehalose degradation or repression of trehalose biosynthesis. Moreover, since wild-type USDA 110 cells overexpressing OtsA accumulated levels of trehalose similar to those of the control strain containing the empty vector, our results suggest that the concen-

FIG. 3. Trehalose and maltose accumulation by wild-type *B. japonicum* USDA 110, trehalose synthesis mutants, and overexpressing strains grown in AG medium supplemented with 60 mM NaCl. (A) Trehalose contents in *Bradyrhizobium* cells. (B) Maltose contents in *Bradyrhizobium* cells. Error bars indicate standard deviations of data for three biological replicates.

tration of trehalose in salt-stressed *Bradyrhizobium* cells may be controlled by the combined activities of trehalose biosynthesis and degradation systems.

The *B. japonicum* *treS* gene is involved in degradation of trehalose to maltose. In order to investigate whether TreS catalyzes the degradation of trehalose in *B. japonicum*, maltose accumulation levels in wild-type, mutant, and overexpressing strains were determined. The accumulation of maltose by all of the tested strains is shown in Fig. 3B. The strains carrying a mutation in *treS* ($\Delta treS$, $\Delta otsA \Delta treS$, $\Delta treS \Delta treY$, and $\Delta otsA \Delta treS \Delta treY$) accumulated amounts of maltose smaller than those of the wild-type strain, and the strain overexpressing

TreS resulted in an increase in the intracellular concentration of maltose. In addition, the mutant $\Delta treS$ strain overexpressing TreS (via pTE3-*treS*) accumulated an amount of maltose significantly larger than that of the $\Delta treS$ control strain (pTE3). The single and double *otsA* mutant strains, the $\Delta otsA$ and $\Delta otsA \Delta treY$ mutants, respectively, which accumulated relatively low levels of trehalose, also produced relatively less maltose than did the wild-type strain. These results indicated that TreS is likely involved in the degradation of trehalose to maltose in *B. japonicum* strain USDA 110.

Influence of trehalose on expression of *otsA* and *treS*. Since the *OtsA*-overexpressing strain did not accumulate much trehalose (Fig. 3A), our results strongly suggested that the intracellular concentration of trehalose in *Bradyrhizobium* cells is controlled by the combined action of the trehalose biosynthesis and degradation systems. To determine if genes controlling trehalose synthesis and degradation are regulated by trehalose, real-time qRT-PCR analyses were done using primers specific for *otsA* and *treS* transcripts. The absolute expression level of the *otsA* and *treS* genes was initially examined with the wild-type *B. japonicum* strain cultured in AG medium supplemented with 0.1, 1, or 10 mM filter-sterilized trehalose. However, the expression levels of both genes were not different relative to cells grown without trehalose supplementation (data not shown). In contrast, there were significant differences in the expression levels of *otsA* and *treS* in strains overexpressing the *otsA* and *treS* genes. The expression of *otsA* in USDA 110(pTE3-*otsA*) was 119-fold greater than that of the control wild-type strain containing the empty vector, and *treS* gene expression in wild-type cells overexpressing *OtsA* (pTE3-*otsA*) was 1.9-fold greater than that seen with wild-type cells containing pTE3 (Table 2). In addition, *treS* gene expression in USDA 110(pTE3-*treS*) was 300-fold greater than that seen with the control strain, USDA 110(pTE3), and *otsA* gene expression in wild-type cells overexpressing TreS (pTE3-*treS*) was 2.8-fold greater than that seen with vector control cells (Table 2). Results of these studies suggested that expression of the trehalose synthesis gene (*otsA*) and degradation gene (*treS*) is likely modulated by the intracellular concentration of trehalose in *B. japonicum*.

Effects of trehalose accumulation on salinity stress survival. To determine if trehalose accumulation influenced growth of salt-stressed cells, the various trehalose synthesis mutants were grown on agar plates containing AG medium or AG medium containing 60 mM NaCl. While all the tested strains

TABLE 2. qRT-PCR analysis of *otsA* and *treS* genes in *B. japonicum* strains overexpressing trehalose-6-phosphate synthetase and trehalose synthase

Inoculated strain containing indicated plasmid	Absolute gene expression value ^a		Expression ratio ^b	
	<i>otsA</i>	<i>treS</i>	<i>otsA</i>	<i>treS</i>
pTE3	0.36 ± 0.06	0.09 ± 0.008	1.0	1.0
pTE3- <i>otsA</i>	43 ± 3.6	0.18 ± 0.02	119	1.9
pTE3- <i>treS</i>	1.0 ± 0.07	27 ± 8.2	2.8	300

^a The absolute expression values were normalized to those of the housekeeping gene *parA*. Values are means ± standard deviations of data for three biological replicates.

