Role of Trehalose Transport and Utilization in *Sinorhizobium meliloti*–Alfalfa Interactions

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Genes *thuA* and *thuB* in *Sinorhizobium meliloti* Rm1021 code for a major pathway for trehalose catabolism and are induced by trehalose but not by related structurally similar disaccharides like sucrose or maltose. *S. meliloti* strains mutated in either of these two genes were severely impaired in their ability to grow on trehalose as the sole source of carbon. *ThuA* and *ThuB* show no homology to any known enzymes in trehalose utilization. *ThuA* has similarity to proteins of unknown function in *Mesorhizobium loti*, *Agrobacterium tumefaciens*, and *Brucella melitensis*, and *ThuB* possesses homology to dehydrogenases containing the consensus motif AGKHVXCEKP. *thuAB* genes are expressed in bacteria growing on the root surface and in the infection threads but not in the symbiotic zone of the nodules. Even though *thuA* and *thuB* mutants were impaired in competitive colonization of *Medicago sativa* roots, these strains were more competitive than the wild-type Rm1021 in infecting alfalfa roots and forming nitrogen-fixing nodules. Possible reasons for their increased competitiveness are discussed.

Additional keywords: nitrogen fixation, nodule occupancy, symbiosis.

Sinorhizobium meliloti is a gram-negative bacterium that can live as a nitrogen-fixing symbiont inside root-nodule cells of alfalfa and related legumes or as a free-living saprophyte in the rhizosphere. As a free-living bacterium, *S. meliloti* lives in an environment in which both the biotic and abiotic factors are in a state of flux (Miller and Wood 1996). It responds to a variety of stresses by synthesizing and importing compounds that can serve as compatible solutes (Breedveld et al. 1993; Gouffi et al. 1999; Hoelzle and Streeter 1990a; Miller and Wood 1996). The disaccharide trehalose (α -D-glucopyranosyl-[1,1]- α -D-glucopyranoside) serves as an osmoprotectant and stress protectant in many organisms (Elbein 1974; Thevelein 1964), and it is found in high amounts in the nitrogen-fixing nodules formed by rhizobia (Fariás-Rodríguez et al. 1998; Streeter

1985). Besides serving as a stress protectant, trehalose also serves as a carbon and energy source for growth of many bacteria and fungi (Bhumiratana et al. 1974; Boos et al. 1990; Foster et al. 2003; Maréchal 1984; Rimmele and Boos 1994), including rhizobia. In soils, trehalose originates from nodules during nodule senescence (Streeter 1981) and as an excretion product from fungi (Foster et al. 2003; Gaballa et al. 1997) that accumulate trehalose as an essential storage compound in their vegetative cells and spores (Thevelein et al. 1984).

There are three known pathways for trehalose catabolism in microorganisms: i) Trehalose is hydrolyzed into two glucose moieties by the enzyme trehalase, a pathway found in *Escherichia coli*, *Bacillus subtilis*, and many other microorganisms, including fungi (Boos et al. 1987). ii) Trehalose is transported across the membranes either by a permease or by a phosphotransferase system (PTS), leaving trehalose unmodified or phosphorylated as trehalose 6-phosphate (T6P) inside the cell (Andersson et al. 2001). Further degradation of the trehalose or T6P involves hydrolyzing enzymes such as trehalase, T6P-hydrolase, phospho-(1-1)-glucosidase, or phosphotrehalase (Helfert et al. 1995; Rimmele and Boos 1994). Trehalose phosphorylase may also split trehalose by exerting a phosphate attack on the bond joining the glucose moieties (Andersson et al. 2001; Helfert et al. 1995). iii) Trehalose is taken up via the PTS system as T6P, and the enzyme trehalose-6-phosphate phosphorylase, phosphorylates the T6P and splits the molecule to yield β -glucose-1-phosphate and glucose-6-phosphate. Phosphorylated glucose residues, rather than glucose, are the products of trehalose metabolism in this pathway found in *Lactococcus lactis* (Andersson et al. 2001; Levander et al. 2001). Except for α -glucosidases, which work poorly on trehalose (Berthelot and Delmotte 1999), other enzymes involved in trehalose catabolism have not been identified unequivocally in free-living rhizobia (Hoelzle and Streeter 1990b). Trehalase, trehalose phosphorylase, and trehalose-6-phosphate hydrolase were detected in soybean nodules formed by *Bradyrhizobium japonicum* (Hoelzle and Streeter 1990b; Salminen and Streeter 1986; Streeter 1982). Orthologous gene sequences to these enzymes have not been found in the genome of *S. meliloti*.

