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A Novel *Sinorhizobium meliloti* Operon Encodes an α-Glucosidase and a Periplasmic-Binding-Protein-Dependent Transport System for α-Glucosides

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The most abundant carbon source transported into legume root nodules is photosynthetically produced sucrose, yet the importance of its metabolism by rhizobia in planta is not yet known. To identify genes involved in sucrose uptake and hydrolysis, we screened a *Sinorhizobium meliloti* genomic library and discovered a segment of *S. meliloti* DNA which allows *Ralstonia eutropha* to grow on the α-glucosides sucrose, maltose, and trehalose. Tn*5* mutagenesis localized the required genes to a 6.8-kb region containing five open reading frames which were named *agl*, for α-glucoside utilization. Four of these (*aglE*, *aglF*, *aglG*, and *aglK*) appear to encode a periplasmic-binding-protein-dependent sugar transport system, and one (*aglA*) appears to encode an α-glucosidase with homology to family 13 of glycosyl hydrolases. Cosmid-borne *agl* genes permit uptake of radiolabeled sucrose into *R. eutropha* cells. Analysis of the properties of *agl* mutants suggests that *S. meliloti* possesses at least one additional α-glucosidase as well as a lower-affinity transport system for α-glucosides. It is possible that the Fix$^+$ phenotype of *agl* mutants on alfalfa is due to these additional functions. Loci found by DNA sequencing to be adjacent to *aglEFGAK* include a probable regulatory gene (*aglR*), *zwf* and *edd*, which encode the first two enzymes of the Entner-Doudoroff pathway, *pgl*, which shows homology to a gene encoding a putative phosphogluconolactonase, and a novel *Rhizobium*-specific repeat element.

Photosynthetically derived sucrose is the main source of carbon for legume root nodules. In fact, sucrose is the first radiolabeled compound found in the root nodules and bacteroids of nodulated plants which are incorporating $^{14}CO_2$ via photosynthesis (2, 53, 62). The identification of radiolabeled sucrose in the bacteroids in these studies suggests that sucrose is being transported across the symbiosome membrane.

Based on these observations, we thought it possible that sucrose uptake and hydrolysis may be required for, or induced during, symbiosis. However, the possible importance of sucrose metabolism during symbiosis has not yet been evaluated. To date, research on carbon metabolism in indeterminate nodules, such as those formed in the *Medicago sativa-Sinorhizobium meliloti* symbiosis, has focused on the role of dicarboxylic acids in nitrogen fixation (9, 16, 54, 70). Although it has been demonstrated that transport of dicarboxylic acids is required for nitrogen fixation, it seems unlikely that dicarboxylic acids are required by bacteroids as a carbon source per se. *S. meliloti dct* mutants, which fail to transport dicarboxylic acids, cannot utilize carbon sources such as succinate in the free-living state (20) and are Fix$^-$ (16, 70). However, these strains are able to induce and invade nodules, and they proceed through several stages of bacteroid development, becoming blocked just prior to active nitrogen fixation (64). The efficiency of the tricarboxylic acid cycle is reduced in *dct* strains, and the defect in nitrogen fixation may be due to failure to produce enough ATP to power the nitrogenase holoenzyme.

Sucrose metabolism has been examined at the biochemical level in both fast- and slow-growing rhizobia (30, 44). No evidence of sucrose phosphorylase activity, required for sucrose uptake via the phosphoenolpyruvate phosphotransferase system utilized by enteric bacteria, has been found in fast- or slow-growing rhizobia (44). Studies of disaccharide metabolism have demonstrated that sucrose hydrolysis and uptake activities are inducible in *S. meliloti* (30). The results of competition studies suggest that *S. meliloti* possesses at least three systems for disaccharide uptake: one system that transports sucrose, maltose, and trehalose; a second which transports lactose; and a third which transports cellobiose (30). Transport of sucrose in the infection thread has not been investigated.

In these experiments, we sought to identify *S. meliloti* genes involved in sucrose transport or hydrolysis, so that we could begin to address the question of whether sucrose is utilized during nodule invasion or bacteroid development. Mutants of *S. meliloti* which cannot utilize sucrose (11) or grow poorly on sucrose (3) have been isolated, but these strains fail to utilize several carbon sources, and the defects in metabolism were found to be downstream of sucrose uptake or cleavage. No genes involved in *S. meliloti* sucrose uptake or hydrolysis have been identified, nor have mutants of *S. meliloti* that are unable to utilize sucrose yet retain the ability to utilize fructose and glucose, a key phenotype predicted for strains defective in sucrose hydrolysis or transport, been reported.

Since no *Rhizobium* mutants that were specifically defective in sucrose utilization had been reported, and we had not succeeded in isolating them by direct screening, we turned to a different strategy. This involved introducing a cosmid library of *S. meliloti* DNA into a heterologous host unable to utilize sucrose and selecting for derivatives that could grow on sucrose. This type of approach has been used successfully to identify sucrose utilization genes in the phosphoenolpyruvate phosphotransferase system by screening in *Escherichia coli* (25). In our case, *E. coli* proved to be unsuitable, possibly because its G+C content is so much lower than that of *S. meliloti*; therefore, we instead used *Ralstonia eutropha*, a gram-negative soil bacterium with a high (~66%) G+C content that

