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The *thuEFGKAB* Operon of Rhizobia and *Agrobacterium tumefaciens* Codes for Transport of Trehalose, Maltitol, and Isomers of Sucrose and Their Assimilation through the Formation of Their 3-Keto Derivatives

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The *thu* operon (*thuEFGKAB*) in *Sinorhizobium meliloti* codes for transport and utilization functions of the disaccharide trehalose. Sequenced genomes of members of the *Rhizobiaceae* reveal that some rhizobia and *Agrobacterium* possess the entire *thu* operon in similar organizations and that *Mesorhizobium loti* MAFF303099 lacks the transport (*thuEFGK*) genes. In this study, we show that this operon is dedicated to the transport and assimilation of maltitol and isomers of sucrose (leucrose, palatinose, and trehalulose) in addition to trehalulose, not only in *S. meliloti* but also in *Agrobacterium tumefaciens*. By using genetic complementation, we show that the *thuAB* genes of *S. meliloti*, *M. loti*, and *A. tumefaciens* are functionally equivalent. Further, we provide both genetic and biochemical evidence to show that these bacteria assimilate these disaccharides by converting them to their respective 3-keto derivatives and that the *thuAB* genes code for this ketodisaccharide-forming enzyme(s). Formation of 3-ketotrehalose in real time in live *S. meliloti* is shown through Raman spectroscopy. The presence of an additional ketodisaccharide-forming pathway(s) in *A. tumefaciens* is also indicated. To our knowledge, this is the first report to identify the genes that code for the conversion of disaccharides to their 3-ketodisaccharide derivatives in any organism.

Rhizobia are facultative symbionts which form nitrogen-fixing nodules on leguminous plants (1). Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside), which serves as an osmoprotectant in many organisms (2, 3), is found in these symbiotic structures as well as in other structures formed due to interactions between plants and microorganisms (4, 5). In addition to being an osmoprotectant, trehalose is an important source of carbon for microorganisms in agricultural soils. It can originate from nodules during nodule senescence (6) or as an excretion product (7) from mycorrhizal fungi, in which trehalose is an essential storage compound in vegetative cells and spores (8), or from insects and other soil fauna. There are three well-documented pathways for trehalose catabolism in microorganisms: (i) trehalose is hydrolyzed into two glucose moieties by the enzyme trehalase found in *Escherichia coli*, *Bacillus subtilis*, and many other microorganisms, including fungi (9); (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 (10), and the imported trehalose or T6P is hydrolyzed by enzymes such as trehalase, T6P-hydrolase, phospho-(1-1)-glucosidase or phosphotrehalase (11, 12) to yield both glucose and phosphorylated glucose as products (trehalose phosphorylase may also split trehalose by exerting a phosphate attack on the bond joining the glucose moieties [11; reference 13 and references therein]); and (iii) trehalose is taken up via the PTS as T6P as described above, but in this pathway, the enzyme trehalose-6-phosphate phosphorylase phosphorylates the T6P and splits the molecule to yield β-glucose-1-phosphate (βG1P) and glucose-6-phosphate (phosphorylated glucose residues rather than glucose are end products of trehalose catabolism in this pathway found in *Lactococcus lactis* [13, 14]). Because rhizobia do not possess a complete PTS for carbohydrate uptake (15), the two latter pathways described are unlikely to exist in rhizobia. Trehalase has been detected in *Glycine max* nodules (5, 16), but except for α-glucosidases, which work poorly on trehalose (17), enzymes involved in trehalose catabolism have not been unequivocally identified in free-living rhizobia (18). Previously, we identified and characterized the trehalose-inducible *thu* operon, *thuEFGKAB*, involved in the transport and assimilation of trehalose in *Sinorhizobium meliloti* and demonstrated that the genes *thuEFGK* of this operon code for an ATP-binding cassette for transport of trehalose and that disruption of *thuAB* sequences results in accumulation of trehalose in the cells, implying their role in trehalose utilization. In addition, we showed that disruption of the *thuAB* genes increases the nodulation competitiveness of *S. meliloti*, depending on the interacting host genotype (19–21). However, identification of the biochemical function(s) of the *thuAB* genes and elucidation of the pathway for trehalose utilization in *S. meliloti* have remained elusive.

The deduced amino acid sequence of ThuB revealed similarity to known and putative dehydrogenases which display the characteristic dinucleotide-binding G-X-G-X-X-G or G-X-G-X-X-A

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TABLE 1 Bacterial strains used in this study

Strain or plasmid	Relevant characteristics[a]	Source[b] or reference
A. tumefaciens strains		
MAFF301001	Rif^r	NIAS, GenBank, and this work
At7023	MAFF301001 *thuA*::pThuA409, ϕ(*thuA′-lacZ*) transcriptional fusion, Rif^r Km^r	This work
E. coli strains		
S17-1/λpir	RK2 *tra* regulon, λ*pir*, host for *pir*-dependent plasmids	57
SY327/λpir	λ*pir*, host for *pir*-dependent plasmids	58
M. loti strains		
MAFF303099	Pho^r	NIAS, GenBank
Ml7023	MAFF303099 *thuA*::pThuA597, ϕ(*thuA′-gfp*) transcriptional fusion, Pho^r Km^r	This work
S. meliloti strains		
Rm1021	SU47 str-21 Str^r	59
Rm9628	Rm1021::*aglE192*::Tn*5* Str^r Km^r	38
Sm7019	Rm1021 *thuE*::pThuE629, ϕ(*thuE′-lacZ*) transcriptional fusion, Str^r Km^r	19
Sm7023	Rm1021 *thuA*::Tn*5-1062*, ϕ(*thuA′-luxAB*) transcriptional fusion, Str^r Km^r	19
Sm7024	Rm1021 *thuB*::pThuB582, ϕ(*thuB′-lacZ*) transcriptional fusion, Str^r Km^r	20
Sm7025	Rm9628 *thuE*::pThuE690 Str^r Km^r Tet^r	19
Plasmids		
pRK2013	ColE1 Tra$^+$ Km^r	28
pRK7813	IncP1, broad-host-range cosmid vector, Tet^r	30
pRK7813::MthuAB	pRK7813 clone carrying full-length *M. loti thuAB*	This work
pRK7813::thuA	pRK7813 clone carrying full-length *S. meliloti thuA*	20
pRK7813::thuB	pRK7813 clone carrying full-length *S. meliloti thuB*	20
pRK7813::thuAB	pRK7813 clone carrying full-length *S. meliloti thuAB*	20
pThuA597	pVIK165, 597-bp *M. loti thuA* internal fragment, ϕ(*thuA′-gfp*) transcriptional fusion, Km^r	This work
pThuA409	pVIK112, 409-bp *A. tumefaciens thuA* internal fragment, ϕ(*thuA′-lacZ*) transcriptional fusion, Km^r	This work
pVIK112	*lacZY* for transcriptional fusion, Km^r	27
pVIK165	*gfp* for transcriptional fusion, Km^r	27

[a] Km, kanamycin; Pho, phosphomycin; Str, streptomycin; Rif, rifampin; Tet, tetracycline.
[b] NIAS, National Institute of Agrobiological Sciences, Tsukuba, Ibanaki, Japan.

motifs that have been suggested to be part of a βαβ fold, enabling proteins to bind the ADP moiety of NAD(H) or NADP(H) and/or FAD cofactors, respectively (22, 23). The highest similarity was found to be to a group of dehydrogenases containing the consensus motif AGKHVXCEKP, all of which are known to, or can be expected to, react with substrates that are structurally similar to glucose (24). The deduced product of *thuA* is a protein of unknown function with an amidotransferase motif. Both of these predicted products lack functional as well as sequence homology to enzymes currently well documented to be involved in the utilization of trehalose.