The biological role of trehalose catabolism in prokaryotes and eukaryotes is mostly limited to the study of free-living organisms and little is known about its role in symbiotic and pathogenic interactions. In a recent study, it has been shown that trehalose metabolism plays a crucial role during early infection in rice blast disease (Foster et al. 2003). Since it is becoming increasingly evident that, during early interactions, rhizobia encounter stress, trehalose metabolism can play an important role in the physiology of both free-living and symbiotic rhizobia. Therefore, we have studied the role of trehalose in symbiotic interactions of *S. meliloti*. In this report, we present the identification of two trehalose-inducible genes that are

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Nucleotide sequence data for *thuA* and *thuB* are available in the GenBank (National Center for Biotechnology Information) database under accession number AF175299.

involved in trehalose utilization. We also show that *S. meliloti* strains that carry mutations in these genes become more competitive than wild-type Rm1021 in nodulating alfalfa, in spite of being less competitive in colonizing alfalfa roots. Some possible reasons for their increased nodulation competitiveness are discussed.

RESULTS

DNA sequence analysis of the trehalose utilization mutants.

In an earlier study on trehalose transport in *S. meliloti* 1021, we reported the isolation of a strain, Sm7023, that was unable to utilize trehalose as its sole carbon and energy source (Fig. 1A). The mutated gene was labeled *thuA* for trehalose utilization (Jensen et al. 2002). Here, we report a detailed characterization of this gene and its downstream sequences.

Computer-aided analysis of the DNA sequence surrounding the Tn5 insertion site revealed that the insertion disrupted the first of two open reading frames (ORF) transcribed in the same direction. According to the published sequence data of the *S. meliloti* genome, the *thuA* and *thuB* genes were found to be located on the pSymB megaplasmid (Finan et al. 2001). Gene *thuA* is located 190 base pairs (bp) downstream of the stop codon of *thuK* of the *thuEFGK* operon found to encode a trehalose-inducible periplasmic binding protein-dependent ABC transport system for the disaccharides trehalose, maltose, and sucrose (Jensen et al. 2002). Immediately downstream of gene *thuA* is ORF *thuB*. A nearly perfect palindromic sequence of 17 bp with three intervening base pairs, which can form the stem-loop structure expected for a prokaryotic transcriptional terminator (Platt 1986), was found downstream of ORF *thuB*. Both *thuA* and *thuB* start with an ATG initiation codon that is preceded by a potential ribosome-binding site. Comparison of the deduced 263-amino acid sequence of *thuA* revealed high sequence identity and similarity to proteins with one or more unknown functions encoded by genes mll3015 of *Mesorhizobium loti* (70% identity and 88% similarity), AG_L2958 of *Agrobacterium tumefaciens* (82% identity and 93% similarity) and BMEII0939 of *Brucella melitensis* (81% identity and 87% similarity).

Alignment of the deduced 365-amino acid sequence of *thuB* with other protein sequences in GenBank revealed high similarity and identity to the deduced proteins encoded by the *M. loti* mll3104 gene, AG_L2956 of *A. tumefaciens*, and BMEII0938 of *Brucella melitensis*, which are located downstream of genes encoding the ThA-like proteins in these organisms. In addition, identity to functional motifs in known and putative dehydrogenases, which display the characteristic dinucleotide-binding G-X-G-X-X-G or G-X-G-X-X-A motifs, was also detected. These motifs have been suggested to be part of a $\beta\alpha\beta$ -fold, enabling these proteins to bind the ADP moiety of NAD(H) or NADP(H) cofactors, respectively (Thompson et al. 1997). The highest similarity was found to a group of dehydrogenases containing the consensus motif AGKHVXCEKP, all of which are known or expected to react with substrates that are structurally similar to glucose (Wiegert et al. 1997). Among these are the myo-inositol dehydrogenases from *Bacillus subtilis* (Fujita et al. 1991) and the glucose-fructose oxidoreductase from *Zymomonas mobilis* (Wiegert et al. 1997). BMEII0938 from *Brucella melitensis* is annotated as a myo-inositol dehydrogenase.

Characterization of *thuB* function.

To test whether *thuB* also codes for a protein involved in trehalose utilization, we constructed strain Sm7024, in which the wild-type *thuB* gene is replaced with a transcriptional fusion of *thuB* to a promoterless *lacZ* gene. Sm7024 was tested for growth in M9 liquid medium containing glucose, sucrose, maltose, or trehalose. As shown in Figure 1A, Sm7024 was clearly impaired in its ability to grow on trehalose but not in its ability to grow on glucose, maltose, or sucrose. These results confirm that, along with *thuA*, *thuB* also encodes a protein that is involved in the utilization of trehalose.

Complementation of *thuA* and *thuB*.

Complementation tests of Sm7023 were performed with cosmids expressing *thuA* alone, *thuB* alone, or both *thuA* and *thuB* from the *E. coli lac* promoter. As shown in Figure 1B, cosmids containing *thuA* and *thuB* but not *thuA* or *thuB* alone,

Fig. 1. A, Growth of *Sinorhizobium meliloti* wild type and trehalose utilization mutants on various disaccharides. Shown is the growth of Rm1021 (○), Sm7023 (▲), and Sm7024 (■) in M9 medium containing various carbon sources at 0.4% (wt/vol), as indicated in the individual panels. **B,** Complementation analysis of trehalose utilization *S. meliloti* mutants Sm7023 and Sm7024, containing cosmid pRK7813 expressing *thuA*, *thuB*, or *thuA* and *thuB* from the *lac* promoter. The cells were grown in M9 medium containing trehalose 0.4% (wt/vol). Each curve represents the mean of three different growth experiments.

complemented the Sm7023 phenotype. Cosmids expressing *thuB* alone from the *lac* promoter complemented Sm7024 but not Sm7023. These results indicate that the transposon insertion in Sm7023 has a polar effect on the downstream sequences and that *thuA* and *thuB* genes together encode for a major pathway for trehalose utilization in *S. meliloti*.