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
Sinorhizobium meliloti		
Rm1021	SU47 *str-21*	45
SG1001	Rm1021 *agpA*::Tn*phoA*	24
Rm9620	Rm1021 *aglK2*::Tn*5*	This work
Rm9621	Rm1021 *aglE49*::Tn*5*	This work
Rm9622	Rm1021 *aglF95*::Tn*5*	This work
Rm9623	Rm1021 *aglA112*::Tn*5*	This work
Rm9624	Rm1021 *aglG127*::Tn*5*	This work
Rm9625	Rm1021 *aglA279*::Tn*5*	This work
Rm9626	Rm1021 *aglA115*::Tn*5*	This work
Rm9627	Rm1021 *aglA182*::Tn*5*	This work
Rm9628	Rm1021 *aglE192*::Tn*5*	This work
Rm9631	Rm1021 *aglA112*::Tn*5-233*[a]	This work
Rm9632	Rm1021 *aglE49*::Tn*5-233*[a]	This work
Rm9633	Rm9631 *agpA*::Tn*phoA*	This work
Rm9634	Rm9632 *agpA*::Tn*phoA*	This work
Ralstonia eutropha H16	Wild type, Sm^s	50
Agrobacterium tumefaciens		
At123	GMI9023 ≡ GMI9050 cured of pAtC58 Sm^r, Rif^r	55
At125	GMI9023 pRmeSU47bΩ5007::Tn*5-oriT* Nm^r/Km^r	19
At128	GMI9023 pRmeSU47aΩ30::Tn*5-11* Gm^r Sp^r/Km^r Sm^r	19
Escherichia coli		
C2110	*polA* Nal^r	B. Staskawicz
DH5α	*supE44* Δ*lacU169*(ϕ*80lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Clontech
MT614	MM294 *malE*::Tn*5*	T. M. Finan
MT609	*polA1 thy* Sp^r	T. M. Finan
Plasmids		
pBluescript SK+	Amp^r, ColE1 cloning vector	Stratagene
pBluescript II KS+	Amp^r, ColE1 cloning vector	Stratagene
pRK600	pRK2013 *npt*::Tn*9*, Cm^r	19
pPH1JI	IncP, Gm^r, Sp^r	8
pR751	IncP, Tp^r	46
pLAFR1	Tc^r, IncP broad-host-range cosmid vector	23
pSW213	Tc^r, IncP broad-host-range vector	12
pLW200	pLAFR1 derivative carrying *S. meliloti agl* region	This work
pLW249	pLW200 *aglE49*::Tn*5*	This work

[a] Obtained by replacement of the corresponding Tn*5* insertion with Tn*5-233* (14).

we have shown in previous work expresses the *phbC* gene of *S. meliloti* (68).

We report here the identification of five *S. meliloti* genes which permit the growth of *R. eutropha* on sucrose, maltose, or trehalose. These genes evidently encode an α-glucosidase and a system for the transport of α-glucosides. A cosmid carrying these genes permits uptake of radiolabeled sucrose by *R. eutropha*. In addition, we report the sequence of a putative regulatory locus (*aglR*), the Entner-Doudoroff genes of *S. meliloti*, a putative phosphogluconolactonase gene, *pgl*, and a novel *Rhizobium*-specific repeat element.

MATERIALS AND METHODS

Strains and growth media. Strains and plasmids used in this study are listed in Table 1. Bacterial strains were routinely grown in LB medium (42), which was supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ in the case of *S. meliloti*. Minimal medium M9 (40) supplemented with 1 mM $MgSO_4$, 0.25 mM $CaCl_2$, 1 mg of D-biotin per liter, and 0.4% filter-sterilized carbon source was used to assay the growth of *S. meliloti* strains. Defined medium MM1N [medium MM1 (50) with the concentration of $(NH_4)_2SO_4$ increased to 0.2%] supplemented with 0.5% (wt/vol) filter-sterilized fructose or 0.4% (wt/vol) filter-sterilized sucrose was used to assay growth of *R. eutropha* strains. Where noted, NH_4Cl was substituted for $(NH_4)_2SO_4$, keeping constant the final concentration of nitrogen in the medium. Antibiotics were used at the following concentrations: ampicillin, 150 μg/ml; chloramphenicol, 20 μg/ml; gentamicin sulfate, 5 μg/ml for *E. coli* and 50 μg/ml for *S. meliloti*; kanamycin sulfate, 50 μg/ml; nalidixic acid, 50 μg/ml; neomycin sulfate, 200 μg/ml; and tetracycline, 10 μg/ml. To select for Tn*5* kanamycin was used with *E. coli* and *R. eutropha* and neomycin was used with *S. meliloti*.

Genetic techniques. Conjugal transfer of plasmids was accomplished in triparental matings using pRK600 to provide transfer functions. Plasmid-borne insertions were recombined into the *S. meliloti* genome via homogenotization as described (18) previously, using pPH1JI or pR751 as the incompatible IncP plasmid. Insertions were then transduced by using bacteriophage ϕM12 into strain Rm1021 to ensure a clean genetic background. Southern hybridization was performed to check the construction of each strain. To obtain Gm^r Sp^r derivatives of *S. meliloti* Tn*5*-induced mutations, Tn*5* insertions were replaced with Tn*5-233* (14) as previously described (29).

DNA manipulations. Plasmid and cosmid DNA was isolated from overnight cultures of *E. coli* by the alkaline lysis method (42) or by purification over a Qiagen column. DNA-modifying enzymes were used according to the instructions of the supplier (New England Biolabs, Beverly, Mass., or TaKaRa Biomedicals, Shiga, Japan). GeneScreen Plus membranes (Dupont/NEN, Boston, Mass.) were used for Southern hybridizations. Radiolabeled DNA probes were prepared with the NEBlot random labeling kit (New England Biolabs) and [α-^{32}P]dCTP from Dupont/NEN or Amersham.