De Costa et al. (25) reported that mutating the *thuE* ortholog in *Agrobacterium tumefaciens* abolished its ability to use palatinose (6-*O*-α-D-glucopyranosyl-α-D-fructofuranose), an isomer of sucrose (α-D-glucopyranoside-β-D-fructofuranosyl), but not trehalose as a carbon source. In this report, we present genetic evidence to establish that in *S. meliloti*, in addition to transport and utilization of trehalose, the *thuEFGKAB* operon is involved in the transport and utilization of isomers of sucrose (leucrose, palatinose, and trehalulose) and maltitol (4-O-α-glucopyranosyl-D-sorbitol). We provide both genetic and biochemical evidence to show that the *thuAB* genes of *A. tumefaciens*, *S. meliloti*, and *Mesorhizobium loti* code for enzymes involved in the formation of 3-keto derivatives of trehalose, maltitol, and isomers of sucrose (leucrose, palatinose, and trehalulose). In *S. meliloti*, this is the sole pathway for assimilation of all these disaccharides.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are presented in Table 1. *Escherichia coli* was grown at 37°C in Luria broth (26). *Rhizobium* and *Agrobacterium* strains were grown at 28°C in tryptone yeast (TY) or M9 minimal medium (26) with various carbon sources at 0.4% (wt/vol) unless otherwise stated. Selective media were supplemented with antibiotics to the final concentration as stated: kanamycin, 50 μg ml^{-1}; rifampin, 100 μg ml^{-1}; streptomycin, 500 μg ml^{-1}; tetracycline, 5 μg ml^{-1}; and phosphomycin 100 μg ml^{-1}.

Insertional mutagenesis of *thuA* orthologs of *M. loti* and *A. tumefaciens*. For *thuA* mutagenesis in *M. loti*, we cloned a 597-bp *thuA* fragment of *M. loti* MAFF303099 (from nucleotide +34 to +630 with respect to the *thuA* start codon) into a pCR2.1TOPO plasmid vector (Invitrogen Life Technologies) according to the manufacturer's instructions, introducing the indicated (underlined) restriction endonuclease recognition sites in our PCR primers 5′-GAGAGCTCGTCCATGAACAGACCAA-3′ (SacI; forward) and 5′-CATCTAGACTGCACACCCTCATT-3′ (XbaI; reverse). The resultant plasmid, pTOPOthuA, was digested with SacI and XbaI restriction enzymes to excise the *thuA* fragment, and the excised fragment was ligated into plasmid pVIK165 (27). The resulting plasmid, pThuA597 was electroporated into *E. coli* SY327/λ*pir*, a readily transformable *E. coli* strain. pThuA597 was then reisolated from SY327/λ*pir* using

the Qiagen plasmid purification kit (Qiagen), electroporated into *E. coli* S17-1/λ*pir*, which has on the chromosome the *tra* region of RP4, and finally introduced into *M. loti* MAFF303099 by conjugation (28). Transconjugants were selected on TY plates containing phosphomycin (50 μg ml^{-1}) and kanamycin (100 μg ml^{-1}). Disruption in the *thuA* locus was confirmed by PCR using primers 5′-CACCATGCCGATAAAAGCT GTTGTC-3′ (forward) and 5′-TCAACTCAACCCCTTGTCGCCATC-3′ (reverse), which amplify the full *M. loti thuA* gene (786 bp). The site of recombination was determined by PCR and sequencing using primers 5′-TGTTGTCTGGGGCGAGAA-3′ (sequences upstream of but outside the 597-bp *thuA* fragment used for cloning) and 5′-GGGACCACTCCAG TGAAAAG-3′ (5′ sequences of the green fluorescent protein [GFP] gene within plasmid pVIK165). The resultant strain was named Ml7023.

With *A. tumefaciens*, an internal *thuA* fragment of 409 bp (nucleotides 167 to +576 with respect to the *thuA* start codon) was amplified by PCR using *A. tumefaciens* MAFF301001 as the template. The nucleotide sequences of *thuEFGKAB* genes were obtained from the GenBank database, accession no. AB074877.1. Primers used in the PCR were 5′-GCGAATT CGCAGGATCGTCT-3′ (EcoRI; forward) and 5′-CATCTAGAAGATGT TGCCGG-3′ (XbaI; reverse), introducing the indicated (underlined) restriction endonuclease recognition sites. The PCR product was digested with EcoRI and XbaI restriction enzymes and was ligated into vector pVIK112 (27). The resulting plasmid, pThuA409, was electroporated into *E. coli* S17-1/λ*pir* and finally introduced into *A. tumefaciens* MAFF301001 by conjugation (28). Transconjugants were selected on TY plates containing rifampin (100 μg ml^{-1}) and kanamycin (50 μg ml^{-1}). Disruption in the *thuA* locus and the site of recombination was confirmed by PCR and sequencing using primers 5′-ACGAACACATCAATGAGACGGTGC G-3′ (sequences upstream of but outside the 409-bp *thuA* fragment used for cloning) and 5′-GGCGATTAAGTTGGGTAACG-3′ (5′ sequences of *lacZ* within plasmid pVIK112). The resultant strain was named At7023.

Biolog metabolic profiling. The Biolog phenotype microarray system (Biolog Inc., Hayward, CA) is a commercial identification system used for global analyses of phenotypes of strains on sole carbon and nitrogen sources present in a 96-well microtiter plate. A Biolog profile was assayed for an *S. meliloti thuB* mutant (Sm7024) in comparison with the wild-type Rm1021 using the manufacturer's instructions. To compare the metabolic profile of Ml7023 with that of the wild type, we assessed their growth phenotypes on Biolog plates using the procedure prescribed for *S. meliloti* with some modifications. In brief, strains initially cultured on TY agar plates for 2 days were streaked onto R2A agar plates and allowed to grow overnight. Colonies were transferred again onto new R2A agar plates and allowed to grow overnight. Cells were then collected from the surface of the agar plates with a sterile inoculation loop and finally suspended in the inoculation fluid provided by the manufacturer to an optical density at 600 nm (OD_{600}) of approximately 0.15. One hundred microliters of the cell suspension was inoculated into each well, and the plates were incubated at 29°C in a humidity chamber for 96 h. Growth on the carbon sources was observed visually using the intensity of purple color developed in the wells.

Complementation analysis in *A. tumefaciens* and *S. meliloti thuA* mutants. To complement the *thuAB* functions in At7023, plasmids pRK7813::thuA, pRK7813::thuB and pRK7813::thuAB (20) were separately mobilized into At7023 by triparental mating (29), using pRK2013 (28) as a helper plasmid. Transconjugants were selected on TY plates containing 100 μg ml^{-1} rifampin, 50 μg ml^{-1} kanamycin, and 5 μg ml^{-1} tetracycline.

For complementation analysis in the *S. meliloti thuA* mutant (Sm7023), the entire *thuAB* segment of *M. loti* MAFF303099 was amplified from its genomic DNA by PCR with primers 5′-TGAAGCTTAGCG CTTTGGAG-3′ (HindIII; forward) and 5′-CCGGATCCATGTCAGCCC AT-3′ (BamHI; reverse), introducing the indicated restriction endonuclease sites (underlined). The PCR product was cloned into a pCR2.1TOPO plasmid vector (Invitrogen Life Technologies) according to the manufacturer's instructions, resulting in plasmid pTOPOMthuAB. Plasmid pTOPOMthuAB was digested with HindIII and BamHI restriction enzymes to excise the *thuAB* fragment, and the excised fragment was ligated into plasmid pRK7813 (30). The resultant plasmid, pRK7813::MthuAB, was electroporated into *E. coli* DH5α. Plasmid pRK7813::MthuAB in *E. coli* DH5α was mobilized into Sm7023 by triparental mating (29), using pRK2013 as a helper plasmid (28). Transconjugants were selected on TY plates containing 400 μg ml^{-1} streptomycin, 100 μg ml^{-1} kanamycin, and 5 μg ml^{-1} tetracycline. Selected colonies from transformed At7023 and Sm7023 were grown on M9 minimal medium containing 0.4% (wt/vol) trehalose, palatinose, leucrose, trehalulose, or maltitol in comparison with At7023 and Sm7023.