Induction of *thuA* and *thuB*.

In an earlier report, we showed that the trehalose transport operon *thuREFGK* was inducible by trehalose (Jensen et al. 2002). To determine if *thuA* and *thuB* expression also was induced by trehalose, strains Sm7023 and Sm7024 were grown in M9 liquid medium containing mannitol and were transferred to medium containing trehalose or compounds having structural similarities to trehalose. Luciferase activity in Sm7023 and β -galactosidase activity in Sm7024 were measured after 4 h. Results show that trehalose alone induced the expression of the *thuA* and *thuB* gene fusions (Table 1).

Trehalose assimilation.

Transport experiments showed that Sm7023 and Sm7024 were not impaired in uptake of trehalose compared to wild type (data not shown); therefore, pulse-chase labeling experiments were carried out with ^{14}C -labeled trehalose to determine the fate of transported trehalose over time in Sm7023 and in wild type. The results, presented in Figure 2, confirm that trehalose assimilation by Rm1021 requires induction of *thuA* and *thuB* genes with trehalose and that, in mutant Sm7023, ^{14}C -labeled trehalose was metabolized at a much slower rate. No ^{14}C -labeled intermediates or end products were detectable in either in the wild type, Sm7023, or Sm7024 (data not presented), under our test conditions. That the ^{14}C compound ac-

cumulated in these mutants (Fig. 2) was unmodified trehalose was confirmed by digesting the labeled compound with *E. coli* trehalase into glucose (data not presented).

Root colonization.

The ability of Sm7024 to colonize alfalfa roots was investigated as described by Jensen and associates (2002). Root-colonization results show that, even though Sm7024 was able to colonize the roots as well as Rm1021 in a noncompetitive setup, it was significantly impaired in its ability to compete with the wild type (Table 2). Regardless of the proportion of Sm7024 to wild type in the initial inocula, the proportion of Sm7024 in the total bacteria recovered from the roots was always lower than the expected value. The difference between the expected and the observed values increased as the proportion of the wild type to mutant in the inoculum mixtures increased. Ability to use trehalose is thus critical for competitiveness in host root colonization.

Nodulation tests.

Plants inoculated with Sm7023 or Sm7024 developed normal-looking pink nodules similar to those formed on plants inoculated with the wild-type Rm1021 strain. There was no difference in the number of nodules formed per plant or the dry weight of shoots and roots per plant between the wild-type and the mutant treatments (data not presented). These results indicate that impaired trehalose utilization did not influence the ability of *S. meliloti* to infect, nodulate, or fix nitrogen. However, when in competition with their parent strain Rm1021, at saturation levels of 10^6 bacteria per root, trehalose utilization mutants Sm7023 and Sm7024 consistently occupied higher than expected proportions of the nodules formed at all inoculum combinations tested

Table 1. Induction of *thuA::luxAB* and *thuB::lacZ* gene fusions by various carbon sources in Sm7023 and Sm7024

Carbon source	<i>thuA::luxAB</i> expression ^a (Sm7023)		<i>thuB::lacZ</i> expression (Sm7024)	
	4 h/0 h ^b	Fold induction	4 h/0 h ^c	Fold induction
Cellobiose	ND ^d	ND	23/27	0.9
Glucose	1.7 $\pm$ 0.5/2.3 $\pm$ 0.6	0.7	22/23	1.0
Lactose	ND	ND	31/23	1.3
Maltose	2.1 $\pm$ 0.4/1.1 $\pm$ 0.1	1.9	21/22	1.0
Maltopentaose	ND	ND	24/28	0.9
Mannitol	2.0 $\pm$ 0.4/1.2 $\pm$ 0.1	1.7	21/25	0.8
Sucrose	ND	ND	18/24	0.8
Trehalose	7.5 $\pm$ 0.2/1.3 $\pm$ 0.4	5.8	98/25	3.9

^a Each value represents the mean for three different induction experiments $\pm$ standard error.

^b luciferase activity (relative light units/optical density at 600 nm).

^c β -galactosidase activity (Miller Units).

^d ND = not determined.

Fig. 2. Thin-layer chromatographic (TLC) analysis of the ^{14}C trehalose degradation in trehalose-induced and uninduced cultures of Sm7023 and wild-type Rm1021. Cultures were pulsed with ^{14}C trehalose for 5 min, were harvested and chased with minimal medium containing 0.4% (wt/vol) unlabeled trehalose for 1, 4, 8, 16, 36, and 60 min (lanes 1 to 6). Samples were spotted on the TLC plate, developed with butanol/ethanol/water (5:3:2), and analyzed. Standards: G, ^{14}C glucose; T, ^{14}C trehalose. TLC plates were exposed overnight in a phosphorimager system (BioRad Laboratories).