DNA sequencing and analysis. Plasmids were purified for sequencing by using a Qiagen plasmid mini kit. The sequencing strategy was based on a detailed restriction map of pLW200. Each of the 10 *Eco*RI fragments of pLW200 was subcloned into pBluescript SK+. These plasmids and defined subfragments cloned into pBluescript SK+ or pBluescript II KS+ were subjected to fluores-

cently labeled dideoxy termination reactions at the MIT Biopolymers laboratory or in an MJ Research thermal cycler. The sequencing reactions were then separated on an ABI Prism apparatus at the MIT Biopolymers laboratory or at the Molecular Biology facility at Dartmouth. Contigs were prepared using the SeqMan software program (Lasergene). Comparisons of nucleotide sequences and translated nucleotide sequences were performed by using the BLAST algorithms (1, 28) to search the databases maintained by the National Center for Biotechnology Information. Searches of SWISSPROT by using Profile Scan to find PROSITE (5) patterns were performed with the resources maintained by ExPasy in Switzerland. Additional analysis was performed with the package of software developed by the Genetics Computing Group (26), DNA Strider version 1.2, and the DNASTAR programs by Lasergene.

Isolation of cosmids which improve the growth of *R. eutropha* on sucrose. An *S. meliloti* genomic library in pLAFR1 (23) was mated into *R. eutropha* H16, and transconjugants were selected on MM1N plates containing sucrose as the sole carbon source. In the initial screen, half of the selective plates contained NH_4Cl as a nitrogen source and half contained $(NH_4)_2SO_4$, but all subsequent experiments used $(NH_4)_2SO_4$ as a nitrogen source. *R. eutropha* strains carrying pLW200 and related plasmids produced visible colonies after 3 days of incubation and were scored for growth on sucrose after 5 days. After 11 days of incubation at 30°C, these strains produced colonies approximately 4 mm in diameter. A control strain carrying pLAFR1 did not produce visible colonies even after 11 days of incubation at 30°C, although translucent microcolonies could be observed under magnification. All cosmid-containing strains tested produced 4-mm colonies within 4 days on medium containing the permissive carbon source fructose.

Construction of mutagenized plasmids. Cosmids were mutagenized with Tn*5* by using previously described techniques (29). In hundreds of separate matings, pLW200 was conjugally transferred into MT614, which carries a Tn*5* insertion in *malE*. Cosmids were then conjugally transferred into MT609, selecting with spectinomycin, tetracycline, and kanamycin to obtain isolates carrying mutagenized cosmids. Individual colonies were picked and used as donors in triparental matings with *R. eutropha* H16. *R. eutropha* transconjugants were selected on MM1N containing fructose, tetracycline, and kanamycin and then challenged to grow on MM1N containing sucrose, tetracycline, and kanamycin. Approximately 10% of the mutagenized cosmids were unable to promote growth on sucrose. Cosmids were conjugally transferred from *R. eutropha* H16 into *E. coli* C2110 for DNA preparation and analysis. Cosmid-borne Tn*5* insertions were localized by standard restriction mapping techniques.

Sugar transport assays. [U-^{14}C]sucrose (615 mCi mmol^{-1}; 22.8 GBq mmol^{-1}) was obtained from Amersham Life Science (Buckinghamshire, England). *R. eutropha* H16 harboring pLAFR1, pLW200, or pLW249 was grown in MM1-tetracycline supplemented with both 0.5% fructose and 0.4% sucrose until the optical density at 600 nm was approximately 0.8. Cells were pelleted and resuspended in MM1-tetracycline containing 0.4% sucrose and incubated for 2 h at 30°C, after which they were pelleted, resuspended in an equal volume of MM1, and incubated without carbon source for 15 min at 30°C. One milliliter was withdrawn for measurement of optical density, and radiolabeled sucrose was added to 2 ml of cells to a final concentration of 1.6 nM. Samples (0.1 ml) were withdrawn in duplicate at 0.5, 3, 5, 7.5, 30, and 60 min after the addition of label, applied to Millipore HA filters under vacuum, and washed twice with 2 ml of MM1. Filters were dried at 68°C for 15 min, and 5 ml of Hydrofluor scintillant (National Diagnostics, Atlanta, Ga.) was added before disintegrations per minute were determined in a Beckman LS 6000SC scintillation counter.

Genetic mapping techniques. The genes identified in this study were mapped by the method of Finan et al. (19). Genomic DNA from Rm1021 and *Agrobacterium tumefaciens* At123, At125, and At128 was digested with *Eco*RI, subjected to electrophoresis in a 0.6% agarose gel, transferred to a GeneScreen Plus membrane, and probed with the insert from pLW201, which contains the C-terminal half of *aglG* and the majority of *aglA*.

Plant inoculation assays. *M. sativa* cv. Iroquois was obtained from Agway (Plymouth, Ind.). *S. meliloti* strains were tested for the ability to nodulate alfalfa on nitrogen-free Jensen's medium as described elsewhere (39). Plants were grown in a constant temperature room at 25°C with a 20-h light cycle. Observations were made weekly for at least 6 weeks. Each nodulation assay included the control treatments of water, wild-type *S. meliloti* Rm1021, and an *exoA* mutant, Rm7031 (39). The presence of pink, cylindrical nodules on dark green healthy plants was taken as evidence that nitrogen fixation was occurring. Plants lacking nodules or with ineffective nodules were stunted and chlorotic.

Nucleotide sequence accession number. The DNA sequence reported in this work has been submitted to GenBank and assigned accession no. AF045609.

RESULTS

Identification of *S. meliloti* genomic clones which promote growth on sucrose. An *S. meliloti* genomic library was mated into wild-type *R. eutropha*, and transconjugants were selected on MM1N containing abundant nitrogen and sucrose as the sole carbon source. Approximately 24,000 transconjugants were screened from three separate matings. Fewer than 0.1% of the transconjugants were able to grow on the sucrose plates. We scored colonies for growth by comparing the size of colonies with that of an isogenic strain harboring the vector pLAFR1. Although *R. eutropha* cannot utilize sucrose as a carbon source, it does form microcolonies on this medium because it is a facultative chemolithotroph capable of using CO_2 as a carbon source. These translucent microcolonies were seen only when plates were examined under a dissecting microscope.