***thuB′-lacZ* induction assay *in vitro*.** Induction assays were performed with palatinose and maltitol, with trehalose and glucose as positive and negative controls, respectively, as described by Jensen et al. (19). β-Galactosidase activity was assayed in three replicate cultures according to the method of Miller (31).

Ketosugar analysis. For production of 3-ketodisaccharides by wild-type *A. tumefaciens* and At7023, cells were grown overnight in a minimal medium (MM) described by Kurowski and Darbyshire (32) with 1% (wt/vol) trehalose as a carbon source. From overnight culture, 1.5-ml aliquots were harvested by centrifugation (3,000 × *g* for 3 min) at room temperature and washed in 800 μl Kurowski-Darbyshire medium without any carbon source. Two hundred microliters of washed cells was used to inoculate 20 ml Kurowski-Darbyshire medium with various carbon sources at 1% (wt/vol) and incubated overnight at 30°C on a rotary shaker (300 rpm). The presence of ketosugars in the cell-free supernatant was determined by the alkaline method described by Fukui and Hayano (33). The characteristic ketosugar absorption spectrums having a maximum at 340 nm (34) were recorded using SpectroMax 250 Molecular Device (Global Medical Instrumentation Inc., Albertville, MI).

For determining the induction of ketosugar-forming enzymes in wild-type *A. tumefaciens* and At7023, these cells were grown overnight in 150 ml of Kurowski-Darbyshire medium with 1% (wt/vol) trehalose or sucrose as a carbon source. The cells were harvested by centrifugation (4,000 × *g* for 10 min) at an OD_{600} of approximately 0.6 and resuspended in 5 ml of the same medium without any carbon source. Aliquots of the washed cells (450 μl) were transferred to glass tubes containing 3 ml Kurowski-Darbyshire medium with various carbon sources (1% [wt/vol]) (see Table 2) and incubated at 30°C on a rotary shaker (300 rpm). At 0 and 90 min, aliquots were removed for OD_{600} measurement and determination of the presence of ketosugars. The presences of ketosugars in the cell-free supernatants was determined as described above.

Isolation and identification of 3-ketotrehalose. 3-Ketotrehalose was isolated from culture supernatant on a preparative high-pressure liquid chromatography (HPLC) system with a Phenomenex Luna NH_2 column (10 by 250 mm, 10 μm) used in the reversed-phase mode. Ketotrehalose eluted at 14.6 min with a gradient running from 95 to 50% acetonitrile in water, both containing 0.1% formic acid and at a flow rate of 6 ml/min. The preparative HPLC system consisted of a 600 controller separation module, a 2996 photodiode array UV detector, a 3100 mass detector, and a 2767 sample manager (Waters, Milford, MA). MassLynx version 4.1 and the FractionLynx (Waters) application manager were used to control the system. The mass of 3-ketotrehalose was used as fraction trigger. Ultra-high-pressure liquid chromatography (UHPLC)-high-resolution mass spectrometry (HR-MS) analysis of ketotrehalose was performed on an Acquity UPLC and a LCT Premier time-of-flight MS with electrospray ionization (ESI) (Waters), and the data were processed using MassLynx version 4.1. Nuclear magnetic resonance (NMR) spectra were obtained on samples dissolved in D_2O (Sigma) on a Varian (Palo Alto, CA) 400-MHz spectrometer. One-dimensional ^1H and ^{13}C NMR experiments and two-dimensional heteronuclear multiple bond correlation experiments (gHMBC) were performed to verify the structures of the isolated compounds. MestreNova 5.2.4 software was used to process the spectroscopic data.

Laser tweezers Raman spectroscopy (LTRS). The inocula of *S. meliloti* strain Rm1021 and Sm7023 grown overnight at 28°C in TY medium were harvested by centrifugation (3,000 × *g* for 10 min) and washed twice with minimal medium (MM) (32). Thirty microliters of the washed cells was used to inoculate 3 ml of antibiotic-free MM containing either 0.4% (wt/vol) mannitol or 0.2% (wt/vol) both mannitol and trehalose. The cells were harvested at an OD_{600} of approximately 0.3 (mid-exponential phase) and were washed twice in MM without a carbon source prior to Raman spectroscopy measurements.

Laser tweezers Raman spectroscopy of a single bacterial cell in suspension was achieved based on a custom-built Raman microspectroscopy setup (35) using an Olympus IX-71 inverted optical microscope (60×; numerical aperture [NA], 1.2) (MO-Olympus America, Center Valley, PA) and a continuous-wave laser source with an excitation power of 30 mW at a 785-nm wavelength. The Raman signal from the probed cell was detected with a spectrometer (Acton 2300i; Princeton Instruments) equipped with a 300-groove/mm grating.

The bacterial cell suspension was diluted such that only a single cell could be trapped to avoid any interference from neighboring cells. An aliquot of the sample (500 μl) was placed in a 35-mm-diameter culture dish formed by a stainless steel cell chamber (AttoChamber; Invitrogen, Eugene, OR) and an MgF_2 coverslip. Trehalose was added to a final concentration of 0.4% (wt/vol). Bacterial cells were held in the optical trap for 5 min, and repetitive spectra were collected. A spectral integration time as short as 10 s was found to be sufficient to obtain Raman spectra with a good signal-to-noise ratio to identify the characteristic Raman bands of the bacterial cells. All measurements were done at room temperature.

Raman spectra of trehalose, purchased from Sigma-Aldrich (St. Louis, MO), and HPLC-isolated ketotrehalose (as described above) were acquired for 2 min in 5% aqueous solution using the same Raman spectroscopy setup.

All the data were acquired and processed with WinSpec32 software (Roper Scientific, Tucson, AZ). The spectra were background subtracted and normalized to the total area under the spectrum. Data were processed using Excel (Microsoft).

RESULTS

Presence of the trehalose transport and utilization operon (*thuEFGKAB*) in members of the *Rhizobiaceae*. We have reported that in *S. meliloti*, the *thu* operon (*thuEFGKAB*) is the major system for the transport and utilization of the disaccharide trehalose (19–21). The entire *thu* operon is also present in similar organizations with various degrees of homology and identity in the sequenced genomes of several other members of the *Rhizobiaceae*, as shown in Fig. 1.

Sequence analysis reveals that *Rhizobium etli* CFN42 and *Rhizobium leguminosarum* bv. *trifolii* WSM 2304 have two copies each of *thuA* and *thuB*. One copy is located on the plasmid and the other on the chromosome (36, 37). The *thuAB* genes on the plasmid are found as part of the entire *thu* operon (*thuEFGKAB*), while that on the chromosome lacks the transport part (*thuEFGK*). In *M. loti* the *thuAB* genes are found only on the chromosome and the entire *thuEFGK* transport genes are lacking.

