

Redundancy in Periplasmic Binding Protein-Dependent Transport Systems for Trehalose, Sucrose, and Maltose in *Sinorhizobium meliloti*

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We have identified a cluster of six genes involved in trehalose transport and utilization (*thu*) in *Sinorhizobium meliloti*. Four of these genes, *thuE*, *-F*, *-G*, and *-K*, were found to encode components of a binding protein-dependent trehalose/maltose/sucrose ABC transporter. Their deduced gene products comprise a trehalose/maltose-binding protein (ThuE), two integral membrane proteins (ThuF and ThuG), and an ATP-binding protein (ThuK). In addition, a putative regulatory protein (ThuR) was found divergently transcribed from the *thuEFGK* operon. When the *thuE* locus was inactivated by gene replacement, the resulting *S. meliloti* strain was impaired in its ability to grow on trehalose, and a significant retardation in growth was seen on maltose as well. The wild type and the *thuE* mutant were indistinguishable for growth on glucose and sucrose. This suggested a possible overlap in function of the *thuEFGK* operon with the *aglEFGAK* operon, which was identified as a binding protein-dependent ATP-binding transport system for sucrose, maltose, and trehalose. The K_m s for trehalose transport were 8 ± 1 nM and 55 ± 5 nM in the uninduced and induced cultures, respectively. Transport and growth experiments using mutants impaired in either or both of these transport systems show that these systems form the major transport systems for trehalose, maltose, and sucrose. By using a *thuE'-lacZ* fusion, we show that *thuE* is induced only by trehalose and not by cellobiose, glucose, maltopentaose, maltose, mannitol, or sucrose, suggesting that the *thuEFGK* system is primarily targeted toward trehalose. The *aglEFGAK* operon, on the other hand, is induced primarily by sucrose and to a lesser extent by trehalose. Tests for root colonization, nodulation, and nitrogen fixation suggest that uptake of disaccharides can be critical for colonization of alfalfa roots but is not important for nodulation and nitrogen fixation per se.

The gram-negative soil bacterium *Sinorhizobium meliloti* is able to interact with the roots of *Medicago sativa* (lucerne or alfalfa) to form nitrogen-fixing nodules and survive as a free-living saprophytic bacterium in the soil. To successfully survive as a saprophyte, it must have the ability to compete with other soil microorganisms for the limited nutrient resources and to cope with different abiotic stresses, such as osmotic stress. As a soil bacterium, *S. meliloti* can be exposed to many different molecules originating from other bacteria, insects, plants, and fungi. For example, more than 400 different molecules have been identified in alfalfa that may become available to *S. meliloti* through exudation or decomposition (36). One of these compounds is the disaccharide trehalose [α -D-glucopyranosyl-(1,1)- α -D-glucopyranoside] (13, 36), a well-known osmoprotectant found in many different organisms (11). Trehalose occurs in plant tissues colonized by microorganisms, such as mycorrhizal roots (41), and in other symbiotic structures, including nitrogen-fixing nodules formed by legumes in response to rhizobial infection (13, 34). Under nonstress conditions, *S. meliloti* can take up trehalose and use it as its sole carbon and energy source (17). Under various stress conditions, *S. meliloti* synthesizes and accumulates trehalose, which can serve as a compatible solute (7, 22). In cultures grown at high osmolarity, exogenous trehalose stimulates the accumulation of intracel-

lular trehalose (43); this has been found to decrease the generation time (18).

A variety of mechanisms for trehalose transport have been documented for prokaryotes. In *Salmonella enterica* serovar Typhimurium, for example, trehalose is transported via two pathways: the mannose-specific Π^{Man} enzyme of the phosphoenolpyruvate:carbohydrate phosphoenoltransferase system (PTS) and the galactose permease pathway (37). *Escherichia coli* has two systems for trehalose uptake which are differentially used depending upon the osmolarity of the medium. Under conditions of high osmolarity, trehalose is hydrolyzed by a periplasmic trehalase to glucose, which is taken up by enzyme II of the PTS specific for glucose (5). At low osmolarity, trehalose is taken up by the use of the PTS-dependent enzyme Π^{Tre} (27). Evidence for a similar PTS has also been reported for *Vibrio parahaemolyticus* (28). In the hyperthermophilic archaeon *Thermococcus litoralis*, a gene cluster has been found that encodes a maltose/trehalose transport system (24) which has homology to the *malEFGK* cluster of *E. coli*. This cluster belongs to the family of binding protein-dependent ATP-binding cassette transport systems (BPD ABC transport systems) that normally consist of a high-affinity substrate-binding protein located in the periplasm, two hydrophobic membrane proteins, and two cytoplasmic ATP-hydrolyzing subunits (6). Willis and Walker (47) identified the operon *aglEFGAK* from *S. meliloti* which encodes a periplasmic binding protein-dependent sugar transport system involved in the uptake of the α -glucoside disaccharides sucrose, maltose, and trehalose.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain, plasmid, or transposon	Relevant characteristic(s)	Source or reference
<i>S. meliloti</i>		
Rm1021	SU47 <i>str-21</i> Str ^r	31
Sm7019	Rm1021 <i>thuE</i> ::pThuE629, Φ (<i>thuE'</i> - <i>lacZ</i>) transcriptional fusion, Str ^r Km ^r	This work
Sm7023	Rm1021 <i>thuA</i> ::Tn5-1062, Φ (<i>thuA'</i> - <i>luxAB</i>) transcriptional fusion, Str ^r Km ^r	This work
Sm7025	Rm9628 <i>thuE</i> ::pThuE690 Str ^r Km ^r Tet ^r	This work
Rm9628	Rm1021 <i>aglE192</i> ::Tn5 Str ^r Km ^r	47
<i>E. coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	
S17-1/ λ <i>pir</i>	RK2 <i>tra</i> regulon, λ <i>pir</i> , host for <i>pir</i> -dependent plasmids	42
S327/ λ <i>pir</i>	λ <i>pir</i> , host for <i>pir</i> -dependent plasmids	33
Plasmids		
pRL1062	Bearing Tn5-1062, <i>oriT</i> (RK2) Km ^r	48
pRK2013	ColE1 Tra ⁺ Km ^r	9
pApa7023	\approx 20-kb <i>ApaI</i> fragment from <i>S. meliloti</i> Sm7023 containing a <i>thuA</i> ::Tn5-1062, Km ^r	This work
pVIK112	<i>lacZY</i> for transcriptional fusions, Km ^r	25
pThuE629	pVIK112, 629-bp <i>thuE</i> internal fragment, Φ (<i>thuE'</i> - <i>lacZ</i>) transcriptional fusion, Km ^r	This work
pThuE690	pSUP205, 690-bp <i>thuE</i> internal fragment, Tet ^r	This work
pSUP205	<i>mob</i> λ <i>cos</i> Cm ^r Tet ^r	42
Transposon		
Tn5-1062	Tn5 derivative, promoterless <i>luxAB</i> , <i>oriV</i> Km ^r	48

However, since the *agl* mutants were able to utilize these disaccharides, the existence of at least one additional transport system for α -glucosides in *S. meliloti* was suggested (47).