(Table 3). At nonsaturating inoculum levels of 10^2 cells per root (so that bacteria would have to competitively colonize the roots before nodulating), Sm7024 and Rm1021 appeared equally competitive (Table 4). To understand this discrepancy, in one experiment, the roots were inoculated with 100 cells and, after one week, half of the plants were harvested to estimate the relative numbers of competing strains on the root. Remaining plants were harvested after five weeks and were scored for nodulation. The results presented in Table 5 show that strain 7024 had multiplied less on the root but had formed a significantly higher than expected proportion of the nodules based on its actual numbers on the root.

To test if the increased competitiveness of strain Sm7023 and Sm7024 was a direct consequence of their inability to use trehalose, we tested the nodulation competitiveness of strain Sm7019 (Jensen et al. 2002). Unlike strains Sm7023 and Sm7024, which can import trehalose but cannot utilize it for growth, Sm7019 cells can neither import trehalose nor utilize it for growth. Strain Sm7019, formed the expected proportion of nodules at all inoculum combinations tested (Table 3). These results indicate that the accumulation of imported but unused trehalose in Sm7023 and Sm7024 may be the actual reason for their competitiveness.

Expression of *thuA* and *thuB* genes during symbiotic infections.

Since trehalose transport and utilization mutants are significantly less competitive in root colonization, trehalose may be an important source of carbon for growth on the root surface. As the *thuA* and *thuB* genes are induced specifically by trehalose, we tested the induction of this operon at various stages of symbiotic interactions to test if trehalose is available during early symbiotic interactions. Strain Sm7024, which carries a *lacZ* transcriptional fusion in *thuB* gene, was used to inoculate alfalfa roots, and plant roots were examined for *thuB* expression. Results show that the *thuB* gene was induced in bacteria

growing on the root surface (Fig. 3D), in bacteria enclosed within the root-hair curl (Fig. 3C), and in the infection threads (Fig. 3A) but not in the symbiotic zone of the nodules (Fig. 3B). These results confirm that trehalose is available for bacteria as a carbon source both on the root surface and during root-hair infections.

Competition at root-hair infection stage.

To determine if the enhanced competitiveness of trehalose utilization mutants occurred at root-hair infection stage or after infection of the root hairs, we challenged Sm7023 marked with DsRed fluorescence protein with Rm1021 marked with the green fluorescence protein (GFP) and vice versa. Alfalfa roots were inoculated with 10^5 cells containing a mixture of these two strains at 1:1 proportions, and the number of infection threads and nodules expressing DsRed and GFP on each root were scored after a minimum of 10 days. In half of these experiments, the label was switched for each strain. Among 194 infection threads observed in 26 roots, 72% of the infections were formed by Sm7023 and the rest by Rm1021.

Table 4. Competition for nodule occupancy at nonsaturating inoculum density^a

Inoculum ratio Rm1021/Sm7024	Total screened	Sm7024	
		% Observed ^b	% Expected
1:1	149	54.4*	50
1:3	148	75.0*	75
3:1	152	25.7*	25
1:9	157	98.1*	90
9:1	157	12.1*	10

^a Between wild-type *Sinorhizobium meliloti* Rm1021 and trehalose utilization mutant Sm7024 at nonsaturating inoculum density of 10^2 cells per root.

^b Chi square analysis. * indicates observed values were not statistically significant from expected values, $P > 0.05$.

Table 2. Competitive colonization of alfalfa roots by wild type *S. meliloti* Rm1021 and trehalose utilization mutant Sm7024

Strain mixture ratio (Rm1021/Sm7024)	Total cells ^b	Rm1021 cells ^b	Sm7024 cells ^b	Rm1021/Sm7024 (%) ^a	
				Expected	Observed
2:1 ^c	13.4 ± 1.76	11.9 ± 1.75	1.44 ± 0.15	33	14 ± 1.33*
1:2 ^d	18.6 ± 1.96	9.1 ± 1.04	9.5 ± 1.37	66	51 ± 3.40*
1:8	9.5 ± 3.76	2.5 ± 0.35	7.1 ± 0.29	88	75 ± 2.98*
9:1	26.6 ± 2.6	26.1 ± 2.58	0.55 ± 0.12	10	2.1 ± 0.51*
1:0	35.0 ± 2.8 ^e	NA ^f	NA	NA	NA
0:1	28.0 ± 2.3 ^e	NA	NA	NA	NA

^a Chi-square test performed with Minitap statistical software. *Observed values significantly different from expected values, $P < 0.001$.

^b Cell per root (10^5) ± S.E. Numbers followed by same letters are not statistically significant at $P > 0.05$.

^c Results average of three experiments.

^d Results average of two experiments.

^e Samples harvested 10 days after inoculation.

^f NA = not applicable.