In *S. meliloti*, the *thuEFGKAB* operon is also needed for the transport and utilization of palatinose and maltitol, and its expression is induced by these compounds. De Costa et al. (25) reported that in *A. tumefaciens* MAFF301001, the *thuEFGKAB* gene cluster is involved in palatinose rather than trehalose utilization. We therefore tested the ability of *S. meliloti* trehalose transport mutants Sm7019, Rm9628, and Sm7025 for their ability to grow on palatinose. Sm7019 has a mutated *thuE* gene, Rm9628 carries a mutation in *aglE* of the *aglEFGAK* operon, an alternate transport system for sucrose, trehalose, and maltose (38), and Sm7025 carries mutations in both *aglE* and *thuE* (19). The results presented in Fig. 2 show that only Rm9628 is able to grow on palatinose. To determine whether growth on other carbon sources is also affected, we screened the trehalose utilization mutant Sm7024, which carries a mutation in *thuB* (20), for growth with various carbon sources using the commercially available phenotype microarray system Biolog (Biolog Inc., Hayward, CA). The results show that Sm7024 was impaired in the utilization of palatinose and maltitol, in addition to trehalose. These preliminary results were confirmed by following the growth of *thuAB* mutants Sm7023 and Sm7024 and wild-type Rm1021 on M9 minimal medium containing palatinose or maltitol (Fig. 2). Induction of the *thuEFGKAB* operon in Sm7024 (*thuB′-lacZ*) by these carbon sources was also tested by the method of Miller (31). At a 100 μM concentration of trehalose, palatinose, or maltitol, *thuB′-lacZ* was induced 3.1, 3.7, and 3.9 times, respectively, over its expression in medium containing glucose (see Fig. S1 in the supplemental material). These results confirm the report of De Costa et al. (25) that the *thuEFGKAB* operon does indeed code for transport and utilization of palatinose and extend their observation to show that the *thuAB* gene functions are also required for assimilation of maltitol.

The trehalose utilization genes *thuAB* of *S. meliloti* and *M. loti* are functionally equivalent. *M. loti* lacks the *thuEFGK* genes for transport. The *thuAB* genes of *M. loti* are present as part of a putative amino acid transport operon (39), and they possess the least similarity to *thuAB* of *S. meliloti* (Fig. 1). Therefore, we tested whether the *thuAB* genes were functionally equivalent in these two organisms. Both *M. loti* strain MAFF303099 and its mutant strain Ml7023 with a disrupted *thuA* sequence were screened for growth with various carbon sources using the Biolog phenotype microarray system. In addition, we tested the growth of these mutants in leucrose (5-*O*-α-D-glucopyranosyl-β-D-fructose), another isomer of sucrose which is not present on the Biolog phenotype microarray plates. Both the wild type and the mutant were able to utilize trehalose. Neither the parent strain nor the mutant Ml7023 was able to grow on palatinose, maltitol, or leucrose (data not shown). The *thuA* mutant Sm7023 was therefore complemented with cloned *thuAB* genes from *M. loti*, and the transconjugants were tested for growth in medium containing trehalose, palatinose, maltitol, or leucrose. These results, presented in Fig. 3, show that Sm7023 complemented with the *thuAB* genes from *M. loti* could utilize not only trehalose but also maltitol, palatinose, and leucrose. These results demonstrate that the *thuAB* genes in these two rhizobia are functionally equivalent. Further, these results show that *M. loti* possesses an alternate pathway for trehalose assimilation. The lack of growth of wild-type *M. loti* on palatinose and maltitol suggests that it lacks a transport mechanism for these disaccharides, which is consistent with the absence of the transport genes, *thuEFGK*, in the genome.

Biochemical function(s) of *thuAB*. The biochemical functions of the *thuAB* genes are currently unknown. As reported in our earlier study (20), we were also unable to detect glucose as a product of trehalose catabolism in *S. meliloti*. We have also been consistently unsuccessful in our attempts to detect other known intermediates of trehalose breakdown, such as glucose-6-phosphate, in reaction mixtures containing trehalose and induced *S. meliloti* cells or cell extracts, through thin-layer chromatography (TLC), HPLC, or HPLC-MS (results not presented). Previous studies with the related alphaproteobacte-

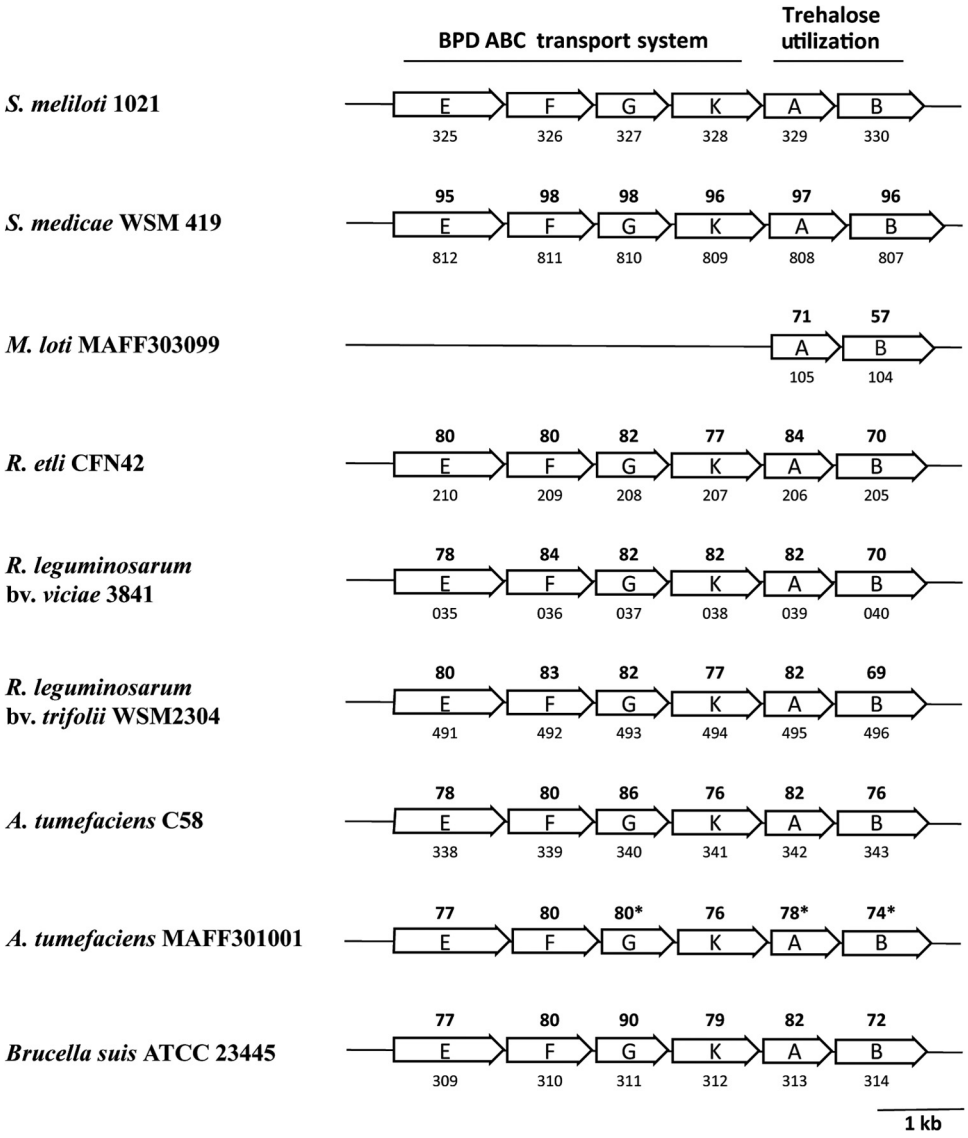

FIG 1 The trehalose transport and utilization operon, *thuEFGKAB*, in members of *Rhizobiaceae*. The proposed biochemical functions encoded by the genes are indicated at the top. Numbers in bold above each gene indicate the percentages of identity with *S. meliloti* 1021 at the amino acid level (or at nucleotide level [*]). Where available, locus tags are indicated directly below clusters: for *S. meliloti* 1021, the prefix is SM_b20; for *S. medicae* WSM 419, it is Smed_3; for *M. loti* MAFF303099, it is mll3; for *R. etli* CFN42, it is RHE_PF00; for *R. leguminosarum* bv. *viciae* 3841, it is pRL120; for *R. leguminosarum* bv. *trifolii* WSM2304, it is Rleg2_5; for *A. tumefaciens* C58, it is Atu3; and for *B. suis* ATCC 23445, it is BSUIS_B0.