^b Ratio of trehalose synthase gene expression in wild-type *B. japonicum* USDA 110 overexpressing trehalose-6-phosphate synthetase and trehalose synthase versus empty plasmid pTE3.

FIG. 4. Influence of salt stress on growth of wild-type *B. japonicum* USDA 110 and mutants with insertions in trehalose synthesis genes. Wild-type *B. japonicum* USDA 110 (A) and mutants Δ otsA (B), Δ treS (C), Δ treY (D), Δ otsA Δ treS (E), Δ otsA Δ treY (F), Δ treS Δ treY (G), and Δ otsA Δ treS Δ treY (H) were inoculated onto AG agar medium and AG medium supplemented with 60 mM NaCl. Plates were photographed after 7 days of growth at 30°C.

grew well on AG medium without added salt, the single, double, and triple mutant strains lacking the OtsAB pathway (the Δ otsA, Δ otsA Δ treS or Δ otsA Δ treY, and Δ otsA Δ treS Δ treY mutants) were inhibited for growth on 60 mM NaCl (Fig. 4). The Δ otsA Δ treY double mutant and the Δ otsA Δ treS Δ treY triple mutant, which lack both the OtsAB and TreYZ pathways, failed to grow on salt-containing medium (Fig. 4). These growth effects were correlated with trehalose accumulation levels (Fig. 3A) but were not correlated with maltose accumulation levels (Fig. 3B). These results indicated that trehalose in *B. japonicum* plays a role as an osmoprotectant for growth under conditions of salt-induced osmotic stress.

Survival phenotypes of trehalose biosynthetic gene mutants under desiccation stress. To examine the effects of trehalose biosynthesis on desiccation stress, *Bradyrhizobium* cells were filtered onto polycarbonate filters and incubated under conditions of 50% RH. Results depicted in Fig. 5 show that the viable cell numbers of the Δ otsA and Δ otsA Δ treY mutants, which are low trehalose accumulators, were not different than that found for the wild-type strain. In contrast, the mutants

TABLE 3. Nodulation phenotype of *Glycine max* cv. Lambert plants inoculated with wild-type and trehalose synthesis and degradation mutants of *B. japonicum* USDA 110^a

Inoculated strain	No. of mature nodules	No. of immature nodules	Nodule dry mass (mg)	Plant dry mass (g)
Wild-type USDA 110	25.5 A	0.5 B	53.0 A	1.5 A
Δ otsA Δ treY mutant	12.5 B	37.8 A	39.8 AB	1.3 AB
Δ otsA Δ treS Δ treY mutant	12.0 B	33.5 A	31.0 B	1.0 B
Δ treS mutant	23.5 A	2.3 B	49.0 A	1.5 AB
Uninoculated control	0 C	0 B	0 C	1.2 B

^a Values are means of four replicate experiments. Numbers of nodules and dry mass values are per plant. Within a column, numbers not followed by the same letter differ significantly at $P = 0.05$, as tested by analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD).

lacking the TreS pathway (the Δ treS, Δ otsA Δ treS, Δ treS Δ treY, and Δ otsA Δ treS Δ treY mutants) survived poorly under desiccation stress conditions relative to the wild-type strain, although the initial cell numbers were not different. The survival of the TreS overexpressing strain, USDA110 (pTE3-*treS*), was slightly greater than that of the wild-type control strain containing pTE3. Moreover, the decreased survival caused by a lack of the TreS pathway was recovered by complementing the mutant strain with *treS*. It should be noted, however, that the growth responses of mutant and wild-type strains to desiccation stress were not correlated with intracellular accumulation levels of trehalose or maltose (Fig. 3). Thus, trehalose and maltose accumulation in *B. japonicum* did not enhance survival against desiccation stress, and trehalose metabolism via the TreS pathway is likely important for survival under desiccating conditions.