In contrast, some transconjugant strains produced visible colonies after 3 days of incubation, and we scored for ability to grow on sucrose after 5 days of incubation. These colonies have the same color and colony morphology as strains grown on fructose. No cosmids which allowed *R. eutropha* to grow at the same rate on sucrose plates as on fructose were identified, and strains carrying cosmids of interest reached only 4 mm in diameter after 11 days of incubation. Seventeen colonies which permit the growth of the recipient on sucrose were selected for further study.

Identification of a segment of *S. meliloti* DNA that improves the growth of *R. eutropha* on sucrose. Restriction mapping revealed that the 17 cosmids chosen appear to contain the same region of the *S. meliloti* genome. Only two restriction patterns were represented in the candidate cosmids. A 19.4-kb region of DNA is present in each of the cosmids, and two of the cosmids contain an additional 3 kb of DNA. We chose a representative cosmid carrying the smaller insert (pLW200) for further study (Fig. 1A). No single *Eco*RI restriction fragment from pLW200, when cloned into a broad-host-range vector, was able to confer the growth advantage on sucrose. To identify the region(s) of importance, pLW200 was subjected to transposon Tn*5* mutagenesis and subsequently mated into *R. eutropha*. Transconjugants were selected on MM1N-fructose plates and then tested for the ability to grow on sucrose. Approximately 10% of the transconjugants carrying mutagenized cosmids were unable to grow on sucrose. In all, we isolated more than 70 mutagenized cosmids which failed to promote growth on sucrose. The Tn*5* insertions in these cosmids map to a central 6.8-kb region which is required for the improved growth phenotype (Fig. 1C).

The same segment of *S. meliloti* DNA also permits growth of *R. eutropha* on maltose and trehalose. To relate our observations to the previous study of disaccharide metabolism by *S. meliloti* (30), we tested whether the cosmid conferring the ability to grow on sucrose influenced the ability of *R. eutropha* to grow on maltose, trehalose, or the galactosides lactose and melibiose. The cosmid pLW200 improved the growth of *R. eutropha* on the α-glucoside disaccharides but not on lactose or melibiose, nor did it influence growth on fructose. pLW249, one of the Tn*5* insertion mutants of pLW200 identified because it fails to improve the growth of *R. eutropha* on sucrose, is also unable to promote growth on maltose or trehalose as a sole carbon source. These results are consistent with the suggestion (30) that *S. meliloti* uses the same transport system for sucrose, maltose, and trehalose.

DNA sequence suggests this region encodes a binding-protein-dependent transport system for α-glucosides. Analysis of transposon insertions and DNA sequence data has led to the detection of five loci within the 6.8-kb region identified by Tn*5* mutagenesis (Fig. 1B). Since they have been implicated in α-glucoside utilization, we have named these genes *agl*. The genes are arranged in the order *aglEFGAK*. On the basis of sequence homologies, *aglA* appears to encode an α-glucosidase, whereas *aglK*, *aglF*, *aglG*, and *aglE* appear to encode a periplasmic-binding-protein-dependent transport system. The proposed functions of the deduced gene products and their

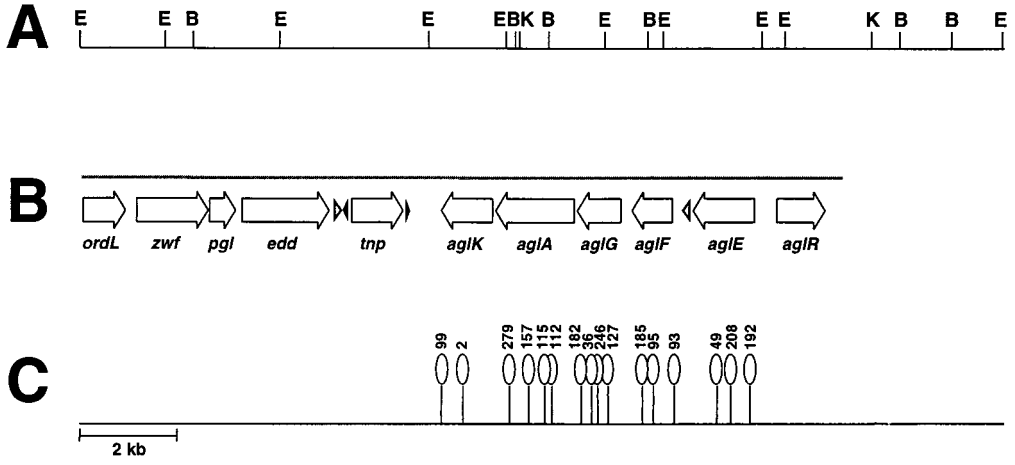

FIG. 1. Genetic organization of the *agl* region of *S. meliloti*. (A) Restriction map of the insert of pLW200. Restriction sites indicated: *Eco*RI (E), *Bam*HI (B), and *Kpn*I (K). (B) Positions and extents of the putative protein coding regions identified by sequence analysis are shown by open arrows. Open triangles indicate the positions and orientation of RIME1. Black triangles indicate the positions of the 39-bp repeat element. The grey line above the arrows indicates the region sequenced. (C) Positions of Tn*5* insertions which eliminate the ability of pLW200 to promote the growth of *R. eutropha* on the α-glucosides sucrose, maltose, and trehalose.

closest database homologues are summarized in Table 2. Transport of the α-glucosides sucrose, maltose, and trehalose followed by their cleavage by AglA can account for how this set of genes confers the ability to utilize these three sugars.