rium *A. tumefaciens* suggested that disaccharide utilization pathways involving dehydrogenase activity may lead to the formation of 3-ketosugars (33, 40, 41). *A. tumefaciens* has also been shown to possess the unique ability not only to oxidize sucrose into its 3-keto derivative but also to secrete this compound into the culture medium (41). The gene(s) coding for this enzyme, however, has not been identified either in *A. tumefaciens* or in any other organism to date. We have therefore investigated the possibility that the *thuAB* genes of *S. meliloti* and *A. tumefaciens* code for enzymes that oxidize trehalose and other disaccharides into their respective 3-keto derivatives, first with *A. tumefaciens* strain MAFF301001.

A *thuAB* mutant of *A. tumefaciens* is unable to grow with sucrose isomers and to form 3-ketotrehalose and 3-ketodisaccharides of sucrose isomers. In their report, De Costa et al. (25) claimed that the *thuAB* orthologs (*palAB* according to their suggested nomenclature) may code for truncated and possible nonfunctional proteins. Our results presented here, however, establish that these genes code for fully functional proteins. The *thuA* sequence of *A. tumefaciens* MAFF301001 was disrupted by site-directed mutagenesis as described in Materials and Methods. The resultant mutant strain, At7023, and the wild-type parent were grown in medium containing either trehalose, sucrose, isomers of sucrose (leucrose, palatinose, or trehalulose), or other related disaccharides (Table 2). Wild-type *A. tumefaciens* MAFF301001 was able to use all of the tested sugars as sole carbon sources, whereas the mutant At7023 showed no growth in medium containing leucrose, maltitol, palatinose, or trehalulose (Table 2). Where bacterial growth was present, cell-free supernatants of the cultures were analyzed for the presence of keto derivatives of these saccharides

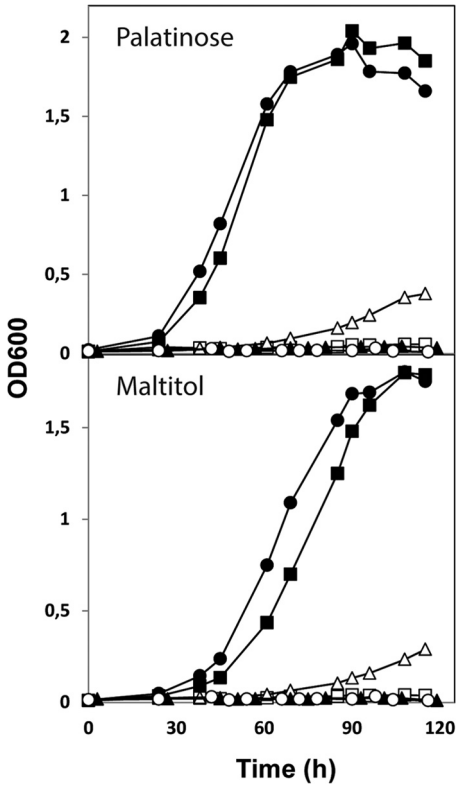

FIG 2 Growth of wild-type *S. meliloti* Rm1021 (●), the trehalose utilization mutants Sm7023 (▲) and Sm7024 (○), the trehalose transport mutant Sm7019 (△), the sucrose transport mutant Rm9628 (■), and the trehalose and sucrose transport mutant Sm7025 (□) on M9 minimal medium containing palatinose or maltitol at 0.4% (wt/vol).

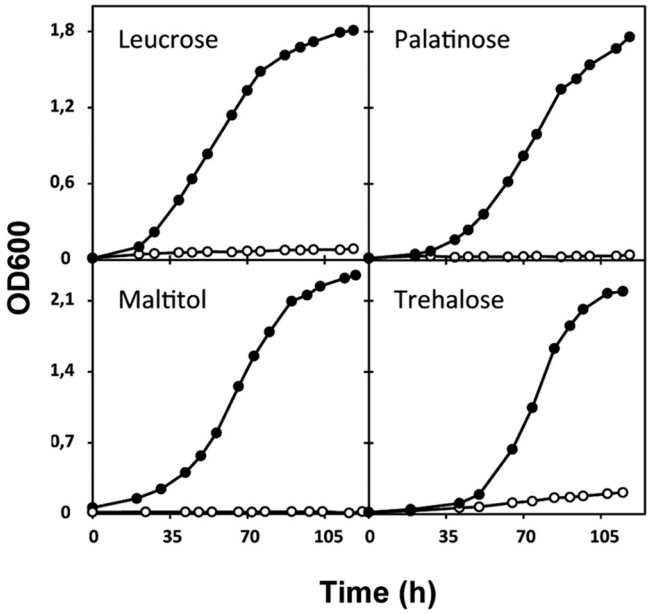

FIG 3 Complementation analysis for utilization of leucrose, maltitol, palatinose, and trehalose in *S. meliloti* Sm7023. Shown is the growth of Sm7023 (○) and of Sm7023 containing plasmid pRK7813::MthuAB (●) expressing the *thuAB* genes of *M. loti* from the *lac* promoter in pRK7813. The cells were grown in M9 medium containing various carbon sources at 0.4% (wt/vol) as indicated.

by the alkaline method (33). The presence of ketodisaccharide is indicated by a characteristic spectrum with an absorption maximum at 340 nm (Fig. 4). The detection of the 340-nm peak always coincided with the presence of potential ketodisaccharides in the culture supernatant with an accurate mass of 340.1006 Da (or 342.1162 Da for ketomaltitol) corresponding to the elemental composition $C_{12}H_{20}O_{11}$ (or $C_{12}H_{22}O_{11}$ for ketomaltitol) as determined by HR-MS (results not shown). In lactose-grown cultures, however, the keto spectrum peak shifted to 345 nm as shown before (42), and we were also unsuccessful in adequately separating the ketolactose from lactose under our test conditions. The results presented in Table 2 show that disruption of the *thuAB* gene sequence abolishes growth of *A. tumefaciens* in palatinose, leucrose, maltitol, and trehalulose but not in trehalose, sucrose, maltose, and cellobiose. Figure 4 shows that even though *thuAB* disruption does not prevent growth of *A. tumefaciens* in trehalose, it stops the formation of 3-ketotrehalose. Moreover, these results also establish that *thuAB* disruption is inconsequential for production of 3-keto derivatives of sucrose, maltose, cellobiose, and lactose.

The observation that *thuAB* disruption did not prevent the formation of 3-keto derivatives of sucrose, maltose, cellobiose, and lactose implies that in addition to *thuAB*, there may be one or more coding sequences for this function. The 3-ketosucrose-forming enzyme purified and characterized by Hayano and Fukui (34) and Vanbeeum and Deley (43) was reported to have activity on sucrose, maltose, and cellobiose as well as activity, albeit reduced, on trehalose and palatinose. On the other hand, if there is an alternative pathway(s) for formation of ketodisaccharides, the lack of ketotrehalose in trehalose-grown At7023 culture filtrates needs to be reconciled. One possibility may be that the genes coding for ketosucrose-forming enzymes may be inducible only by sucrose, lactose, or maltose and not by trehalose. To test for the presence of two such distinctly inducible coding sequences, we grew the *A. tumefaciens* wild-type strain and At7023 in medium containing either sucrose or trehalose as an inducer, incubated the washed live cells with various disaccharide substrates as described in Materials and Methods, and followed the formation of their keto derivatives in the reaction mixtures over a period of 90 min. The results presented in Table 2 show that when induced with trehalose, only the wild-type *A. tumefaciens* was able to form 3-ketodisaccharide derivatives from all substrates tested. When induced with sucrose, on the other hand, both wild-type and At7023 cells were able to form keto derivatives of all the substrates tested (Table 2; see Fig. S2 in the supplemental material).