Table 3. Competition for nodule occupancy at saturating inoculum density^a

Strain mixture	Inoculum ratio	Total nodules screened	Expected mutant nodules (%)	Observed mutant nodules (%) ^b
Rm1021 + Sm7024	1:1	147	50	93.8*
	1:3	150	75	97.7*
	3:1	150	25	55.0*
	9:1	147	10	35.7*
Rm1021 + Sm7023	1:1	150	50	96.7*
	9:1	150	10	56.0*
Rm1021 + Sm7019	1:1	150	50	56.0#
	1:9	140	90	87.1#
	9:1	150	10	10.0#

^a Between wild-type *Sinorhizobium meliloti* Rm1021 and trehalose utilization and transport mutants at saturating inoculum density of 10^6 cells per root.

^b Chi-square analysis. * indicates that observed values significantly different from expected values, $P < 0.001$; # indicates that observed values statistically not significant, $P > 0.05$.

Among the 83 nodules formed, 63 (76%) nodules were occupied by Sm7023 and 20 (24%) by Rm1021. Regardless of the fluorescent tag used, we consistently observed more infection threads and nodules occupied by Sm7023 than by Rm1021, indicating that the competitiveness of Sm7023 was determined at the root-hair infection stage.

DISCUSSION

Observations reported here provide genetic evidence for the fact that the *thuA* and *thuB* genes code for a major assimilatory pathway for trehalose in *S. meliloti*. This, however, may not be the only pathway for trehalose catabolism in Rm1021, because the mutants assimilated ¹⁴C-labeled trehalose, albeit slowly (Fig. 2). Trehalose can act as a poor substrate for α-glucosidases in *S. meliloti* (Berthelot and Delmotte 1999). Therefore, it is not clear why Sm7023 and Sm7024 are unable to utilize trehalose for growth. Perhaps α-glucosidases need to be induced by other carbon substrates before exposure to trehalose. The function of *thuA* could not be deduced, as the gene does not possess sequence similarity to any known enzymes of trehalose assimilatory pathways, and since we were unable to detect any labeled intermediates or products, the trehalose assimilatory pathway in

S. meliloti currently remains unknown. Based on the bioinformatics, we suggest that ThuB catalyzes a NADP(H)-dependent dehydrogenase reaction in utilization of trehalose. It is interesting to note that, to date, besides *S. meliloti*, most of the other microorganisms that possess genes having high overall sequence similarity to *thuA* and *thuB* also belong to the family Rhizobiaceae and associate intimately with eukaryotic systems. *M. loti* is a nitrogen-fixing symbiont of *Lotus* spp., *A. tumefaciens* is a plant pathogen, and *Brucella melitensis* is a facultative intracellular pathogen of both animals and humans. In *A. tumefaciens* and *Brucella melitensis*, organization of the whole *thuEFGK* operon and *thuA* and *thuB* is identical to that of *S. meliloti*, suggesting an identical function for these genes. Functional comparisons of genes between *Brucella melitensis* and *S. meliloti* by other workers (Campbell et al. 2002; Ferguson et al. 2002; Karbarz et al. 2003; Scupham and Triplett 1997) have shown that the genes compared were functionally homologous. In *M. loti*, however, *thuA* and *thuB* genes are located proximal to an amino acid transport operon rather than to its trehalose transport operon (Kaneko et al. 2000).

Our plant tests show that, even though the *thuA* and *thuB* genes are induced at the root surface and in the infection threads, their functions are not critical for infection, nodula-

Table 5. Competitive colonization and nodulation of alfalfa by *Sinorhizobium meliloti* Rm1021 and Sm7024 at nonsaturating inoculum concentration

Inoculum ratio (Rm1021:Sm7024)	Total ^a	Rm1021 ^a	Sm7024 ^a	Sm7024 cells per root (%)		Total screened	Sm7024 nodules (%)	
				Expected	Observed		Expected	Observed
1.5:1	11.2 ± 1.26	9.2 ± 1.26	2.0 ± 1.32	39	18 ^{*b}	140	18	57.1 [*]
1:2	24.2 ± 1.7	15.8 ± 10.7	8.3 ± 6.9	66	34 [*]	129	34	59.7 [*]
1:0	15.0 ± 0.51	NA ^c	NA	NA	NA	ND	NA	ND
0:1	12.0 ± 0.57	NA	NA	NA	NA	ND	NA	ND

^a Cells per root (10⁴). Data are means ± standard deviation
^b Chi square distribution. An * indicates observed values significantly different from the expected values, *P* < 0.001.
^c NA = not applicable. ND = not determined.

Fig. 3. Microscopic observations of *thuB::lacZ* expression in alfalfa root inoculated with Sm7024 and stained for β-galactosidase expression. **A**, *thuB::lacZ* expression in infection thread (it) leading to a nodule (n). **B**, Longitudinal section (50 μm thick) of a nodule formed by Sm7024, showing the absence of *thuB::lacZ* expression in the symbiotic zone (S) of the nodule. **C**, Sm7024 cells trapped in the curled portion of the root hair (rh). **D**, Sm7024 colony growing on the root epidermis (ep).