In the genome of *S. meliloti* there are a predicted 430 ABC transport systems and over half are found on the megaplasmid pSymB (12). In this report, we demonstrate that an operon of four genes located in pSymB, called *thuEFGK*, encoding proteins belonging to the superfamily of BPD ABC transporters, is the second system suggested by Willis and Walker (47). By mutational analysis and transport and growth studies, we show that the *thuEFGK* operon is also involved in transport of trehalose, maltose, and sucrose. Despite their substrate overlap for these disaccharides, *thuEFGK* is induced only by trehalose, whereas *algEFGAK* is induced by sucrose and presumably also by trehalose. Furthermore, we show here that an *S. meliloti* mutant of these two transport systems is severely impaired in its ability to colonize alfalfa roots, whereas nodulation and nitrogen fixation are not affected.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains and plasmids used in this study are presented in Table 1. *E. coli* strains DH5 α , S17-1/ λ *pir*, and S327/ λ *pir* were grown in Luria broth (39). *S. meliloti* strain Rm1021 and *S. meliloti* strain Rm9628 were grown in tryptone yeast (TY) medium or M9 minimal medium with an appropriate carbon source (39). Unless otherwise stated, antibiotics were added, when appropriate, at the following final concentrations: kanamycin, 50 μ g/ml; streptomycin, 500 μ g/ml; tetracycline, 5 μ g/ml.

Transposon mutagenesis. Mutagenesis of *S. meliloti* Rm1021 was performed using pRL1062. pRK2013 was used as helper plasmid and triparental matings were performed according to the method of de Bruijn and Rossbach (8).

DNA sequencing. To recover genomic sequences contiguous with the inserted transposon, DNA extracted from Sm7023 was digested with *ApaI* restriction enzyme (which does not cut within the transposon), ligated, and transformed into DH5 α by electroporation (48). Selection of transformants on TY-kanamycin plates resulted in the isolation of plasmid pApa7023, which carried a 6.1-kb genomic *S. meliloti* DNA and was sequenced by primer walking from the Tn5 insertion using the primer 5'-CTAAGCTGCCTCCATCCATG-3' for the first sequencing reaction. The entire nucleotide sequence was determined on both strands by using *Taq* FS DNA polymerase and fluorescent dideoxy terminators in

a cycle sequencing method, and the resultant DNA fragments were separated by electrophoresis and analyzed using an automated Applied Biosystems 377 DNA Sequencer.

Sequence data analysis. Putative open reading frames (ORFs) and translation products were determined using the programs MacVector 5.0.1 (Eastman Kodak) and CodonUse 3.1 (Conrad Halling, Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, Ill.). Protein searches were run with the program BLASTP (1) on the National Center for Biotechnology Information server (Bethesda, Md.). Sequence alignments were done using the program ClustalX (44).

Insertional inactivation of *thuE*. For insertional mutagenesis of *thuE* in *S. meliloti* Rm1021, an internal *thuE* fragment of 629 bp (from bp +301 to +929 of the *thuE* ORF) was amplified by PCR using plasmid pApa7023 as a template. Primers used in the PCR were 5'-TGGAAATTCGTCGACCTGACC-3' (*EcoRI*; forward) and 5'-GATCTAGACCGCGAGGTTC-3' (*XbaI*; reverse), introducing the indicated (underlined) restriction endonuclease recognition sites. The PCR product was digested with *EcoRI* and *XbaI* restriction enzymes and cloned into vector pVIK112 (25), resulting in plasmid pThuE629, which was electroporated into *E. coli* SY327/ λ *pir*, a readily transformable *E. coli* strain. pThuE629 was reisolated, electroporated into *E. coli* S17-1/ λ *pir*, which has on the chromosome the *tra* region of RP4, and finally introduced by conjugation into *S. meliloti* strain Rm1021 (8). For insertional mutagenesis of *thuE* in *S. meliloti* strain Rm9628, an internal *thuE* fragment of 690 bp (from bp +301 to +990 of the *thuE* ORF) was amplified by PCR using plasmid pApa7023 as a template. Primers used in the PCR were 5'-TGGAAATTCGTCGACCTGACC-3' (*EcoRI*; forward) and 5'-ATGAATTCGGCCGAGCCCAG-3' (*EcoRI*; reverse), introducing the indicated restriction endonuclease recognition sites. The PCR product was digested with *EcoRI* restriction enzyme and cloned into vector pSUP205 (42), resulting in plasmid pThuE690, which was electroporated into *E. coli* DH5 α and finally introduced by conjugation into *S. meliloti* strain Rm9628. Transconjugants were screened by replica plating for their ability to grow on TY or M9 minimal medium with 0.4% (wt/vol) trehalose and containing appropriate antibiotics. Disruption of the *thuE* locus was confirmed by Southern blot hybridization using the internal fragment of *thuE* as a probe; the resulting strains were called Sm7019 and Sm7025, respectively.

For plasmid DNA isolation, restriction enzyme digestion, and ligation, standard methods were used as previously described (39). All synthetic oligonucleotides used in this study were purchased from MedProbe (Oslo, Norway). Electroporation was performed with a Bio-Rad Gene Pulser according to the manufacturer's specifications.