Symbiotic phenotypes of trehalose synthesis mutants on soybeans. To determine if trehalose accumulation in *B. japonicum* influences its ability to form symbiotic interactions with its host legume, soybean (*G. max* cv. Lambert) seeds were inoculated independently with the Δ otsA Δ treY, Δ otsA Δ treS Δ treY, and Δ treS mutants and the wild-type strain. Results represented in Table 3 show that the number of mature nodules

FIG. 5. Viable cell numbers of trehalose synthesis mutants, *treS*-overexpressing strains, and wild-type *B. japonicum* after incubation under desiccating condition (50% RH). Plate count data are expressed as log₁₀ CFU/ml for desiccated cells at time of inoculation (white bars) and after growth for 72 h (black bars). Error bars represent the standard deviation of data for three biological replicates.

FIG. 6. Symbiotic phenotypes of wild-type and mutant *B. japonicum* strains inoculated onto *G. max* cv. Lambert plants. (A) Nodules formed on soybean roots inoculated with the wild-type strain. (B) Mature and immature nodules formed on soybean roots inoculated with the $\Delta otsA \Delta treS \Delta treY$ mutant. (C) Plants were inoculated with wild-type *B. japonicum* USDA 110, the $\Delta otsA \Delta treY$ double mutant, or the $\Delta otsA \Delta treS \Delta treY$ triple mutant and analyzed after 31 days. Bars, 0.2 mm.

produced by the $\Delta otsA \Delta treY$ or $\Delta otsA \Delta treS \Delta treY$ mutant strains was less than that seen with soybean seeds inoculated with the wild-type strain. However, the mutant strains induced a large number of immature, ineffective nodules on the roots of soybean (Fig. 6A and B; Table 3). The dry masses of soybean plants and nodules produced when plants were inoculated with the $\Delta otsA \Delta treY$ and $\Delta otsA \Delta treS \Delta treY$ mutants were less than those seen with plants inoculated with the wild-type USDA 110 strain. Moreover, the leaves of soybean plants inoculated with the mutants were chlorotic and looked similar to the uninoculated control plants (Fig. 6C). Thus, the nitrogen fixation ability of nodules induced by the $\Delta otsA \Delta treY$ and $\Delta otsA \Delta treS \Delta treY$ mutants was likely severely impaired relative to that found with the wild-type strain. In contrast, the nodulation and nitrogen fixation phenotype of the $\Delta treS$ mutant strain was not different from that of the wild-type strain (Table 3). These results indicated that trehalose accumulation in *B. japonicum* is linked to the development of soybean nodules and that TreS-mediated trehalose degradation has a limited role in the symbiotic interaction of this bacterium with its legume host.

DISCUSSION

While four trehalose biosynthetic pathways (OtsAB, TreS, TreYZ, and TreT) have thus far been described for prokaryotes, the combination of pathways possessed by a single bacterium appears to be bacterial species dependent. In *B. japonicum* strain USDA 110, three independent trehalose pathways (OtsAB, TreS, and TreYZ) were detected by enzymatic assays (38), and the *otsAB*, *treS*, and *treYZ* genes have been identified by genome sequence analyses (17). In this study, the presence and role of the putative trehalose biosynthesis genes in *B. japonicum* USDA 110 were determined using genetic and molecular approaches.

Results of the studies presented here show that disruption of *otsA* caused a decrease in trehalose accumulation levels in cells, especially those of the *otsA treY* double mutant, which accumulated extremely low levels of trehalose when grown under conditions of salinity stress. In contrast the single *treY* mutation had little effect on trehalose accumulation. Thus, trehalose biosynthesis in *B. japonicum* appears to be mediated mainly by the OtsAB pathway and only marginally by the

FIG. 7. Proposed trehalose biosynthetic and metabolic pathways in *B. japonicum*. AgIA, alpha-glucosidase; MalQ, 4-alpha-glucanotransferase; OtsA, trehalose-6-phosphate synthase; OtsB, trehalose-phosphatase; TreS, trehalose synthase; TreY, malto-oligosyltrehalose synthase; and TreZ, malto-oligosyltrehalose trehalohydrolase.

TreYZ pathway (Fig. 7). Our results also indicated that despite construction of double (Δ otsA Δ treY) and triple (Δ otsA Δ treS Δ treY) mutations in the trehalose biosynthetic genes, low levels of trehalose were still detected with these strains. This is likely due to the existence of other yet-defined or -annotated genes. For example, bll0902 is annotated as *treS*-like and may play a role in trehalose synthesis in *B. japonicum*.