tion, and nitrogen fixation per se. Their functions, nevertheless, play a significant role in root colonization. That the *thuA* and *thuB* genes are induced in bacteria growing on the root surface, in the root-hair curl, and infection threads imply that trehalose is available in the root environment and in the infection threads. The source or sources of carbon available for the invading bacteria in the infection thread is currently unknown (Gage and Margolin 2000; Ma et al. 2004). Even though our results indicate that trehalose is available in the infection threads, we contend that it is not an important source of carbon for bacteria in the infection thread, because the inability of strain Sm7019 to transport and utilize trehalose did not influence its symbiotic functions or its competitiveness in any way. Both the trehalose utilization mutants and the transport mutant, on the other hand, were impaired in competitive colonization of alfalfa roots, indicating that trehalose may be an important source of carbon for saprophytic colonization of the root. More significantly and interestingly, *S. meliloti* strains disrupted in *thuA* and *thuB* functions were actually more competitive in nodule occupancy. The results of the competitive colonization experiments (Table 5) show that these strains were competitive in spite of their impaired colonization potential. Competition experiments with fluorescent-tagged Sm7023 and Rm1021 show that competitiveness was determined at the root-hair infection stage and that disruption of *thuA* and *thuB* function causes the bacteria to become more efficient in symbiotic infections. Whether in the related pathogenic bacteria, disruption of these gene functions will also lead to reduced saprophytic competence and enhanced pathogenic virulence remains to be seen. The phenotype of Sm7023 and Sm7024 suggests that it may be more than a coincidence that many bacteria that possess *thuA* and *thuB* associate with eukaryotic systems. Since strain Sm7019 did not out-compete Rm1021 in nodulation, we conclude that the increased infectivity and competitiveness of Sm7023 and Sm7024 is not due to their inability to use trehalose in planta but due to the incidental accumulation of trehalose in these cells. In plant-fungal interactions, on the other hand, ability to mobilize stored trehalose has been shown to increase the pathogenic virulence (Foster et al. 2003). One way or the other, trehalose catabolism seems to play a critical role in early interactions between plants and microorganisms.

In pathogenesis, many virulence-associated functions, including host colonization and infection-related development, require pathogens to respond to rapid changes in the environment and to cope with adverse growing conditions (Foster et al. 2003). Wright and Beattie (2004) demonstrated that *Pseudomonas syringae* pv. *tomato* cells are exposed to osmotic stress in the apoplast and that its ability to withstand osmotic stress is critical to its virulence on its host. As in pathogenesis, in root-nodule symbiosis, it is becoming increasingly evident that *S. meliloti* is exposed to osmotic, oxidative, and ethylene-related stresses during infections. We found that *S. meliloti* cells are osmotically stressed in the infection threads because, in the infection threads formed by Rm1021 containing the pPROGREEN plasmid, GFP expression was induced (data not presented). Santos and associates (2000) have demonstrated that in alfalfa infections, bacteria in the infection threads encounter stress due to reactive oxygen species generated during infections. *S. meliloti* mutants with impaired mechanism to cope with oxidative stress are unable to multiply in the infection threads, even though their ability to withstand oxidative stress in vitro is not influenced (Herouart et al. 2002). Rhizobia possess and require mechanisms to overcome ethylene-induced stress during infection (Duodu et al. 1999; Yuhashi et al. 2000). *S. meliloti* engineered to overcome the inhibitory effect of the ethylene during infections was shown to possess enhanced nodulation competitiveness (Ma et al. 2004). Accumulation of trehalose in cells has been shown to aid microorganisms in coping with oxidative stress (Benaroudj et al. 2001). It is also well-documented that trehalose accumulation in cells aids microorganisms to contend with stress due to starvation, desiccation, and heat shock. Based on all these observations, we suggest that the competitive success of Sm7023 and Sm7024 is due to the incidental accumulation of trehalose in the cells during the infection process, which enables them to contend with infection-related stresses and proliferate better than their parent strain Rm1021 during root-hair invasion stages of nodulation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media.

A list of the bacterial strains and plasmids used in this study is provided in Table 6. *E. coli* strains were grown on Luria