Disaccharide uptake assay. Cells were grown to exponential phase in M9 minimal medium with 0.2% (wt/vol) mannitol in the presence of 0.2% (wt/vol) trehalose, 0.2% (wt/vol) maltose, or 0.2% (wt/vol) sucrose. The cells were har-

vested by centrifugation ($3,000 \times g$ for 10 min) at room temperature and washed three times with M9 salts without any carbon source and adjusted with the same medium to a turbidity at 600 nm of 0.2. [^{14}C]trehalose (1.1 GBq/mmol), [^{14}C]maltose (2.3 GBq/mmol), or [^{14}C]sucrose (2.1 GBq/mmol) was added to a final concentration of 1 μM and the suspension was incubated at 25°C. At various time points after addition, 110- μl aliquot samples were removed and filtered through 0.45- μm -pore-size filters (Millipore) under vacuum. The filters were washed with 5 ml of M9 salts and transferred to scintillation vials containing 4 ml of Ultima Gold scintillation liquid (Packard). The radioactivity trapped in the cells was measured with a scintillation counter (2300TR; Packard Instruments Co., Meriden, Conn.). [^{14}C]maltose and [^{14}C]sucrose were purchased from Amersham-Pharmacia (Little Chalfont, Buckinghamshire, England), whereas [^{14}C]trehalose was prepared according to the method of Horlacher et al. (23). The protein content of the cultures was estimated using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif.) with bovine serum albumin as the standard. Uptake rates were calculated for the first 4 min, during which time uptake was found to be linear.

Competition experiment. Mid-log-phase cells were harvested as described above, washed three times with M9 salts without any carbon source, and resuspended in the same medium to an optical density at 600 nm (OD_{600}) of approximately 0.2. Aliquots of the washed cells (650 μl) were transferred to 1.5-ml microcentrifuge tubes containing M9 medium with various carbon sources (see Table 2) at a final concentration of 100 μM along with [^{14}C]trehalose (1.1 GBq/mmol) or [^{14}C]sucrose (2.1 GBq/mmol) at a final concentration of 1 μM . At 2 min after addition, 110- μl aliquot samples were removed and treated as described above.

Induction assay. Cells were grown in 100 ml of M9 minimal medium with 0.4% (wt/vol) mannitol at 28°C to an OD_{600} of between 0.2 and 0.3. The culture was divided into 10-ml aliquots in 50-ml flasks and incubated at 28°C on a rotary shaker (150 rpm), and various carbon sources were added to a final concentration of 0.4% (wt/vol). At different time points following the addition of carbon sources, samples were removed and assayed for β -galactosidase activity by the method of Miller (32).

Plant nodulation tests. Nodulation assays were performed in Leonard jar assemblies containing sterile vermiculite and nitrogen-free Hoagland's medium (45). Five *M. sativa* (Moapa 69) seedlings were planted in each jar. Each treatment had three replicates, with three jars per replicate. Plants were grown with photosynthetically active photon (400 to 700 nm) flux density of 250 $\text{mol m}^{-2} \text{s}^{-1}$ using a 12-h photoperiod at 25 and 20°C and 30 to 40% relative humidity. Nodulation was assessed 5 weeks after planting. A random sampling of nodules from each jar was checked for *S. meliloti* identity on TY extract plates with appropriate antibiotic selections. Symbiotic nitrogen fixation was assessed by dry weight determination. Roots and shoots were harvested separately from each jar and dried to a constant weight in an oven set at 75°C.

Root colonization test. Root colonization assays were performed in Mejanta jars (Sigma Chemical Company) containing washed sterile vermiculite saturated with nitrogen-free Hoagland's medium (45). Vermiculite was washed two times with tap water and soaked overnight in nitrogen-free Hoagland's medium and used to fill the jars after the excess liquid was decanted. Five sterile alfalfa seedlings (48 h old) were planted in each jar and grown as described above. Each treatment had three replicates with three jars per replicate. The plants were inoculated at the time of planting with 100 to 200 bacterial cells per plant. The inoculum was prepared from early-stationary-phase cultures of *S. meliloti* grown in Vincent's medium (45) with glucose as the carbon source. Cells were harvested, washed once with sterile water, and suspended in sterile water diluted to contain approximately 10^3 cells/ml, and 100 μl was used to inoculate each plant. Roots were harvested 1 week after inoculation under sterile conditions and sonicated for 10 min in ice-cold buffer (0.05 M phosphate [pH 7.2]) containing 0.01% (wt/vol) Tween and vortexed for 30 s at maximum speed. Appropriate dilutions of these suspensions were plated onto selective media. All colonization experiments were repeated at least twice.

Statistical analysis. Statistical analysis of the root colonization data was done using statistical software from Minitab Inc. Chi-square tests were performed to assess the significance between the observed and expected ratios of wild type to mutant cells in the harvested suspensions (46).

Nucleotide sequence accession number. Sequence data for *thuR*, *thuEFGK*, and *thuA* have been deposited in the GenBank (National Center for Biotechnology Information) database under accession no. AF175299.

RESULTS

Identification of trehalose transport genes. To isolate trehalose transport genes, transposon mutagenesis was performed using a derivative of Tn5 containing promoterless *luxAB* genes (48). Approximately 5,000 *S. meliloti* Rm1021 mutants were tested for their ability to grow on trehalose as sole carbon and energy source. One Tn5 insertion mutant, Sm7023, was isolated which was severely reduced in its ability to utilize trehalose as its sole carbon and energy source. Sm7023 was tested for its ability to utilize other carbon sources and was found to grow as well as the wild type in sucrose, maltose, glucose, succinate, and mannitol (data not presented). Computer-aided analysis of the DNA sequences surrounding this Tn5 insertion site revealed that the insertion disrupted an ORF. Comparison of the deduced amino acid sequence encoded by this ORF revealed high sequence identity (70%) and similarity (88%) to a protein with unknown function encoded by the *mll3015* gene of *Mesorhizobium loti* (26). Since the phenotype of the mutant Sm7023 suggested that this gene is involved in trehalose utilization, we named the mutated ORF *thuA*. Upstream of the Tn5 insertion site, four ORFs that have strong homology to BDP ABC transport systems and a putative regulatory protein were detected (Fig. 1). Because of the similarity to trehalose transport genes of *T. litoralis* and the proximity to the gene *thuA* required for trehalose utilization, we have given this operon the provisional name *thu* for trehalose utilization. According to the published sequence data of the *S. meliloti* genome, the *thu* locus was found to be located on the pSymB megaplasmid (12).