The trehalose biosynthesis and degradation systems present in *B. japonicum* are similar to those reported for *Rhodobacter sphaeroides* (19), a bacterium taxonomically related to *B. japonicum*. Thus, the alphaproteobacteria likely contain evolutionarily conserved systems controlling trehalose levels in cells. In contrast, osmoregulated trehalose biosynthesis in the Gram-positive bacterium *Corynebacterium glutamicum* is mediated by the TreYZ pathway and not by the OtsAB pathway (43).

The functional redundancy of multiple genes and pathways for trehalose biosynthesis in *B. japonicum* likely points to the overall importance of this sugar for the survival of this bacterium growing in soils and plants. This redundancy, however, is lacking in several tested fast-growing *Rhizobium* strains, and only trehalose-6-phosphate activity has been reported for *S. meliloti* and *Mesorhizobium loti* (38). However, like *B. japonicum*, an *otsA* mutant of *R. leguminosarum* accumulated relatively less trehalose than did a *treY* mutant (20). The OtsAB pathway has also been shown to be essential for trehalose biosynthesis in other Gram-negative bacteria, including *Escherichia coli* and *Rhodobacter sphaeroides* (14, 19). This suggests that the OtsAB pathway may be a dominant trehalose biosynthetic pathway in Gram-negative bacteria, including rhizobia.

Our studies suggested that the TreS pathway is involved in trehalose catabolism in *B. japonicum*. The maltose which is produced by the TreS protein in *B. japonicum* might be further metabolized to glucose by 4-alpha-glucanotransferase (MalQ) and alpha-glucosidase (AgIA), which are enzymes catalyzing maltose degradation (Fig. 7) (2, 3). Therefore, it would seem logical that trehalose can be used to support growth of this bacterium. However, earlier studies have shown that *B. japonicum* cannot utilize trehalose as a sole source of carbon for growth (34), despite the fact that *B. japonicum* bacteroids have also been reported to have trehalase, an enzyme catalyzing degradation of trehalose into two molecules of glucose (27, 28, 33). Interestingly, strain USDA 110 contains *malQ* (bll6765)

and *aglA* (blr0901), and these genes, as well as the trehalose biosynthetic genes, were highly induced by desiccation stress (6). However, genome sequence analyses indicated that the *B. japonicum* USDA 110 genome did not contain homologs of *treA* and *treF*, coding for trehalase, and *thuAB*, which codes for trehalose utilization enzymes. Thus, other studies are needed to fully understand trehalose and maltose metabolism in this bacterium.

Gene expression studies reported here indicate that the trehalose biosynthesis genes *otsA*, *treS*, and *treY* were induced by salinity and desiccation stresses. This correlates well with previous whole-genome microarray data of desiccation-stressed cells (6). However, Chang et al. (4) reported that expression of the trehalose synthesis genes in *B. japonicum* were not induced by salt stress. This discrepancy is likely due to the fact that different concentrations of NaCl were used in both studies. Our studies also suggested that expression of the *otsA* and *treS* genes were likely modulated by the intracellular concentration of trehalose (Table 2; Fig. 7). Although the *otsA*-overexpressing strain did not accumulate much trehalose (Fig. 3A), the expression of the trehalose degradation gene (*treS*) was induced due to overexpression of the *otsA* gene. In contrast, overexpression of the *treS* gene led to repression of the trehalose biosynthetic gene *otsA*. Interestingly, while our results suggested that trehalose accumulation in the triple mutant should be more than that seen with the double *otsA* *treY* mutant, this was not observed (Fig. 3A). Since TreS utilizes trehalose as its substrate, this may be due in part to a lack of effectiveness of the TreS degradation pathway with the lower levels of trehalose present in the *otsA* *treY* mutant strain. Thus, it seems logical that *B. japonicum* controls trehalose accumulation by modulating gene expression levels. Recently, the PhyR/EcfG regulon was found to be a stress response regulator in *B. japonicum* USDA 110 (12). The authors of that recent study reported that expression of *otsA* and *treS* was downregulated in *phyR* and *ecfG* mutants, and this involves an ECF-type sigma factor. A conserved σ^{ecfG} -type promoter region is located upstream of *otsA* in USDA 110. Thus, the PhyR/EcfG regulon may also be involved in regulation of trehalose synthase genes in *B. japonicum* cells growing under physiological stress conditions. In contrast, *otsA* expression in *E. coli* is under the control of *rpoS*, which is the stationary-phase sigma factor (14). Moreover, a deletion mutant of another stationary-phase sigma factor, *sigD*, in *Myxococcus xanthus* did not accumulate trehalose in cells and was more sensitive to salinity stress compared to the wild-type strain (40). Since the *B. japonicum* USDA 110 strain has two copies of *sigD*, but no *rpoS*, it is possible that *sigD* may also modulate expression of the trehalose synthesis genes in this bacterium.