Mutants of *S. meliloti* disrupted by insertions in genes carried on pLW200 can utilize sucrose and are Fix$^+$. All of the Tn*5* insertions shown in Fig. 1 were transferred from the mutagenized cosmid into the Rm1021 genome by homogenotization, and the Tn*5* insertions were subsequently transduced into Rm1021 to ensure a clean genetic background. The resulting mutants were tested for the ability to utilize various carbon sources. All of the mutants are able to utilize glucose and succinate as carbon sources. *S. meliloti aglA* mutants, which are disrupted in the gene encoding the putative α-glucosidase, grow as well as or almost as well as wild type in sucrose and trehalose but grow more slowly in liquid cultures containing maltose. *S. meliloti aglE* mutants, which are disrupted in the putative periplasmic binding protein, and *aglF* and *aglG* mutants, which are disrupted in the putative inner membrane permeases, grow more slowly than the wild type or *aglA* mutant in liquid cultures containing sucrose, maltose, or trehalose and fail to grow in liquid cultures in which the concentration of trehalose or maltose is reduced to 1 mM. *aglE*, *aglF*, and *aglG* mutants form weakly growing colonies on M9 plates containing sucrose, maltose, or trehalose. These data strongly suggest that *S. meliloti* possesses at least one additional α-glucosidase activity besides the one proposed to be encoded by *aglA*. Because the mutants defective in the putative transport system are more severely affected than those affected in the putative α-glucosidase, we propose that *aglE*, *aglF*, *aglG*, and *aglK* encode the major transport system for import of α-glucosides into the cell and that there is at least one other, possibly lower-affinity, pathway for import of these sugars. In addition, because the growth defect is more severe in the *aglE* and *aglF* mutants than the *aglA* mutant, these data suggest that the proposed additional α-glucosidase is not an extracellular enzyme. All of the *agl* mutants are able to elicit Fix$^+$ nodules on *M. sativa*.

AglA shares homology with α-glucosidases. The predicted amino acid sequence of AglA shares homology with many members of family 13 of glycanases, also referred to as the α-amyase family (31–33). The family is composed of proteins with diverse substrate specificities and products (63). Significantly, AglA shares homology with proteins that have been demonstrated to cleave sucrose, trehalose, and oligosaccharides composed of glucose.

***aglK*, *aglF*, and *aglG* appear to encode components of an ATP-dependent inner membrane permease.** The deduced protein AglK shows strong homology to several members of the ATP-binding cassette (ABC) family of cytoplasmic ATP-hydrolyzing peripheral membrane proteins. AglF and AglG are homologous to members of the MalF/MalG family of inner membrane sugar permeases. The *E. coli* protein MalG has been proposed to be involved in protein-protein interactions with MalK, a cytoplasmic ATP-hydrolyzing peripheral membrane protein and homologue of AglK (13, 48). The hydrophobicity traces for AglF and AglG suggest that they could form six transmembrane domains, consistent with the hypothesis that they are integral membrane proteins. Taken together, it seems likely that AglF and AglG are integral membrane proteins which may be involved in transport of sugar substrates across the inner membrane.

AglE shares homology with periplasmic solute binding proteins. AglE appears to be a member of the MalE periplasmic solute binding protein family. The closest homologue of AglE is Slr0529, a hypothetical protein of *Synechocystis* sp. strain PCC6803, which in turn shares homology with two periplasmic sugar binding proteins: the maltose/maltodextrin binding protein MalE of *E. coli* (15, 58) and MsmE of *Streptococcus mutans*, which is involved in the uptake of melibiose, raffinose, and isomaltotriose (56). The highest conservation between MalE, MsmE, Slr0529, and AglE is in a region which in MalE forms the hinge between N- and C-terminal domains and is adjacent to residues which have been shown to contact the substrate in the ligand-bound crystal (60). The structure of maltose binding protein has been compared to those of other periplasmic substrate binding proteins, and although they tend to have very different primary sequences, their three-dimensional structures show many similarities (52).

Support for the inference that AglE is a periplasmic binding protein is provided by gene order in the *agl* region. In almost every reported case (10), genes encoding periplasmic solute

TABLE 2.

Deduced protein	Closest database homologue	Protein family	Sequence features
AglK	*Agrobacterium radiobacter* LacK (66)	ABC ATP-binding cassette proteins	ATP/GTP-binding motif (P-loop) and ABC transporter signature (5)
AglA	*Bacillus coagulans* oligo-1,6-glucosidase (65)	Family 13 of glycosyl hydrolases (α-glucosidases) (31–33)	Contains 7 invariant amino acids found at the active site of α-amylase enzymes (63)
AglG	*Synechocystis* Slr0531 (35, 37)	MalFG family of inner membrane permeases	Inner membrane permease signature (5)
AglF	*Synechocystis* Slr0530 (35, 37)	MalFG family of inner membrane permeases	Inner membrane permease signature (5)
AglE	*Synechocystis* Slr0529 (35, 37)	MalE family of periplasmic sugar binding proteins	
AglR	*Escherichia coli* RafR (4)	LacI family of transcriptional regulators	
Zwf	*Pseudomonas aeruginosa* Zwf (41)	Glucose-6-phosphate dehydrogenase	Glucose-6-phosphate dehydrogenase signature (5)
Pgl	*P. aeruginosa* Pgl (41)	6-Phosphogluconolactonase (41) and SOL/DevB family of putative oxidoreductases (57)	
Edd	*Agrobacterium tumefaciens* MocB (38)	6-Phosphogluconate dehydratase	6-Phosphogluconate dehydratase signatures (5)

FIG. 2. Uptake of [^{14}C]sucrose by *R. eutropha* H16 harboring pLAFR1 (▲), pLW200 (■), or pLW249 (◆). OD_{600}, optical density at 600 nm.

binding proteins are directly upstream of their associated inner membrane permease genes. It seems possible that *aglE* encodes a periplasmic binding protein which would interact with the putative sugar permeases encoded by *aglF* and *aglG*.