The predicted amino acid sequence of another ORF exhibits similarity to various solute-binding protein components of BPD ABC transport systems. In particular, the putative protein has 37% identity and 71% similarity with the MalE trehalose/maltose-binding protein of *T. litoralis* (24). After the convention established for *E. coli* and *T. litoralis* maltose/trehalose uptake systems, we designate this ORF as *thuE*.

Downstream from *thuE* are two ORFs, *thuF* and *thuG*, whose protein products have a high degree of similarity to hydrophobic transmembrane components in the BPD ABC transport systems. Hydropathic profiles, determined according to the method of Kyte and Doolittle (29), and analysis for predicted transmembrane helices (3) suggest that both *ThuF* and *ThuG* contain six hydrophobic regions, corresponding to six membrane-spanning α -helical segments found in the inner membrane proteins of BPD ABC transport systems (6, 20). In addition to these six hydrophobic regions, the highly conserved sequence motif termed the EAA loop (EAA-X₃-G-X₉-I-X-LP), present in all inner membrane proteins from BPD ABC transport systems (6), was identified in *ThuF* and *ThuG* with minor variations. Downstream from *thuG* is *thuK*, which encodes a polypeptide whose predicted amino acid sequence reveals similarity to various ATP-binding proteins known to be components that energize the ABC transport systems (2, 21). Similarities between these proteins include conserved regions known as the Walker motif A (consensus G-X₂-G-X-G-K-S/T), Walker motif B (consensus hhhh-D-E-P, where h stands for hydrophobic), and the linker peptide (consensus L-S-G-G-Q-Q/R/K-Q-R) that is unique to the ABC transport family (6, 40). An ORF upstream of *thuE*, designated *thuR*, extends in

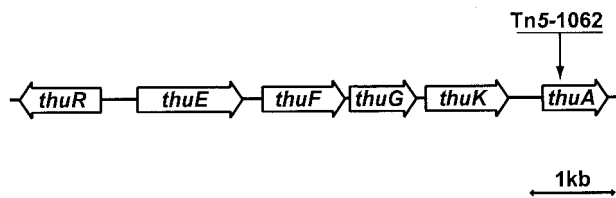


FIG. 1. Gene map of the trehalose utilization region of *S. meliloti* Rm1021. The insertion site of transposon Tn5-1062 in mutant Sm7023 is indicated.

the opposite orientation from a GTG initiation codon (Fig. 1). The putative translation product of *thuR* is a polypeptide with an N-terminal sequence (KLKEFAKOLGLSPTTVS) that contains a helix-turn-helix motif characteristic of many DNA-binding proteins. Alignment of ThuR with other protein sequences present in GenBank revealed high homology to many of the known and putative transcriptional regulators involved in carbohydrate utilization which belong to the LacI-GalR family of regulatory proteins (35). The highest homology was found with the *S. meliloti* AlgR protein (49% identity and 78% similarity), a putative repressor involved in the regulation of the *aglEFGAK* operon encoding an α -glucosidase and a periplasmic BPD ABC transport system for α -glucosides (47).

***thuEFGK* is involved in trehalose, maltose, and sucrose uptake.** Due to the similarity between ThuE and the high-affinity trehalose/maltose transport system found in *T. litoralis*, we analyzed the parameters of trehalose transport in the wild-type strain, Rm1021. It was grown in M9 medium containing either mannitol or mannitol and trehalose as carbon sources. [14 C]trehalose uptake was measured at concentrations ranging from 0.01 to 0.5 μ M. Rm1021 had K_m values of 8 ± 1 and 55 ± 5 nM in the uninduced and trehalose-induced cultures, respectively. The V_{max} s for trehalose transport were 1.0 ± 0.1 and 15 ± 0.1 nmol/min/mg of protein in these cultures. K_m values in the submicromolar range confirmed that Rm1021 has a high-affinity transport system for trehalose.

To test if *thuE* is involved in the uptake of trehalose and/or maltose in *S. meliloti*, we disrupted the *thuE* locus by creating a transcriptional fusion to the *E. coli lacZ*. The resultant strain, Sm7019, and the wild-type strain Rm1021 were tested for their ability to utilize trehalose as sole carbon and energy source. They were grown in M9 liquid medium containing 0.4% (wt/vol) glucose, sucrose, maltose, or trehalose. As shown in Fig. 2, the wild type and Sm7019 had indistinguishable growth curves on glucose. However, the mutant was clearly impaired in its ability to grow with trehalose as carbon source. Growth on maltose was also impaired, albeit to a lesser extent. No significant growth retardation was observed in sucrose. These results indicate that *thuE* encodes a protein that is involved in utilization of trehalose and maltose.

Uptake experiments were carried out with [14 C]trehalose or [14 C]maltose with *S. meliloti* Rm1021 and Sm7019 that had been grown in M9 minimal medium containing mannitol and various carbon sources. Because Sm7019 does not grow well on trehalose, Sm7019 and wild-type strains were cultured on mannitol in order to measure basal transport rates. Basal transport rates of trehalose and maltose for the wild type and Sm7019 were found to be the same when these strains were grown in

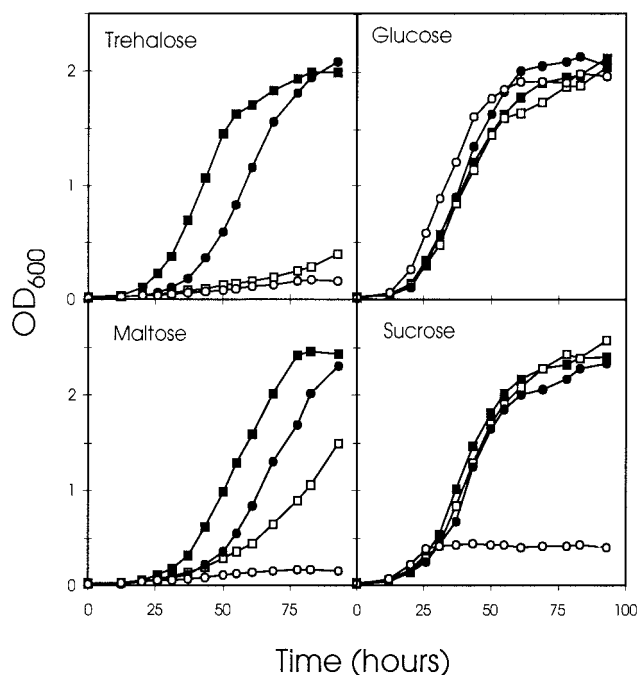


FIG. 2. Growth of *S. meliloti* wild type and transport mutants on various disaccharides. Shown is the growth of Sm7019 (\square), Sm7025 (\circ), Rm9628 (\bullet), and Rm1021 (\blacksquare) in M9 medium containing various carbon sources at 0.4% (wt/vol), as indicated in the individual panels.