Cloned *S. meliloti thuAB* genes restore the ability of the *A. tumefaciens thuA* mutant to grow on sucrose isomers and form ketodisaccharides of these isomers as well as of trehalose. Complementation tests of the *A. tumefaciens thuA* mutant (At7023) were performed with cosmids containing the *thuA*, *thuB*, or *thuAB* genes of *S. meliloti*. The resultant transconjugants were tested for growth and ketodisaccharide production in medium containing trehalose, palatinose, leucrose, trehalulose, or maltitol. The results presented in Fig. 5 and Table 2 show that the wild-type phenotype was restored only in transconjugants containing both *thuAB* genes of *S. meliloti*. At7023 complemented with a cosmid containing *thuA* alone showed very limited and delayed growth in leucrose but no 3-ketodisaccharide production.

TABLE 2 Growth and ketosugar production in wild-type *A. tumefaciens* MAFF301001 and mutant At7023

Sugar	Cells grown for 18 h				Ketosugar production				At7023 with pRK7813::thuAB	
	MAFF301001		At7023		Trehalose induced		Sucrose induced			
	Growth	Ketosugar production	Growth	Ketosugar production	MAFF301001	At7023	MAFF301001	At7023	Growth	Ketosugar production
Cellobiose	+	+	+	+	+	+	+	+	ND[a]	ND
Lactose	+	+	+	+	+	−	+	+	ND	ND
Leucrose	+	+	−	−	+	−	±[b]	±	+	+
Maltitol	+	+	−	−	+	−	+	+	+	+
Maltose	+	+	+	+	+	−	+	+	ND	ND
Palatinose	+	+	−	−	+	−	+	+	+	+
Sucrose	+	+	+	+	+	−	+	+	ND	ND
Trehalose	+	+	+	−	+	−	+	+	+	+
Trehalulose	+	+	−	−	+	−	+	+	+	+

[a] ND, not determined.
[b] ±, 3-ketodisaccharide spectrum barely detectable (see Fig. S2 in the supplemental material).

Isolation and identification of 3-ketotrehalose by HR-MS and NMR. Since three different monoketo derivatives of trehalose theoretically can be formed, HR-MS and nuclear magnetic resonance (NMR) experiments were employed to assess the structure of the ketotrehalose isolated. Supernatants of wild-type *A. tumefaciens* MAFF301001 cultures grown with trehalose as the sole source of carbon were used for the isolation of ketotrehalose by semipreparative HPLC with mass-guided fractionation. High-resolution ESI-MS of the isolated compound in the negative mode gave *m/z* 385.0996 $(M + FA - H)^-$ and calculated *m/z* 385.0988 for $C_{13}H_{21}O_{13}$ $[(M + FA - H)^-]$, corresponding to the elemental composition of ketotrehalose ($C_{12}H_{20}O_{11}$). Adducts of ketotrehalose and chlorine [*m/z* 375.0702 $(M + Cl)^-$, calculated *m/z* 375.0700] as well as the dimer of ketotrehalose and formate [*m/z* 725.1995 $(2M + FA - H)^-$, calculated *m/z* 725.1994] could also be observed (see Fig. S3 in the supplemental material). The ^{13}C NMR experiments clearly showed a single carbonyl signal at 206.70 ppm (see Fig. S4 in the supplemental material), indicating the formation of a single keto isomer. A two-dimensional gradient heteronuclear multiple bond correlation (gHMBC) experiment con-

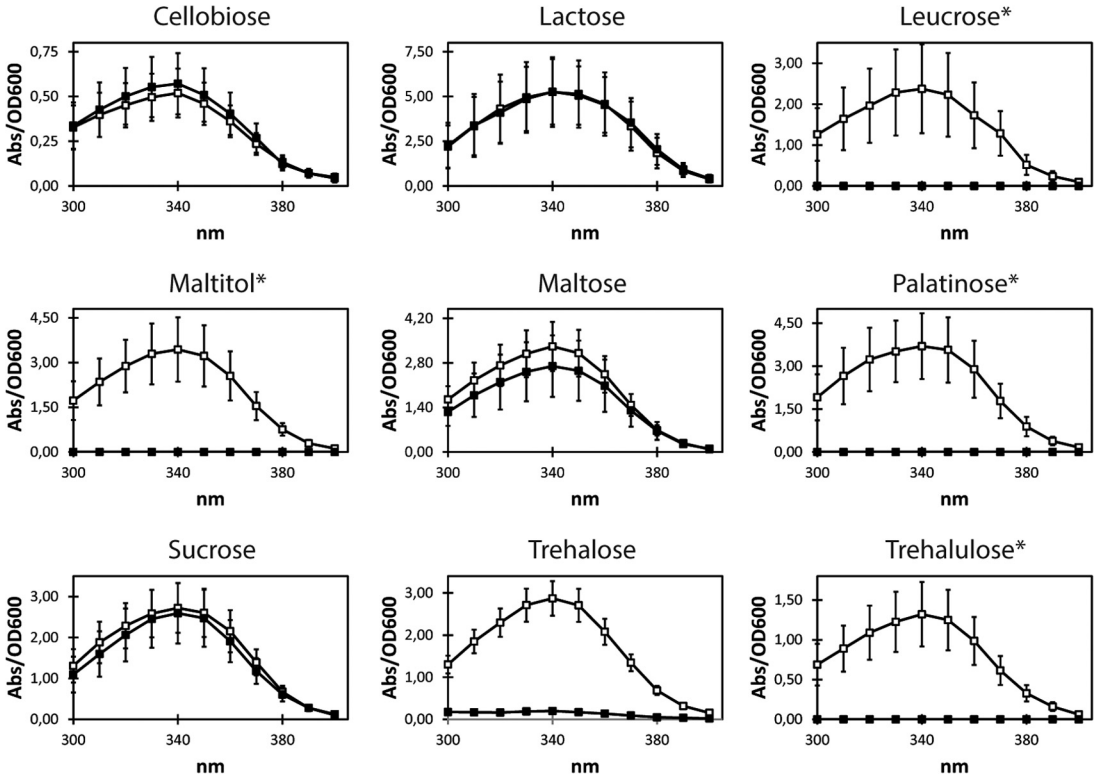

FIG 4 Absorption spectra of ketosugars formed by *A. tumefaciens* MAFF301001 (□) and mutant At7023 (■) grown overnight with various disaccharides (1%, wt/vol) as indicated. An asterisk indicates that no growth of At7023 was observed. The presence of ketosugars in the cell-free supernatant was determined by the alkaline method described by Fukui and Hayano (33). Each value represents the mean from at least three different experiments ± standard error.