Table 6. Bacterial strains and plasmids used in this study^a

Strain or plasmid	Relevant characteristics	Reference or source
<i>Escherichia coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (Φ 80 <i>lacZAM15</i>) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Sambrook et al. 1989
SY327/ λ pir	λ pir, host for <i>pir</i> -dependent plasmids	Miller and Mekalanos 1988
S17-1/ λ pir	RK2 <i>tra</i> regulon, λ pir, host for <i>pir</i> -dependent plasmids	Simon et al. 1983
<i>Sinorhizobium meliloti</i>		
Rm1021	SU47 <i>str</i> -21, Str ^r	Meade et al. 1982
Sm7019	Rm1021 <i>thuE</i> ::pThuE629, Φ (<i>thuE'</i> - <i>lacZ</i>) transcriptional fusion, Str ^r , Km ^r	Jensen et al. 2002
Sm7023	Rm1021 <i>thuA</i> ::Tn5-1062, Φ (<i>thuA'</i> - <i>luxAB</i>) transcriptional fusion, Str ^r , Km ^r	Jensen et al. 2002
Sm7024	Rm1021 <i>thuB</i> ::pThuB582, Φ (<i>thuB'</i> - <i>lacZ</i>) transcriptional fusion, Str ^r , Km ^r	This study
Plasmids		
pRK7813	IncP1, broad-host-range cosmid vector, Tc ^r	Jones and Gutterson 1987
pRK2013	ColE1, Tra ⁺ , Km ^r	Ditta et al. 1980
pMB393	Broad-host-range vector, Sp ^r	Gage et al. 1996
pVIK112	<i>lacZY</i> for transcriptional fusions, Km ^r	Kalogeraki and Winans 1997
pPROGREEN	<i>P_{proli}gfp</i> fusions, Km ^r	Axtell and Beattie 2002
pDG70	XL1Blue MRF ⁺ Km ^r (ptrp driving GFP(mut1) in parent vector pHC41)	Gage 2002
pDG77	XL1Blue MRF ⁺ Km ^r (ptrp driving DsRed in parent vector pHC41)	Gage 2002
pSac7023	Approximately 20-kb SacI fragment from <i>S. meliloti</i> Sm7023 containing a <i>thuA</i> ::Tn5-1062, Km ^r	This study
pRK7813::thuA	pRK7813 clone carrying full length <i>thuA</i>	This study
pRK7813::thuB	pRK7813 clone carrying full length <i>thuB</i>	This study
pRK7813::thuAB	pRK7813 clone carrying full length <i>thuAB</i>	This study
pThuB582	pVIK112, 582-bp <i>thuB</i> internal fragment, Φ (<i>thuB'</i> - <i>lacZ</i>) transcriptional fusion, Km ^r	This study

^a Km, kanamycin; Sp, spectinomycin; Str, streptomycin; and Tc, tetracycline.

broth (LB) and *S. meliloti* Rm1021, and derivatives thereof were grown on tryptone yeast medium (TY) or M9 minimal medium with various carbon sources at 0.4% (wt/vol) or as specified. Unless otherwise stated, antibiotics were added, when appropriate, to the following final concentrations: ampicillin, 50 $\mu\text{g ml}^{-1}$; kanamycin, 50 $\mu\text{g ml}^{-1}$; streptomycin, 500 $\mu\text{g ml}^{-1}$; and tetracycline, 5 $\mu\text{g ml}^{-1}$ (Sambrook et al. 1989).

DNA sequencing.

To recover genomic sequences contiguous with the inserted transposon, DNA extracted from Sm7023 was digested with *SacI* restriction enzyme, was ligated, and was transformed into *E. coli* DH5 α by electroporation. Selection of transformants on TY kanamycin plates resulted in the isolation of plasmid pSac7023. *S. meliloti* genomic DNA present in plasmid pSac7023 was sequenced by primer-walking, using primers 5'-CTAAGCTGCCTCCATCCATG-3' and 5'-GAGGTCACATGGAATATCAG-3' for the two first sequencing reactions out of the Tn5-1062 insertion site. Nucleotide sequence was determined on both strands using *Taq* FS DNA polymerase and fluorescent dideoxy terminators in a cycle-sequencing method and an automated DNA sequencer. Sequence data analysis and alignments were done using a multialign program (Corpet 1988).

Complementation analysis.

DNA fragments containing *thuA*, *thuB*, and *thuA* and *thuB* were amplified by polymerase chain reaction (PCR), using genomic DNA of Rm1021 as template. Primers used to amplify *thuA* were 5'-TGAAGCTTCCAAATCACGAG-3' (*HindIII*, forward) and 5'-GGGAATTCATCGATATCCGG-3' (*EcoRI*, reverse). To amplify *thuB*, the primers used were 5'-GATCTAGACTGCACCGAGCC-3' (*XbaI*, forward) and 5'-CCGGATCCTTACCCCCACAG-3' (*BamHI*, reverse). The primers for *thuA* and *thuB* were 5'-TGAAGCTTCCAAATCACGAG-3' (*HindIII*, forward) and 5'-CCGGATCCTTACCCCCACAG-3' (*BamHI*, reverse), introducing the indicated restriction endonuclease recognition site. The PCR products were digested with the designated restriction enzymes, were ligated into vector pRK7813 (Jones and Gutterson 1987), and were electroporated into *E. coli* DH5 α , according to standard protocols. Plasmids pRK7813, pRK7813::*thuA*, pRK7813::*thuAB*, and pRK7813::*thuB* were mobilized into Sm7023 and Sm7024 by triparental mating (de Bruijn and Rossbach 1994), using pRK2013 (Ditta et al. 1980) as helper plasmid. Transconjugants containing pRK7813 derivatives were selected on TY plates containing either streptomycin, kanamycin (100 $\mu\text{g/ml}$), or tetracycline (5 $\mu\text{g/ml}$). Growth of transconjugants was tested in liquid M9 minimal medium containing trehalose.

Insertional inactivation of *thuB*.