minimal medium (Fig. 3A and B). When grown in minimal medium plus maltose, both the wild type and Sm7019 mutant showed a slightly enhanced rate of transport for both trehalose and maltose compared to cells grown on mannitol alone (Fig. 3A and B). However, the most significant change in transport rate was observed in the wild-type strain when it was grown in minimal medium containing mannitol plus trehalose. Uptake of both trehalose and maltose was enhanced eightfold in these treatments (Fig. 3A and B). The Sm7019 cultures grown in mannitol plus trehalose did not show this eightfold enhancement, suggesting that the *thuEFGK* operon is indeed responsible for this enhanced transport of both trehalose and maltose in Rm1021.

Due to earlier findings suggesting that sucrose, maltose, and trehalose share a common uptake system in *S. meliloti* strains (17, 47), we wished to test the involvement of *thuE* in the uptake of sucrose. Uptake experiments were carried out with [14 C]sucrose with *S. meliloti* Rm1021 and Sm7019 cells that had been grown in M9 media containing mannitol alone or with maltose, trehalose, or sucrose in addition to mannitol. Both the wild type and Sm7019 took up sucrose at a basal rate when grown on medium containing mannitol alone. Maltose enhanced the rate of sucrose transport twofold over the uninduced basal level (Fig. 3C). The presence of sucrose in the medium, however, resulted in a sixfold induction of sucrose uptake in the wild type and fourfold induction in the *thuE* mutant (Fig. 3C). Significant differences were also apparent when the *thuE* mutant and wild-type strains were grown in medium containing mannitol and trehalose (Fig. 3C). In Sm7019 cultures, the sucrose transport rate was comparable to the uninduced basal rate, while in the wild-type strain the

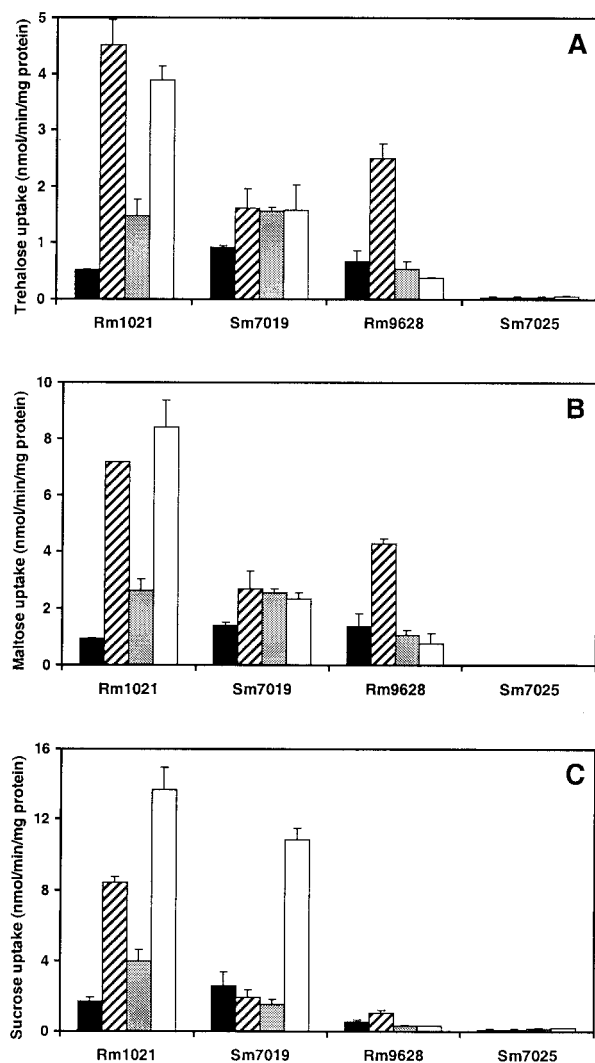


FIG. 3. Uptake of [^{14}C]trehalose, [^{14}C]maltose, or [^{14}C]sucrose by Rm1021, Sm7019, Rm9628, and Sm7025. Cultures were grown on mannitol plus trehalose (hatched), mannitol plus maltose (stippled), mannitol plus sucrose (white), or mannitol only (black). Each uptake value represents the mean for three different uptake experiments \pm standard error.

presence of trehalose induced sucrose transport to up to 3.5 times over the basal rate (Fig. 3C). These results suggest that the *thuEFGK* operon may be involved in sucrose uptake as well as in the uptake of trehalose and maltose.

Sucrose-inducible trehalose uptake system. The eightfold increase in maltose and trehalose uptake in cells cultured in the presence of sucrose indicates the presence of a sucrose-inducible uptake system for trehalose, maltose, and sucrose. Genes encoding such a sucrose-inducible uptake system could be the *aglEFGAK* operon from *S. meliloti*, shown by Willis and Walker (47) to be involved in uptake of the α -glucoside disaccharides sucrose, maltose, and trehalose. To test this hypothesis, uptake experiments with [^{14}C]trehalose or [^{14}C]maltose were carried out with *S. meliloti* Rm9628, a strain containing a mutation in the *aglE* gene that encodes the binding protein of the *AgIEFGAK* transport system (47). Uptake experiments

showed that Rm9628 cells grown in a trehalose-containing medium had increased rates of uptake of trehalose and maltose (four- and threefold, respectively) compared to the basal uninduced level (Fig. 3A and B). In contrast, only a twofold increase in the uptake of [^{14}C]sucrose was seen in Rm9628 cells that had been grown on trehalose. No increase was apparent in maltose- or sucrose-containing media (Fig. 3C). These results indicate that even though the *thuEFGK* operon is involved in sucrose uptake, *aglEFGAK* is the operon which codes for the major transport system for sucrose. The results of the uptake experiments from Rm9628 and Sm7019 also indicate that sucrose and trehalose are the primary inducers of the *aglEFGAK* and *thuEFGK* operons, respectively. The fivefold increase in sucrose uptake by Rm1021 grown in trehalose-containing media suggests that trehalose may also induce the *agl* operon, albeit rather less efficiently (compare sucrose uptake between Rm1021 and Rm9628 grown in trehalose medium in Fig. 3C).