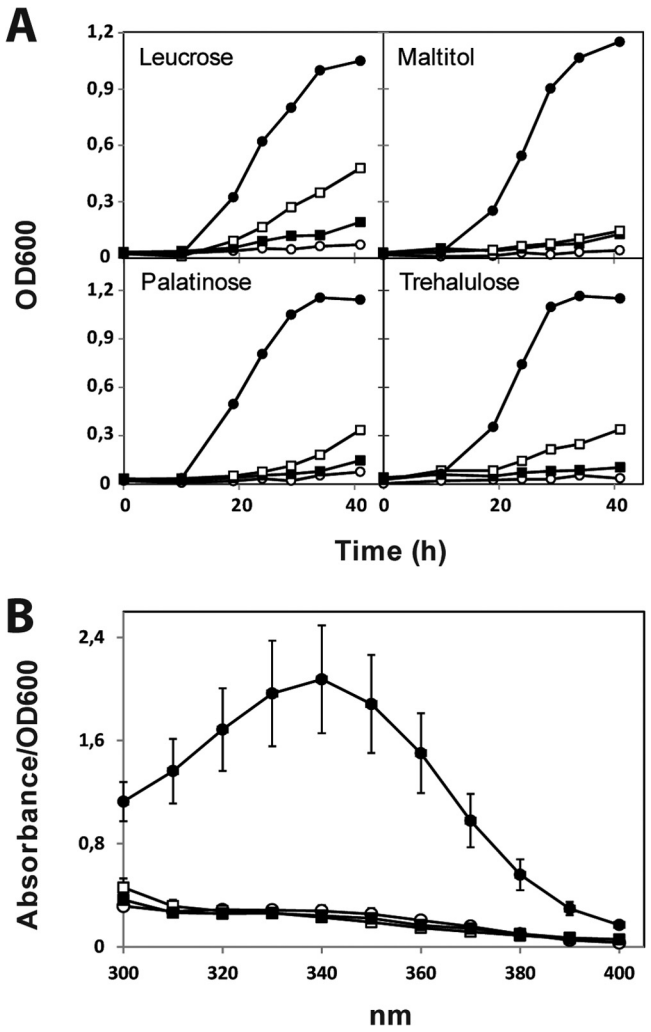

FIG 5 (A) Complementation analysis for utilization of leucrose, maltitol, palatinose, and trehalulose in *A. tumefaciens* At7023 containing cosmid pRK7813 expressing *thuA* (□), *thuB* (■), or *thuAB* (●) of *S. meliloti* from the *lac* promoter. (○), At7023 without cosmid. The cells were grown in M9 medium containing various carbon sources at 0.4% (wt/vol) as indicated. (B) Absorption spectra of 3-ketotrehalose formed by *A. tumefaciens* At7023 containing cosmid pRK7813 expressing *thuA* (□), *thuB* (■), or *thuAB* (●) of *S. meliloti* from the *lac* promoter. (○), At7023 without cosmid. The cells were grown overnight in M9 medium containing trehalose (1%, wt/vol), and the presence of ketosugars in the cell-free supernatant was determined by the alkaline method described by Fukui and Hayano (33). Each value represents the mean from three different experiments ± standard error.

firmed the involvement of the carbonyl in the structure (see Fig. S5 in the supplemental material). The final structure of the ketotrehalose was verified by analysis of the typical doublet splitting pattern of all the downfield-shifted signals seen in the ^{1}H spectrum of ketotrehalose compared with the proton spectrum of trehalose (see Fig. S6 and S7 in the supplemental material). 2-Ketotrehalose would generate a singlet signal for the 1′ proton, and 4-ketotrehalose would, in analogy, generate a triplet for the 2′ proton not seen. The gHMBC data further confirmed the 3-ketotrehalose structure.

***In vivo* detection of 3-ketotrehalose formation in *S. meliloti* cells metabolizing trehalose.** In spite of the fact that the complementation of At7023 with *S. meliloti thuAB* genes restores its ability to form keto derivatives of trehalose and isomers of sucrose (leucrose, palatinose, and trehalulose), we were unsuccessful in our repeated attempts to detect ketotrehalose *in vitro*, either in cell-free supernatants of trehalose-grown *S. meliloti* cultures or in reaction mixtures containing trehalose and extracts of induced *S. meliloti* cells, with HPLC-MS or TLC. It is reported that ketodisaccharides are reactive compounds (44, 45) that can serve as intermediates for synthesis of other compounds in the cell. We hypothesized that unlike *A. tumefaciens*, *S. meliloti* may not accumulate ketotrehalose in high enough concentrations in the cells and that it may be only transiently present in the cells. Therefore, we attempted to follow the formation of ketotrehalose in real time in optically trapped live cells of *S. meliloti* Rm1021 and the trehalose utilization mutant Sm7023 through confocal laser tweezers Raman spectroscopy (LTRS). LTRS allows chemical identification of cellular components due to the excellent sensitivity of detection possible with this technique, enabling real-time measurements even of individual cells of microorganisms (46–48).

The rationale behind this experiment was based on the fact that the Raman spectrum of 3-ketotrehalose extracted by HPLC-MS from trehalose-grown *A. tumefaciens* showed a peak around the 1,734 cm^{-1} region, which is absent in trehalose (Fig. 6A). This peak can be attributed to a vibration of the carbonyl group in the 3-ketotrehalose molecule. The 3-ketotrehalose Raman spectrum was verified by a normal-mode analysis using the software package Gaussian (Gaussian, Inc., Wallinford, CT) (data not shown). The keto conversion of trehalose in the cells was therefore traced by the differences in the peak intensities at 1,734 cm^{-1} of the wild-type Rm1021 and the Sm7023 strain upon trehalose application (Fig. 6B). Here, the initial spectrum obtained 1.5 min after adding the trehalose solution was subtracted from each subsequent spectrum to obtain the difference spectra that represent changes of the peak intensity inside the cell with time. The increased peak intensity in the induced Rm1021 cells and its absence in uninduced Rm1021 and Sm7023 indicate the role of *thuAB* in the conversion of trehalose to 3-ketotrehalose in *S. meliloti* as well.

DISCUSSION

Together, the results presented show that the *thuEFGK* genes of *S. meliloti* and *A. tumefaciens* code for transport of trehalose, maltitol, and isomers of sucrose (leucrose, palatinose, and trehalulose) and that the *thuAB* genes of these two bacteria as well as those of *M. loti* code for an enzyme(s) involved in the conversion of these disaccharides to their respective 3-keto derivatives. Disruption of *thuAB* resulted in lack of growth in medium containing isomers of sucrose (Fig. 2 and 5; Table 2), so this is the only pathway for assimilation of these disaccharides in these members of *Rhizobiaceae*. Since *thuAB* disruption did not influence the ability of *M. loti* and *A. tumefaciens* to utilize trehalose, an alternate and yet-unidentified pathway(s) must exist for utilization of trehalose in these organisms. In *S. meliloti*, on the other hand, *thuAB* disruption abolishes growth in trehalose-containing medium as well (20), indicating that trehalose assimilation in *S. meliloti* occurs only via 3-ketotrehalose formation. Arellano et al. have reported a dehydrogenase-dependent assimilatory pathway for trehalose in *Caulobacter crescentus* (40).