AglR, a putative regulatory protein, is homologous to DNA binding proteins. The *aglR* open reading frame, upstream of *aglE* and divergently transcribed, encodes a deduced protein homologous to transcriptional regulators of the *lacI* family of repressors. Many proteins in this family are involved in catabolite repression of sugar utilization operons. *aglR* lies outside the 6.8-kb region of pLW200 shown to be required for the utilization of α-glucoside disaccharides by *R. eutropha*, as would be expected if it serves as a negative regulator of the expression of the other *agl* genes.

The *agl* genes permit uptake of sucrose by *R. eutropha*. To test our model that the *aglE*, *aglF*, *aglG*, and *aglK* gene products are involved in transport of α-glucosides, we examined the ability of an *R. eutropha* strain harboring pLW200 to incorporate radiolabeled sucrose. These experiments were conducted with *R. eutropha* in order to observe the activity of the *agl* genes outside the context of other *S. meliloti* sucrose transport or hydrolysis systems. As shown in Fig. 2, pLW200, which carries the *agl* genes, is able to promote the uptake of [U-^{14}C]sucrose. This effect is not seen in an isogenic strain carrying the vector pLAFR1 or in a strain harboring pLW249, a derivative of pLW200 which has a Tn*5* insertion in the *aglE* gene. These results suggest that the genes carried on pLW200 encode a functional system for the transport of sucrose.

The *agl* region maps to the *S. meliloti* chromosome. *S. meliloti* has three replicons, the chromosome and two megaplasmids of 1.4 and 1.7 Mb (59). The *agl* region was mapped by Southern hybridization using the method of Finan et al. (19). *Eco*RI-digested genomic DNA from Rm1021, Rm9623, Rm9624, Rm9625, and *A. tumefaciens* strains cured of the Ti plasmid and carrying (i) *S. meliloti* megaplasmid pRmeSU47a, (ii) *S. meliloti* megaplasmid pRmeSU47b, or (iii) no megaplasmid was probed with the insert from pLW201, which contains the C-terminal half of *aglG* and the majority of *aglA*. A strongly hybridizing band of 2.1 kb was observed in the lane containing DNA isolated from Rm1021, and strongly hybridizing bands of approximately 8 kb were present in the lanes containing DNA

from the three *S. meliloti* Tn*5* insertion mutants. No bands were seen in the lanes containing DNA from the *A. tumefaciens* strains, indicating that the locus maps to the *S. meliloti* chromosome and not to one of the megaplasmids.

Identification of Entner-Doudoroff genes. Because all of the cosmids identified in our screen had such a large (~20-kb) overlap, we were interested in whether additional loci involved in sugar metabolism mapped nearby, and we determined the DNA sequence of 7.3 kb downstream of the *agl* region. Several open reading frames were identified. Of particular interest are two loci, *zwf* and *edd*, which appear to encode the first two enzymes of the Entner-Doudoroff pathway. The Entner-Doudoroff pathway is known to be the major pathway for glucose utilization in *S. meliloti* (61), but these loci have not been previously cloned from *S. meliloti*. The activities encoded by *zwf* and *edd* have been detected in the free-living state and in the bacteroid fraction of alfalfa nodules (36). A nitrosoguanidine-induced mutant of *S. meliloti* lacking glucose-6-phosphate dehydrogenase activity has been isolated (11) and found to be Fix^+ on alfalfa, suggesting that this enzymatic activity is not essential during symbiosis or that there may be an additional, developmentally regulated locus encoding this activity.

A small open reading frame between *zwf* and *edd* encodes a protein with homology to *Pseudomonas aeruginosa* Pgl, which is identified in GenBank accession AF029673 as 6-phosphogluconolactonase (41), and we have provisionally named this locus *pgl*. The location of the *pgl* gene between *zwf* and *edd* is interesting, because 6-phosphogluconolactonase acts between glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydratase in the Entner-Doudoroff pathway. *S. meliloti* Pgl also shows homology to the DevB/SOL family of oxidoreductases (57).

Repeat elements and an insertion element in the *agl* region. An unusual feature of this region is the presence of two copies of RIME1, a *Rhizobium*-specific intergenic mosaic element. This repeat element was first identified between *chvI* and *exoS* of *S. meliloti* (49). RIME1 contains two large inverted repeats and was named for its structural similarity to BIME, a repeat element found in enteric bacteria (27). The 5′ copy of RIME1 is found between *aglE* and *aglF*, and the 3′ copy is immediately upstream of the insertion sequence described below.

RIMEs have been previously identified by DNA sequencing in *S. meliloti*, *Rhizobium* sp. strain NGR234, and *Rhizobium leguminosarum* (49). In addition, RIME sequences hybridized to DNA from *Agrobacterium rhizogenes* but not that of *A. tumefaciens* or *Bradyrhizobium japonicum* (49). Although Østerås et al. (49) reported that no copies of RIME were identified on the *Rhizobium* sp. strain NGR234 megaplasmid by Southern hybridization, we were able to identify three copies of RIME1 by performing a BLASTN search of the symbiotic megaplasmid of *Rhizobium* sp. strain NGR234 (22). We also identified additional copies in *Rhizobium trifolii* and the *phoCDET* (6) and *exp* (7) regions of *S. meliloti*. All copies of RIME1 found to date are located in intergenic regions or overlapping the coding region by a few bases.