In order to test if there were additional transport systems for trehalose, maltose, and sucrose uptake in *S. meliloti*, we constructed an *S. meliloti* Rm9628 strain disrupted in the *thuE* locus as well. As expected, the resultant double mutant, Sm7025, was more strongly impaired in its ability to import these three disaccharides than was its parent strain, Rm9628, or the *thuE* mutant Sm7019 (Fig. 3). Regardless of the carbon sources supplied, the quantities of trehalose and maltose imported by Sm7025 amounted to less than 10% of the amounts imported by the wild type, its parent strain Rm9628, or the *thuE* mutant Sm7019 cultured under similar conditions. Sucrose import by Sm7025 was reduced to 25, 12, 55, and 60% of the amount imported by Rm9628 cells grown in mannitol or mannitol and trehalose, maltose, and sucrose, respectively (Fig. 3C). These results confirm that *thuEFGK* and *aglEFGAK* together code for the two major transport systems in *S. meliloti* involved in the uptake of the disaccharides trehalose, maltose, and sucrose.

Inhibition of trehalose and sucrose uptake by other disaccharides. The fact that trehalose induces both its own uptake, the uptake of maltose, and to a lesser extent uptake of sucrose suggests that these two uptake systems have an overlapping specificity for uptake and induction. Glenn et al. (16) had shown in *S. meliloti* strain WU60 that sucrose, maltose, and trehalose shared a common uptake system, but we wished to confirm this finding in *S. meliloti* Rm1021. To determine if trehalose, maltose, and maybe other structurally related disaccharides use a common uptake system, inhibition experiments were carried out with cultures of Rm1021, Rm9628, and Sm7019 grown in M9 minimal medium containing mannitol and sucrose or trehalose. Mid-log-phase cells were assayed for [^{14}C]trehalose or [^{14}C]sucrose uptake in the presence of a 100-fold excess of unlabeled competitor. As shown in Table 2, the disaccharides cellobiose, sucrose, and maltose acted as strong inhibitors of [^{14}C]trehalose uptake, and cellobiose, trehalose, and maltose inhibited [^{14}C]sucrose uptake when Sm7019 and wild-type Rm1021 were grown in medium containing sucrose to induce the *aglEFGAK* operon. Lactose and the monosaccharides mannitol and glucose did not exhibit such strong inhibitory effects. These results suggest that *AgIEFGAK* transports cellobiose, sucrose, maltose, and trehalose. When Rm1021 and Rm9628 were grown in media containing treha-

lose to induce the *thuEFGK* operon, [¹⁴C]trehalose uptake was strongly inhibited by the disaccharide maltose but not by the disaccharides cellobiose and sucrose. The monosaccharide glucose also inhibited trehalose uptake to a lesser extent (Table 2). These results indicate that the *ThuEFGK* system is involved primarily in the uptake of trehalose and maltose but not of sucrose or cellobiose.

The uptake of sucrose in cultures in which the *thuEFGK* operon was induced, on the other hand, presented a more complex picture. [¹⁴C]sucrose uptake by wild-type strain Rm1021 cultured in medium containing trehalose was inhibited >90% by maltose and trehalose and 65% by cellobiose (Table 2). This inhibition pattern suggests that both the *agl* and *thu* uptake systems are induced in these cultures. Uptake of [¹⁴C]sucrose by Rm9628 cultured in the presence of trehalose was inhibited only by maltose and trehalose, and not by cellobiose. These observations confirm that the *AglEFGAK* system is involved in the uptake of cellobiose. That strain Rm9628 had a much longer lag phase (>24 h) than Sm7019 and Rm1021 (<13 h) when cultured in the presence of cellobiose as the only source of carbon further supports this conclusion.

Growth tests. When testing the Sm7025 strain for its ability to utilize trehalose, maltose, or sucrose as sole carbon and energy source, it was found that the mutant showed almost no growth on trehalose and maltose and sucrose (Fig. 2). These results confirm that, even though the *ThuE* system is not primarily involved in sucrose transport, *ThuEFGK* may be the alternative transport system suggested by Willis and Walker (47) for sucrose uptake.

Induction of *thuE* operon. Since expression of a binding protein in *S. meliloti* may be regulated by its substrate (4, 30), we tested whether *thuE* expression is induced by trehalose and/or other sugars. Induction experiments were carried out with strain Sm7019 grown in M9 liquid medium containing 0.4% (wt/vol) mannitol to which trehalose, sucrose, or maltose was added to a final concentration of 0.4% (wt/vol). We assumed that residual uptake of the disaccharides in strain Sm7019 (Fig. 3) would allow for induction of the *thuEFGK* operon. β -Galactosidase activity was measured over time. In Fig. 4, it is shown that 1 h after transfer to trehalose-containing medium, *thuE'-lacZ* gene expression increased, whereas bacteria in the maltose- or sucrose-containing medium did not

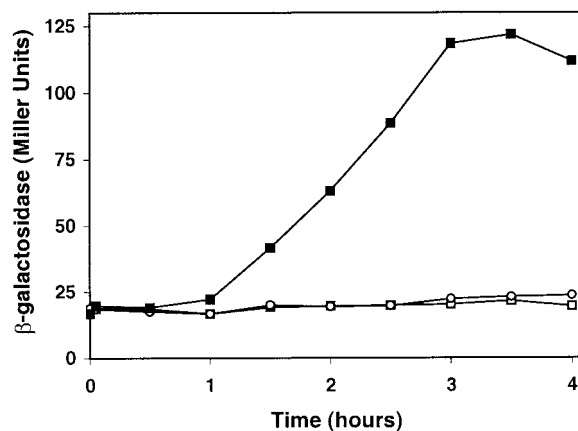


FIG. 4. Induction of *thuE'-lacZ* gene expression by trehalose (■), maltose (□), and sucrose (○) in *S. meliloti* Sm7019.

show any increased expression of β -galactosidase activity even after 4 h of incubation. To study the specificity of induction of this operon, similar experiments were carried out with Sm7019 cells exposed to compounds having structural similarities to trehalose. Among the nine different sugar compounds tested, only trehalose and lactose induced *lacZ* expression after the 4-h incubation period (by a factor of eight and two, respectively). Cellobiose, sucrose, glucose, lactose, maltopentaose, maltose, and mannitol failed to induce this operon (results not shown).