The accumulation of trehalose is a common response to salinity- and desiccation-induced stress in several bacteria (5, 21, 42). Our results also indicate that trehalose accumulation in *B. japonicum* affects tolerance of salinity stress. Surprisingly, cells of the mutants lacking the trehalose degradation pathway mediated by TreS survived poorly under desiccation stress conditions relative to the wild-type strain. Similarly, mutant strains of *Saccharomyces cerevisiae* and *Corynebacterium glutamicum* lacking trehalose degradation activity were severely impaired in their ability to recover from physiological stresses (22, 43). Singer and Lindquist postulated that high concentra-

tions of trehalose inhibited the refolding and reactivation of denatured proteins by molecular chaperones (32). Thus, TreS-mediated trehalose degradation in *B. japonicum* cells subjected to desiccation stress may improve refolding and reactivation of denatured proteins and play an important role in the bacterium's recovery from dehydrating conditions.

Trehalose accumulation in *B. japonicum* also positively influenced nodulation of *G. max* cv. Lambert, since nodulation studies indicated that mutants lacking both the OtsAB and TreYZ pathways induced only a small number of mature nodules on soybean plants (Table 3). This is similar to what has been reported for the *Rhizobium etli*-*Phaseolus vulgaris* symbiotic interaction (39). However, the effect of trehalose accumulation by rhizobial cells on their symbiotic phenotype appears to be dependent on the rhizobial species and host genotype examined and is not consistent across symbiotic systems. For example, *R. leguminosarum* mutants lacking the OtsAB and TreYZ pathways were reported to form effective nitrogen-fixing nodules on *Trifolium repens*, although the mutants had enhanced competitiveness relative to the wild-type strain (20). In contrast, *thuB* mutants, which lacked trehalose catabolism, showed an enhanced competitiveness phenotype only on certain host genotypes in the *Sinorhizobium-Medicago* symbiotic interaction (1). Similarly, trehalose concentration in bacteroids has been shown to vary in different host genotypes that have been inoculated by *Bradyrhizobium* strains (34, 38). Taken together, our results indicate that trehalose accumulation in *Bradyrhizobium* cells influences its symbiosis with soybeans, perhaps by acting as a compatible solute to overcome host-induced osmotic stress that occurs during the nodulation process.

ACKNOWLEDGMENTS

This study was supported, in part, by grant 2004-35604-14708 from the USDA/CSREES/NRI and grant 0543238 from the National Science Foundation and by the University of Minnesota Agricultural Experiment Station.