For insertional mutagenesis of *thuB*, an internal *thuB* fragment of 582 bp (nucleotide +197 to +779 with respect to the *thuB* start codon) was amplified by PCR, using the plasmid pSac7023 as DNA template. Primers used in the PCR reaction were 5'-GAGAATTCGACGCGGCCACC-3' (*EcoRI*, forward) and 5'-CATCTAGATGGCCTGTGCGCC-3' (*XbaI*, reverse), introducing the indicated restriction endonuclease recognition site. The PCR product was digested with *EcoRI* and *XbaI* restriction enzymes and was ligated into vector pVIK112 (Kalogeraki and Winans 1997), resulting in plasmid pThuB582, which was electroporated into *E. coli* SY327/ λ pir, a readily transformable *E. coli* strain. pThuB582 was reisolated, was electroporated into *E. coli* S17-1/ λ pir, which has on the chromosome the *tra* region of RP4, and finally, was introduced by conjugation into *S. meliloti* Rm1021 (de Bruijn and Rossbach 1994). Transconjugants were screened by replica plating for

their ability to grow on TY or M9 minimal medium with 0.4% trehalose and appropriate antibiotics. Disruption of the *thuB* locus was confirmed by Southern blot hybridization using the internal fragment of *thuB* as a probe; the resulting strain was named Sm7024.

For plasmid DNA isolation, restriction enzyme digestion, and ligation, standard methods were used (Sambrook et al. 1989). Synthetic oligonucleotides used in this study were purchased from MedProbe (Oslo, Norway). Electroporation was performed with a BioRad Gene Pulser (Bio-Rad Laboratories, Hercules, CA, U.S.A.) according to the manufacturer's specifications.

Induction assays.

Cells were grown in 100 ml of M9 minimal medium with 0.4% mannitol at 28°C to an optical density at 600 nm (OD₆₀₀) value of between 0.2 and 0.3. The culture was divided into 10-ml aliquots in 50-ml flasks that were incubated at 28°C on a rotary shaker (150 rpm), and various carbon sources were added to a final concentration of 0.4%. At different timepoints following the addition of carbon sources, culture samples were removed and assayed for β -galactosidase activity by the method of Miller (1972). Luciferase activity was measured by removing 50- μl aliquot samples, which were added to 250 μl luciferase substrate *n*-decanal (0.1% [vol/vol] suspension in water). Thirty seconds after addition of *n*-decanal, light emission was measured over a period of 10 s in a luminometer (Bio-Orbit 1253). Protein content was estimated using the Bio-Rad protein assay (Bio-Rad Laboratories).

Pulse-chase experiment.

Cells were grown to midexponential phase in M9 medium with 0.2% mannitol alone or in the presence of 0.2% trehalose. The cells were harvested by centrifugation (3,000 $\times g$ for 10 min) at room temperature and were washed once with M9 salts without any carbon source and were adjusted to an OD₆₀₀ value of approximately 1.0 with the same medium. [¹⁴C]trehalose (1.1 GBq/mmol) was added to 1,965- μl culture samples to obtain a final concentration of 1 μM and then incubated at 25°C. After a 5-min incubation, cells were harvested and resuspended in M9 minimal medium containing 0.4% unlabeled trehalose. At different timepoints, 200- μl samples were taken out, and the cell pellet was lysed in 10% trichloroacetic acid and cleared of cell debris by centrifugation for 30 min at 13,000 $\times g$. A total of 5 μl from each sample was spotted onto a silica-coated thin-layer chromatography plate (silica gel 60, Sigma, St. Louis), and then, developed with butanol/ethanol/water (5:3:2) and analyzed using Molecular Imager (BioRad). [¹⁴C]trehalose was prepared according to the method of Horlacher and associates (1996).

Root colonization and plant nodulation tests.

Root colonization and plant nodulation tests were performed according to the methods described by Jensen and associates (2002). To determine the strain occupancy in the nodules, the root systems were sterilized with 70% ethanol for 10 s, followed by sterilization in 6% hydrogen peroxide containing a drop of Tween 20 for 10 min. The sterilized roots were washed in sterile water, and nodules were excised aseptically and crushed in 50 μl of sterile water and were screened on selective media.

Fluorescent-marked strains for infection studies.

S. meliloti Rm1021 and Sm7023 were marked with either GFP or DsRed fluorescent protein, by mating plasmids pDG 70 or pDG77 (Gage 2002), respectively, into these strains by triparental mating, as mentioned above. Strain Rm1021 expressing GFP in response to osmotic shock was constructed by

mating the plasmid pPROGREEN (Axtell and Beattie 2002) into Rm1021 by triparental mating with pRK2013 as the helper plasmid.

Root-hair infection studies and microscopy.

Microscopy was done with a Leitz epifluorescence microscope. GFP expression was recorded using the GFP filter cube with BP470/40 excitation filter and BP525/50 suppression filter. DsRed fluorescence was monitored using the excitation filter BP515-560 and suppression filter LP590. Expression of *thuB::lacZ* transcriptional fusion was monitored by staining the roots for β -galactosidase activity as described by Boivin and associates (1990).

Statistical analysis.

Statistical analysis was carried out using the MINITAB statistical program (Minitab Inc., Coventry, U.K.).

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Erratum

A correction was made to this article on October 6, 2005. The labeling in Figure 1 was incomplete.