Effects of transport mutations on plant nodulation. The symbiotic phenotypes of the mutant Sm7019 and the *thuE algE* double mutant were tested on alfalfa plants. The interaction resulted in the formation of pink normal-looking nodules similar to nodules seen on plants inoculated with the wild-type Rm1021 strain. The numbers of nodules formed per plant and plant dry weight were also not significantly different between the mutants and the wild type (data not presented). These nodulation tests indicate that the *thuEFGK* and *aglEFGAK* uptake systems are not critical for nodule development or symbiotic nitrogen fixation in alfalfa. However, these results do not rule out a role for sucrose and trehalose transport in competitive nodulation.

TABLE 2. Effects of various competitors on the uptake of [¹⁴C]trehalose and [¹⁴C]sucrose in *S. meliloti* strains Rm1021, Sm7019, and Rm9628

Competitor	Inhibition (%) of [¹⁴ C]trehalose uptake ^a				Inhibition (%) of [¹⁴ C]sucrose uptake ^a			
	Sucrose-induced		Trehalose-induced		Sucrose-induced		Trehalose-induced	
	Sm7019	Rm1021	Rm9628	Rm1021	Sm7019	Rm1021	Rm9628	Rm1021
None	0	0	0	0	0	0	0	0
Cellobiose	89	70	0	17	84	91	5	65
Glucose	13	18	55	33	39	54	79	68
Lactose	4	0	10	4	9	18	0	23
Maltose	91	90	88	89	84	93	82	94
Mannitol	0	2	11	9	15	0	14	6
Sucrose	81	72	12	26	0	0	0	0
Trehalose	0	0	0	0	81	95	82	95

^a The results are expressed as mean percent inhibition of trehalose or sucrose uptake, and each value represents the mean for three different experiments. The transport rates with 1 μ M concentrations of radiolabeled sugars served as control values. For competition studies, *S. meliloti* cultures were mixed with a 100-fold excess of the unlabeled sugar (100 μ M) plus [¹⁴C]trehalose (1 μ M) or [¹⁴C]sucrose (1 μ M).

TABLE 3. Colonization of alfalfa root by *S. meliloti* strains^a

Strain mixture	Mixture ratio (WT/MU)	Total cells per root (10 ³) ^b	Rm1021 cells per root (10 ³)	Mutant cells per root (10 ³)	WT/MU (% ratio) ^c	
					Expected	Observed
Rm1021 + Sm7019	1:0	44.8 ± 8.0B	NA	NA		
	0:1	35.6 ± 8.3B	NA	NA		
	1:1	29.8 ± 5.1B	20.9 ± 4.5	7.8 ± 1.6	50:50	68:32*
Rm1021 + Rm9628	1:0	38.6 ± 2.2B	NA	NA		
	0:1	64.4 ± 5.6C	NA	NA		
	1:1	70.0 ± 9.8C	43.8 ± 7.5	26.2 ± 3.3	50:50	60:40*
Rm1021 + Sm7025	1:0	23.6 ± 2.0A	NA	NA		
	0:1	22.3 ± 3.5A	NA	NA		
	1:1	36.1 ± 6.2B	28.1 ± 4.7	7.7 ± 2.0	50:50	84:16*

^a WT, wild-type Rm1021; MU, mutant strain; NA, not applicable.

^b Treatments followed by the same letter were not statistically significant at the 95% confidence level. Data are means ± standard errors.

^c Chi-square test performed with Minitab statistical software; *, $P = <0.001$.

Effects of transport mutations on root colonization. Since disaccharides have been shown to be a component of alfalfa root exudate (36), we compared the abilities of mutants Sm7019 and Rm9628 and double mutant Sm7025 to compete with the wild-type Rm1021 for growth on alfalfa root. In spite of considerable scatter in the total number of bacteria recovered from the roots between experiments, the results show (Table 3) that Sm7019, Rm9628, and Sm7025 were able to colonize alfalfa root as well as Rm1021 was when they were not competed against the wild type. In treatments where they were competed with Rm1021, Sm7019 and Rm9628 were significantly impaired in their ability to compete with the wild type, and Sm7025 was even more impaired than Sm7019 and Rm9628 in its ability to compete with the wild type (Table 3). These results suggest that disaccharides may be an important source of energy for *S. meliloti* growth on alfalfa roots.

DISCUSSION

In this paper, we report the identification of an operon in *S. meliloti* strain Rm1021 that is composed of a group of four genes, *thuEFGK*, which encodes a BDP ABC transport system for the disaccharides trehalose, sucrose, and maltose and is inducible by trehalose. To our knowledge this is the first report of a trehalose-inducible transport system for trehalose in *S. meliloti*. Willis and Walker (47) reported the presence of another operon, *aglEFGAK*, for uptake of the disaccharides trehalose, sucrose, and maltose. In spite of the fact that there are two transport systems for trehalose transport, our observations indicate that the *thuE* operon must be functional for optimal growth in trehalose. When the *aglEFGAK* operon alone is active, growth is significantly delayed and reduced, and no growth was observed when both operons were disrupted (Fig. 2). Our results show that the *thuEFGK* operon is involved primarily in the uptake of trehalose and maltose and only to a much lesser extent in sucrose uptake. For example, more than 95% of the total sucrose uptake in *S. meliloti* Rm1021 can be accounted for by the *aglEFGAK* system and only less than 5% of the total sucrose uptake by Rm1021 cultures can be attributed to the *ThuEFGK* system (Fig. 3C). The *aglEFGAK* operon thus has a broader specificity for disaccharide transport. In addition to sucrose, the *aglEFGAK* operon is also involved in the transport of trehalose, cellobiose, and maltose,

as evidenced by results of the competitive inhibition studies on trehalose and sucrose uptake (Table 2).