We have also identified a novel insertion element between *edd* and *aglK*. This insertion element consists of an apparently fragmented reading frame flanked by 39-bp terminal inverted repeats. The nucleotide sequence in this region is highly homologous to the *Rhizobium* sp. strain NGR234 megaplasmid locus *y4zb*, and the *S. meliloti* potential coding region encodes a polypeptide with strong homology (interrupted by in-frame stop codons) to the hypothetical protein Y4zb. Y4zb is thought to be a transposase, but the *y4zb* locus is not flanked by the terminal inverted repeats seen in *S. meliloti*. Both Y4zb and its *S. meliloti* homologue exhibit limited homology to transposases from *Bacillus stearothermophilus* (69) and insertion sequences, and we have therefore tentatively named the locus *tnp*. It is possible that this locus is, or at one time during the evolution of the strain had been, involved in integration or recombination functions.

The 39-bp terminal inverted repeats show homology to intergenic sequences in *S. meliloti* and *R. trifolii*. We observed that this 39-bp sequence is 100% conserved in an intergenic region downstream of *S. meliloti ftsZ* (43). We also found that the same 39 bp are 87% conserved with a sequence between the *S. meliloti* betaine aldehyde dehydrogenase (*betB*) and choline dehydrogenase (*betA*) genes (51) and are 86% conserved with sequence overlapping the stop site of the *Rhizobium trifolii* fructokinase (*frk*) gene (17). These data are shown in Fig. 3. It seems possible that these occurrences of the 39-bp repeat element represent former sites of genomic recombination.

DISCUSSION

We have isolated a cosmid containing *S. meliloti* DNA which is able to promote growth on sucrose, maltose, or trehalose and have obtained Tn*5* insertions in the cosmid which abolish that ability. DNA sequencing led to the identification of five possible protein coding regions within the boundaries defined by Tn*5* insertions. We have demonstrated that the cosmid promotes the uptake of radiolabeled sucrose by *R. eutropha* and that *S. meliloti agl* mutants are affected for growth on α-glucosides. Considering these data and incorporating inferences made about these proteins on the basis of their deduced amino acid sequences, we propose that the AglEFGK proteins form a periplasmic-binding-protein-dependent transport system for α-glucoside disaccharides and that AglA is an α-glucosidase active on the disaccharides trehalose, sucrose, and maltose. Although *agl* mutants grow more slowly on α-glucoside disaccharides, they can still utilize these carbon sources and our model therefore accounts for these observations by including at least one additional mechanism for transporting and cleaving sucrose, maltose, and trehalose.

If our model is correct and *S. meliloti* possesses more than one glycanase which is able to cleave sucrose, maltose, and trehalose, it would not be the first example of redundant glycanase activity in *S. meliloti*. *exoK* mutants, which are deficient in the production of an extracellular glycanase active on the acidic exopolysaccharide succinoglycan, still possess succinoglycan-cleaving activity. Three loci, *exsH*, *prsD*, and *prsE*, were found to encode the second glycanase and the system required to export the glycanase (71). The *S. meliloti* genome is approximately 6.6 Mb, severalfold larger than the genome of *Haemophilus influenzae* (21), and so the existence of redundant functions is perhaps not too surprising.

The *agl* genes encode one of the first periplasmic-binding-protein-dependent transport systems identified in *S. meliloti*. Gage and Long have identified a gene encoding an *S. meliloti* periplasmic binding protein required for transport of α-galactosides (24). The *agpA* (α-galactoside permease) gene that they have found is homologous to *opp* genes encoding oligopeptide permeases (34). A Tn*phoA* insertion in *agpA* renders *S. meliloti* unable to grow on α-galactosides but does not affect the symbiotic properties of the strain. To test whether removal of both the *agp* and *agl* systems would affect symbiosis, we constructed double mutants which are disrupted in the genes encoding the α-galactoside binding protein (*agpA*) and either the proposed α-glucoside binding protein (*aglE*) or glycosyl hydrolase (*aglA*). Both the *agpA aglE* and *agpA aglA* strains are still able to utilize α-glucosides and are Fix^+. In addition to the periplasmic-binding-protein-dependent systems for α-glucosides and α-gal-