Of the two alternate transport systems present for transport of sucrose, maltose, and trehalose in *S. meliloti* (19, 38), sucrose isomers (leucrose, palatinoase, and trehalulose) are transported only

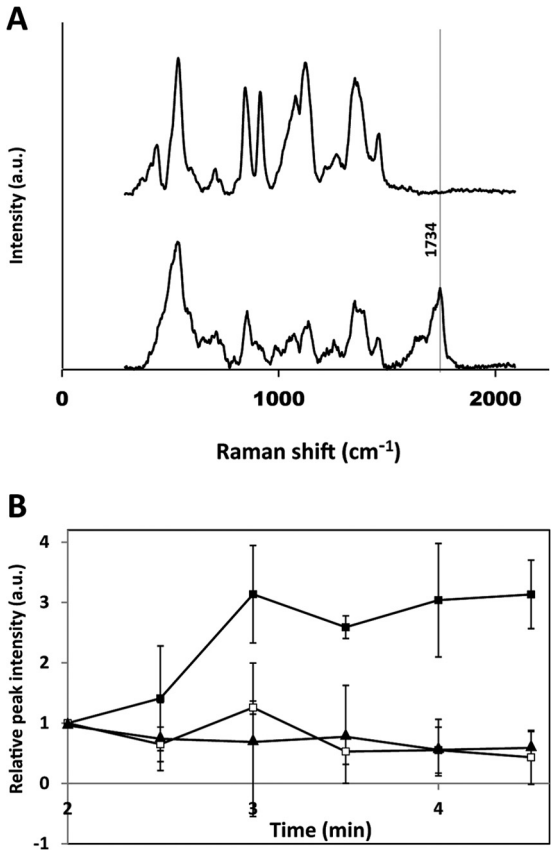

FIG 6 (A) Raman spectra of 5% (wt/vol) aqueous solutions of trehalose (upper spectrum) and 3-ketotrehalose (lower spectrum). The spectra were acquired within a 2-min acquisition time with an excitation power of 30 mW at a 785-nm wavelength. (B) Plot of the difference of normalized intensities of the 1,734-cm^{-1} peak upon trehalose application in the *S. meliloti* wild type (Rm1021) grown in the presence of trehalose (■), *S. meliloti* Rm1021 grown without trehalose (□), and the trehalose utilization mutant Sm7023 grown in the presence of trehalose (▲) versus time. Prior to actual measurements, the safety of the method was evaluated by monitoring the bacterial cells in the optical trap for up to 5 min under experimental conditions (data not shown). Each value represents the mean from three different experiments ± standard error.

through the *thuEFGK*-mediated transport system. Growth of strain Sm9628 (*aglE*192::Tn*5*) in palatinose and the inability of strains Sm7019 and Sm7025 to utilize palatinose (Fig. 2) support this conclusion. It is also significant that utilization of palatinose, which is an isomer of sucrose (α-D-glucosyl-1,6-D-fructose), is coupled with trehalose but not sucrose utilization. The *S. meliloti thu* operon is also inducible only with trehalose, palatinose, and maltitol and not with sucrose (see Fig. S1 in the supplemental material) (20). In *Klebsiella pneumoniae*, for instance, the *aglB* gene, which is necessary for the utilization of all of the sucrose isomers (trehalulose, turanose, maltulose, leucrose, and palatinose) as well as maltitol and maltose, is induced only by sucrose isomers, maltitol, or maltose and not by sucrose (49). The phytopathogenic bacterium *Erwinia rhapontici* and several other human dental plaque bacteria also possess operons dedicated solely to the transport and utilization of sucrose isomers but not sucrose (49–51). The genes which code for utilization of sucrose isomers in these bacteria, however, do not possess any sequence homology to *thuAB*. Like *S. meliloti*, *E. rhapontici* transports sucrose isomers through an ABC transport system, while the other bacteria transport these sugars through the phosphotransferase system (PTS) (50).

Wild-type *M. loti* was unable to grow on palatinose and maltitol. Cloned *thuAB* genes of *M. loti*, however, could restore the ability of Sm7023 to grow not only on trehalose but also on all the isomers of sucrose tested, including palatinose and maltitol (Fig. 3). These observations point to the absence of a transport system for palatinose and maltitol in *M. loti* and to the presence of another gene(s) for the utilization of trehalose. This is consistent with the absence of *thuEFGK* orthologs in *M. loti*.

A. tumefaciens possesses at least two distinct enzymes for the formation of 3-ketodisaccharides: one inducible with sucrose, maltose, and lactose and the other encoded by *thuAB* and induced by trehalose and isomers of sucrose. The inability of trehalose-induced At7023 cells to convert any of the disaccharides to their respective keto derivatives and their ability to do so when induced with sucrose (Table 2) are consistent with the presence of two distinct enzymes with overlapping activities but stringent requirements for their induction. Therefore, we contend that the 3-ketodisaccharide-forming enzyme encoded by *thuAB* is functionally similar to but separate from the D-glucoside 3-hydrogenase purified to homogeneity from crude cell extracts of *A. tumefaciens* by Hayano and Fukui (34) and Vanbeeumen and Deley (43) or the one suggested by Arellano et al. (40) in *C. crescentus*, which was shown to be inducible by lactose. Since the *A. tumefaciens* genome does not possess other coding sequences homologous to *thuAB*, these two proteins may not possess high sequence similarity. Arellano et al. (40) have suggested genes Atu4377, -4378, and -4379 as possible candidates based on the various degrees of sequence similarities between *lacA*, *-B*, and *-C* of *C. crescentus*.

The presence of 3-ketodisaccharide-forming enzymes has been reported in *Flavobacterium sacchrophilum* (52), *Stenotrophomonas maltrophilia* (42), and *Halomonas* (*Deleya*) sp. α-15 (53). Cell membrane preparations of *Flavobacterium* and *Halomonas* have been used for large-scale production of 3-ketodisaccharides *in vitro* (54). *A. tumefaciens*, however, is the most studied, potentially because it is unique in its ability to secrete the 3-ketosugars into the culture medium. Whole-cell preparations of this organism have also been widely used for commercial production of 3-ketosucrose and 3-ketocellobiose (55, 56). None of the other microorganisms which possess this pathway, *S. meliloti* included, secrete the ketodisaccharides formed extracellularly. We have attempted to use live *S. meliloti* cells as well as cell extracts to produce 3-ketotrehalose *in vitro* without success (results not presented). Likewise, our attempts to overexpress the *thuAB* genes (separately as *thuA* and *thuB* or together as *thuAB*) of both *A. tumefaciens* and *S. meliloti* in *E. coli* have so far yielded only catalytically inactive proteins.

The presence of dehydrogenase motif AGKHVXCEKP and the flavin binding motif G-X-G-X-X-G/A in *thuB* is consistent with its role in the dehydrogenation of the disaccharides to their respective keto forms. Both the *thuA* and *thuB* genes, however, are needed to restore the wild-type phenotype to At7023 (Fig. 5 and Table 2), as in *S. meliloti* (20). The role of the amidotransferase motif of *thuA* and its role in the formation of ketodisaccharides are currently unclear. Whether these two proteins act together or sequentially on the substrates to form the 3-ketodisaccharide is also unsolved. As Arellano et al. (40) have suggested for *C. crescentus*,

thuA and *-B* could code for two subunits of this enzyme. A limited and delayed growth was observed in leucrose-grown cultures of At7023 complemented with *thuA* of *S. meliloti* (Fig. 5A), but no 3-ketoleucrose was detected (data not presented). Therefore, we contend that the delayed growth may be due to assimilation of leucrose through an alternate (and inefficient) pathway involving glucosidases. We speculate that *thuA* might code for a cofactor which may function with these glycosidases as well. As reported above, our attempts to overexpress them to resolve these issues have not been successful so far.

S. meliloti does not accumulate or secrete the 3-ketodisaccharide intermediate in a high enough concentration for detection by HPLC-MS, but its formation and transient presence could be detected in real time using LTRS within the cells (Fig. 6). Detection of 3-ketotrehalose in wild-type Rm1021 and its absence in Sm7023 thus establishes the role of *thuAB* in 3-ketotrehalose formation in *S. meliloti*. Complementation of strain At7023 with cosmids containing the *thuAB* genes of *S. meliloti* to restore the ketodisaccharide production in this strain (Fig. 5 and Table 2) indicates that the *thuAB* genes of these two organisms are functionally equivalent as deduced from their sequence homology and identity.

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