REFERENCES

- Ampomah, O. Y., J. B. Jensen, and T. V. Bhuvaneshwari. 2008. Lack of trehalose catabolism in *Sinorhizobium* species increases their nodulation competitiveness on certain host genotypes. *New Phytol.* **179**:495–504.
- Angelov, A., M. Putyrski, and W. Liebl. 2006. Molecular and biochemical characterization of α -glucosidase and α -mannosidase and their clustered genes from the thermoacidophilic archaeon *Picrophilus torridus*. *J. Bacteriol.* **188**:7123–7131.
- Boos, W., and H. Shuman. 1998. Maltose/maltodextrin system of *Escherichia coli*: transport, metabolism, and regulation. *Microbiol. Mol. Biol. Rev.* **62**:204–229.
- Chang, W. S., W. L. Franck, E. Cytryn, S. Jeong, T. Joshi, D. W. Emerich, M. J. Sadowsky, D. Xu, and G. Stacey. 2007. An oligonucleotide microarray resource for transcriptional profiling of *Bradyrhizobium japonicum*. *Mol. Plant Microbe Interact.* **20**:1298–1307.
- Csonka, L. N. 1989. Physiological and genetic responses of bacteria to osmotic stress. *Microbiol. Rev.* **53**:121–147.
- Cytryn, E. J., D. P. Sangurdekar, J. G. Streeter, W. L. Franck, W. S. Chang, G. Stacey, D. W. Emerich, T. Joshi, D. Xu, and M. J. Sadowsky. 2007. Transcriptional and physiological responses of *Bradyrhizobium japonicum* to desiccation-induced stress. *J. Bacteriol.* **189**:6751–6762.
- Egelhoff, T. T., and S. R. Long. 1985. *Rhizobium meliloti* nodulation genes: identification of *nodDABC* gene products, purification of *nodA* protein, and expression of *nodA* in *Rhizobium meliloti*. *J. Bacteriol.* **164**:591–599.
- Elbein, A. D., Y. T. Pan, I. Pastuszak, and D. Carroll. 2003. New insights on trehalose: a multifunctional molecule. *Glycobiology* **13**:17r–27r.
- Elsheikh, E. A. E., and M. Wood. 1990. Rhizobia and Bradyrhizobia under salt stress: possible role of trehalose in osmoregulation. *Lett. Appl. Microbiol.* **10**:127–129.
- Fellay, R., J. Frey, and H. Krisch. 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for in vitro insertional mutagenesis of gram-negative bacteria. *Gene* **52**:147–154.
- Figurski, D. H., and D. R. Helinski. 1979. Replication of an origin-containing derivative of plasmid Rk2 dependent on a plasmid function provided in trans. *Proc. Natl. Acad. Sci. U. S. A.* **76**:1648–1652.
- Gourion, B., S. Sulser, J. Frunzke, A. Francez-Charlot, P. Stiefel, G. Pessi, J. A. Vorholt, and H. M. Fischer. 2009. The PhyR- σ^{EccG} signalling cascade is involved in stress response and symbiotic efficiency in *Bradyrhizobium japonicum*. *Mol. Microbiol.* **73**:291–305.
- Gutierrez, C., M. Ardourel, E. Bremer, A. Middendorff, W. Boos, and U. Ehmann. 1989. Analysis and DNA-sequence of the osmoregulated *treA* gene encoding the periplasmic trehalase of *Escherichia coli* K12. *Mol. Gen. Genet.* **217**:347–354.
- Hengge-Aronis, R., W. Klein, R. Lange, M. Rimmel, and W. Boos. 1991. Trehalose synthesis genes are controlled by the putative sigma factor encoded by *rpoS* and are involved in stationary-phase thermotolerance in *Escherichia coli*. *J. Bacteriol.* **173**:7918–7924.
- Horlacher, R., K. Uhland, W. Klein, M. Ehrmann, and W. Boos. 1996. Characterization of a cytoplasmic trehalase of *Escherichia coli*. *J. Bacteriol.* **178**:6250–6257.
- Jensen, J. B., O. Y. Ampomah, R. Darrah, N. K. Peters, and T. V. Bhuvaneshwari. 2005. Role of trehalose transport and utilization in *Sinorhizobium meliloti*-alfalfa interactions. *Mol. Plant Microbe Interact.* **18**:694–702.
- Kaneko, T., Y. Nakamura, S. Sato, K. Minamisawa, T. Uchiyama, S. Sasamoto, A. Watanabe, K. Idesawa, M. Iriguchi, K. Kawashima, M. Kohara, M. Matsumoto, S. Shimpo, H. Tsuruoka, T. Wada, M. Yamada, and S. Tabata. 2002. Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA 110. *DNA Res.* **9**:189–197.
- Keyser, H. H., and P. B. Cregan. 1987. Nodulation and competition for nodulation of selected soybean genotypes among *Bradyrhizobium japonicum* serogroup 123 isolates. *Appl. Environ. Microbiol.* **53**:2631–2635.
- Makihara, F., M. Tsuzuki, K. Sato, S. Masuda, K. V. P. Nagashima, M. Abo, and A. Okubo. 2005. Role of trehalose synthesis pathways in salt tolerance mechanism of *Rhodobacter sphaeroides* f. sp. *denitrificans* IL106. *Arch. Microbiol.* **184**:56–65.
- McIntyre, H. J., H. Davies, T. A. Hore, S. H. Miller, J. P. Dufour, and C. W. Ronson. 2007. Trehalose biosynthesis in *Rhizobium leguminosarum* bv. *trifolii* and its role in desiccation tolerance. *Appl. Environ. Microbiol.* **73**:3984–3992.
- Miller, K. J., and J. M. Wood. 1996. Osmoadaptation by rhizosphere bacteria. *Annu. Rev. Microbiol.* **50**:101–136.
- Nwaka, S., B. Mechler, M. Destruelle, and H. Holzer. 1995. Phenotypic features of trehalase mutants in *Saccharomyces cerevisiae*. *FEBS Lett.* **360**:286–290.
- Pichereau, V., A. Hartke, and Y. Auffray. 2000. Starvation and osmotic stress induced multiresistances. Influence of extracellular compounds. *Int. J. Food Microbiol.* **55**:19–25.
- Potts, M. 1994. Desiccation tolerance of prokaryotes. *Microbiol. Rev.* **58**:755–805.
- Qu, Q. H., S. J. Lee, and W. Boos. 2004. TreT, a novel trehalose glycosyltransferase of the hyperthermophilic archaeon *Thermococcus litoralis*. *J. Biol. Chem.* **279**:47890–47897.
- Sadowsky, M. J., R. E. Tully, P. B. Cregan, and H. H. Keyser. 1987. Genetic diversity in *Bradyrhizobium japonicum* serogroup 123 and its relation to genotype-specific nodulation of soybean. *Appl. Environ. Microbiol.* **53**:2624–2630.
- Salminen, S. O., and J. G. Streeter. 1986. Enzymes of α , α -trehalose metabolism in soybean nodules. *Plant Physiol.* **81**:538–541.
- Salminen, S. O., and J. G. Streeter. 1987. Uptake and metabolism of carbohydrates by *Bradyrhizobium japonicum* bacteroids. *Plant Physiol.* **83**:535–540.
- Sambrook, J., and D. W. Russell. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schafer, A., A. Tauch, W. Jager, J. Kalinowski, G. Thierbach, and A. Puhler. 1994. Small mobilizable multipurpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145**:69–73.
- Simon, R., U. Priefer, and A. Puhler. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. *Biotechnology (NY)* **1**:784–791.
- Singer, M. A., and S. Lindquist. 1998. Multiple effects of trehalose on protein folding in vitro and in vivo. *Mol. Cell* **1**:639–648.
- Streeter, J. G. 1982. Enzymes of sucrose, maltose, and α , α -trehalose catabolism in soybean root nodules. *Planta* **155**:112–115.
- Streeter, J. G. 1985. Accumulation of α , α -trehalose by *Rhizobium* bacteria and bacteroids. *J. Bacteriol.* **164**:78–84.
- Streeter, J. G. 1987. Carbohydrate, organic acid, and amino acid composition of bacteroids and cytosol from soybean nodules. *Plant Physiol.* **85**:768–773.
- Streeter, J. G. 1994. Failure of inoculant rhizobia to overcome the dominance of indigenous strains for nodule formation. *Can. J. Microbiol.* **40**:513–522.