The *thuEFGK* operon is induced only by trehalose and not by sucrose (Fig. 4). From the results of transport and competition experiments, we can deduce that the *aglEFGAK* operon is induced not only by sucrose but may also be induced by high concentrations of trehalose in the cell. Uptake of sucrose by Rm1021 cultures grown in medium containing trehalose is inhibited by cellobiose (65%), maltose (94%), and trehalose (95%). This pattern of inhibition is consistent with the *aglEFGAK* operon as well as the *thuEFGK* operon being induced by trehalose in these cultures. Uptake of sucrose by Rm9628 cultures grown in the presence of trehalose (where the *agl* operon is not active) is not inhibited by cellobiose. Our contention is that the induction of the *aglEFGAK* operon in Rm1021 grown in the presence of trehalose is effected through the trehalose transported by the functional *ThuEFGK* transport system in these cultures. The following observations are also consistent with this conclusion. (i) Sucrose transport is induced about fivefold in Rm1021 cultures grown with trehalose as the inducer (Fig. 3C). (ii) This induced uptake of sucrose in cultures where trehalose is the inducer requires that both the *thuEFGK* and *aglEFGAK* operons are functional (Fig. 3C); it is not observed in either trehalose-grown Rm9628 or Sm7019.

Our observations that more than 98% of the sucrose uptake in *S. meliloti* is due to the functional *aglEFGAK* system (Fig. 3C) confirm Willis and Walker's observation that this system is the major transporter for sucrose uptake (47). Nevertheless, the growth of Rm9628 in sucrose is not impaired (Fig. 2). This implies that the residual transport of sucrose in these cultures is sufficient for the growth observed and that sucrose must be a very efficient source of carbon for growth of *S. meliloti*. Our results that Sm7025 is unable to grow with sucrose as the sole source of carbon confirms that these two transport systems may be the only transport systems involved in the uptake of sucrose in *S. meliloti*. Willis and Walker (47) reported that the *aglEFGAK* operon encodes a transport system for the α -glucosides sucrose, trehalose, and maltose. Our results, however, suggest that this system also imports cellobiose, which is a β -glucoside (Table 2). Structural characteristics of the four disaccharides that strongly compete with the uptake of sucrose in Rm1021 are that they all contain a 1-linked glucosyl residue

(trehalose, maltose, and cellobiose) or a glucosyl residue linked to a fructosyl residue (sucrose). Lactose, in contrast, which contains a galactosyl residue linked 1–4 to glucose, does not compete (Table 2). Whether the four strong competitors of sucrose uptake are reducing or nonreducing sugars seems to be unimportant, since both reducing (cellobiose and maltose) and nonreducing (trehalose and sucrose) disaccharides are equally competitive. Linkage of the monosaccharide moiety does not seem to play an important role, since the β -glucoside cellobiose is as strong a competitor of maltose and trehalose uptake as the α -glucosides maltose, sucrose, and trehalose. From the results given above, one can deduce that the *aglEFGAK* operon codes for a transport system that takes up a variety of disaccharides containing a 1-linked glucosyl residue. The presence of transporters with broad substrate specificities has been reported in different organisms, including an α -glucoside transporter capable of transporting several α -glucosides that has been documented in *Saccharomyces* (19).

Plant nodulation tests showed that the uptake of trehalose and sucrose does not play a crucial role in nodule development. However, its role in competitiveness for nodule occupancy cannot be ruled out. This finding is similar to the finding that other rhizobial mutants unable to utilize different hexoses formed effective nitrogen-fixing nodules (10, 16, 30, 38), but still, hexose import can be critical for competition for nodule occupancy (15). Lack of a nodulation phenotype for the mutants in disaccharide transport suggests that the functions may be important for the free-living rhizobium. We can only speculate on the possible advantages for *S. meliloti* to have two BPD ABC transport systems for disaccharides and particularly for trehalose sucrose and maltose. *S. meliloti* normally lives in a habitat where both the biotic and abiotic environments are in a state of flux. The availability of carbon sources can suddenly become limited, and therefore the presence of a high-affinity transport system may be necessary for *S. meliloti* to utilize these scarce carbon sources efficiently. It is thus not surprising that disaccharide uptake and utilization appears to be important in rhizosphere competition. Our observations show that Sm7019, Rm9628, and Sm7025 were impaired not in their ability to colonize the root per se (Table 3) but only in their ability to compete with the wild type. Sm7025, which is unable to import trehalose, maltose, and sucrose, was more impaired in competitive colonization than Sm7019 and Rm9628, suggesting that these disaccharides are important sources of carbon in alfalfa root exudate. We have observed induction of the *thuE'-lacZ* fusion when Sm7019 is growing on the root surface (data not presented). This observation and earlier reports (36) show that trehalose is available as a carbon source in the root exudate. The role of maltose in rhizobium-root interactions, however, is less obvious because the presence of maltose in alfalfa root exudates is not documented. To our knowledge this is the first report of disaccharide transport being critical for competitive root colonization by any rhizobium. Trehalose-utilizing *Pseudomonas fluorescens* strains were preferentially found in the mycorrhizosphere of Douglas fir (14). The ability to utilize trehalose likewise endows a competitive edge for root colonization in *S. meliloti*. These observations support the hypothesis of Finan et al. (12) that genes located on the pSymB megaplasmid are important, especially in enhancement of the competitive abilities of *S. meliloti* in the natural habitat.

ACKNOWLEDGMENTS

This work was supported by a grant from the Norwegian Research Council.

The sequencing was performed in the W. M. Keck Facility at Yale University, New Haven, Conn. We also thank Leslie Wanner, U.S. Department of Agriculture, Beltsville, Md., for critically reading the manuscript. Part of this work was done at the Department of Agronomy and Range Sciences at the University of California—Davis. T.V.B. thanks D. A. Phillips and his colleagues for support during her stay at the University of California—Davis. The technical help of Eli Robertsen is gratefully acknowledged.

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