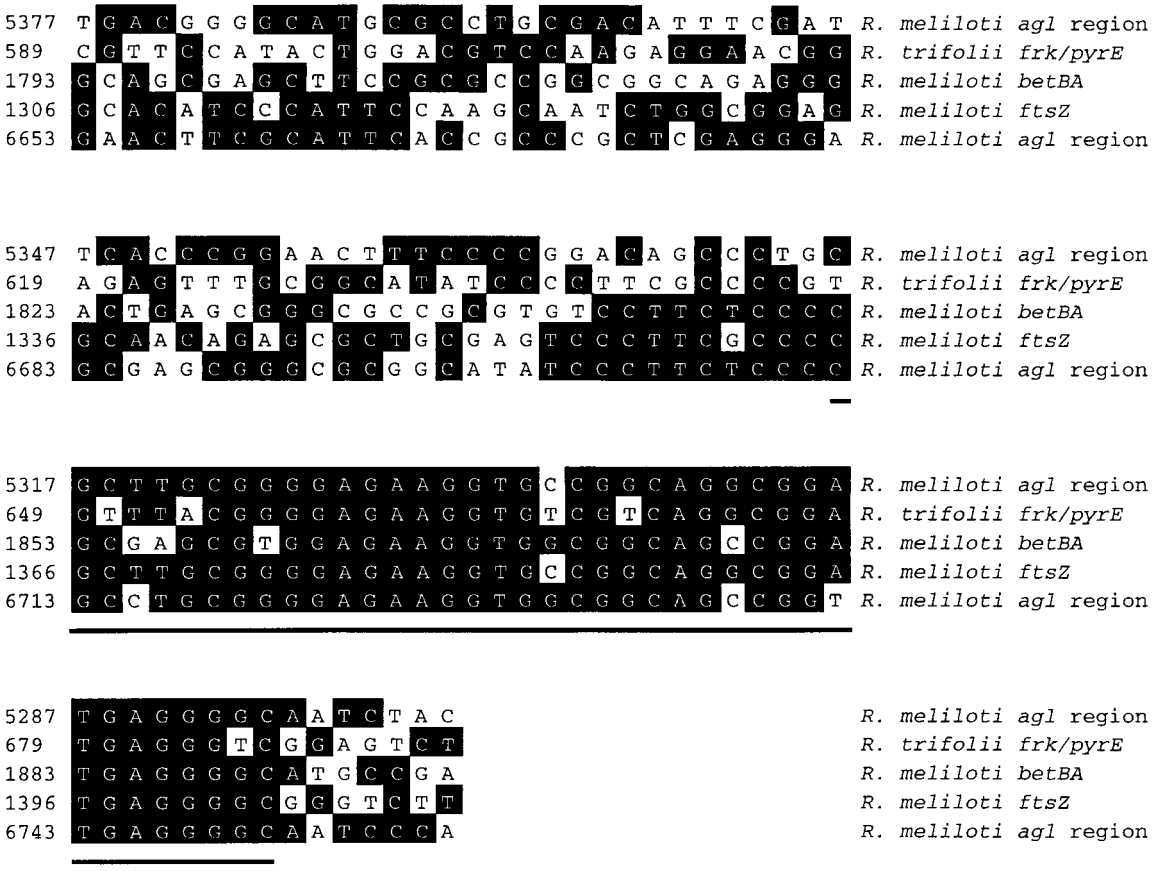

FIG. 3. Alignment of the intergenic regions of *S. meliloti agl* region, *S. meliloti ftsZ* (L25440), *R. trifolii frk pyrE* (U08434) and *S. meliloti betBA* (U39940). The 39-bp terminal repeat element which flanks *tnp* in the *S. meliloti agl* region is underlined.

actosides, *S. meliloti* appears to contain a periplasmic-binding-protein-dependent system for fructose uptake. Williams et al. showed that an *S. meliloti* periplasmic protein cross-reacts with an antibody raised against the *A. radiobacter* fructose binding protein (67). However, the genes encoding the presumed fructose transport system have not been identified.

One of the most novel inferences to come out of this work is that *aglE* may encode a periplasmic binding protein with specificity for sucrose, maltose, and trehalose. If our model is correct, to our knowledge we have identified the first bacterial periplasmic sugar binding protein which specifically binds to sucrose, maltose, and trehalose. Although the deduced protein AglE shares only weak homology with *E. coli* MalE, the region of highest homology is adjacent to residues known to be involved in substrate binding. The region of starch filled cells in interzone II-III of alfalfa nodules (64) raises the possibility that the starch breakdown product maltose could be a carbon source for bacteroids. Our model also implies that the uncharacterized *Synechocystis* proteins Slr0529, Slr0530, and Slr0531 are involved in sugar transport. Sucrose and trehalose are known to be transported by *Synechocystis* sp. strain PCC6803 (47), but no genes have yet been assigned to those functions.

We have characterized the DNA region which lies downstream of the *agl* genes and found several open reading frames which appear to encode enzymes involved in oxidation-reduction reactions. Although only the *agl* genes, located in the central 7 kb of the approximately 20 kb found in all the cosmids isolated in this work, were shown to be required for α-glucoside utilization, the *zwf* and *edd* genes are proposed to be involved in metabolism of the monosaccharides produced after cleavage of sucrose, trehalose, and maltose. *zwf* and *edd* are the first examples of Entner-Doudoroff genes cloned from *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, or *Azorhizobium*. The substrates for the products of *pgl* and *ordL* are unknown.

The presence of two copies of RIME1 in this region is intriguing. No one knows the function or origin of RIME1, but it could represent leftover termini from transposition or cross-species lateral transfer events. The inverted repeats of RIME could act as a binding site for a regulatory protein. If RIME1 is able to form hairpins, and these hairpins act as transcriptional terminators, it is possible that premature termination could occur at RIME1, leading to an abundance of truncated transcript containing only *aglE* and a paucity of full-length transcripts. This hypothesis is interesting because it has been shown that *E. coli* periplasmic substrate binding proteins such as MalE are approximately 30 times as numerous in the cell as their cognate integral membrane permeases (10). This disparity is thought to reflect transcriptional differences, and a REP repeat element found between *malE* and *malF* is believed to be involved. Alternatively, the difference could be accounted for by differences in ribosome binding site strength. As more genomes are sequenced, several repeat elements and mosaic elements will undoubtedly be found and may prove useful as tools for taxonomic studies.

Because *S. meliloti* mutants disrupted in *agl* genes encoding the putative transport system are severely affected in their

growth on α-glucosides, it appears that *aglEFGK* encode the major system for transport of these sugars. These results are consistent with the report that *S. meliloti* uses the same uptake system to transport sucrose, maltose, and trehalose (30). Identification of the sucrose utilization system encoded by the *agl* genes should help to make it possible to screen for mutants defective in sucrose utilization and thus evaluate the importance of sucrose metabolism during the nodulation process. Indeed, preliminary results indicate that it is possible to isolate mutants of either *aglA* or *aglE* strains that are unable to metabolize the α-glucosides sucrose, maltose, and trehalose.

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