37. Streeter, J. G. 2003. Effect of trehalose on survival of *Bradyrhizobium japonicum* during desiccation. *J. Appl. Microbiol.* **95**:484–491.
38. Streeter, J. G., and M. L. Gomez. 2006. Three enzymes for trehalose synthesis in *Bradyrhizobium* cultured bacteria and in bacteroids from soybean nodules. *Appl. Environ. Microbiol.* **72**:4250–4255.
39. Suarez, R., A. Wong, M. Ramirez, A. Barraza, M. D. Orozco, M. A. Cevallos, M. Lara, G. Hernandez, and G. Iturriaga. 2008. Improvement of drought tolerance and grain yield in common bean by overexpressing trehalose-6-phosphate synthase in rhizobia. *Mol. Plant Microbe Interact.* **21**:958–966.
40. Ueki, T., and S. Inouye. 1998. A new sigma factor, SigD, essential for stationary phase is also required for multicellular differentiation in *Myxococcus xanthus*. *Genes Cells* **3**:371–385.
41. Vieira, J., and J. Messing. 1982. The pUC plasmids, an M13mp7 derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**:259–268.
42. Vriezen, J. A. C., F. J. de Bruijn, and K. Nusslein. 2007. Responses of rhizobia to desiccation in relation to osmotic stress, oxygen, and temperature. *Appl. Environ. Microbiol.* **73**:3451–3459.
43. Wolf, A., R. Kramer, and S. Morbach. 2003. Three pathways for trehalose metabolism in *Corynebacterium glutamicum* ATCC13032 and their significance in response to osmotic stress. *Mol. Microbiol.* **49**:1119–1134.
44. Zahran, H. H. 1999. *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol. Mol. Biol. Rev.* **63**:968–989.