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Fructose Uptake in *Sinorhizobium meliloti* Is Mediated by a High-Affinity ATP-Binding Cassette Transport System

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By transposon mutagenesis, we have isolated a mutant of *Sinorhizobium meliloti* which is totally unable to grow on fructose as sole carbon source as a consequence of its inability to transport this sugar. The cloning and sequencing analysis of the chromosomal DNA region flanking the Tn*phoA* insertion revealed the presence of six open reading frames (ORFs) organized in two loci, *frcRS* and *frcBCAK*, transcribed divergently. The *frcBCA* genes encode the characteristic components of an ATP-binding cassette transporter (FrcB, a periplasmic substrate binding protein, FrcC, an integral membrane permease, and FrcA, an ATP-binding cytoplasmic protein), which is the unique high-affinity (K_m of 6 μM) fructose uptake system in *S. meliloti*. The FrcK protein shows homology with some kinases, while FrcR is probably a transcriptional regulator of the repressor-ORF-kinase family. The expression of *S. meliloti frcBCAK* in *Escherichia coli*, which transports fructose only via the phosphotransferase system, resulted in the detection of a periplasmic fructose binding activity, demonstrating that FrcB is the binding protein of the Frc transporter. The analysis of substrate specificities revealed that the Frc system is also a high-affinity transporter for ribose and mannose, which are both fructose competitors for the binding to the periplasmic FrcB protein. However, the Frc mutant was still able to grow on these sugars as sole carbon source, demonstrating the presence of at least one other uptake system for mannose and ribose in *S. meliloti*. The expression of the *frcBC* genes as determined by measurements of alkaline phosphatase activity was shown to be induced by mannitol and fructose, but not by mannose, ribose, glucose, or succinate, suggesting that the Frc system is primarily targeted towards fructose. Neither Nod nor Fix phenotypes were impared in the Tn*phoA* mutant, demonstrating that fructose uptake is not essential for nodulation and nitrogen fixation, although FrcB protein is expressed in bacteroids isolated from alfalfa nodulated by *S. meliloti* wild-type strains.

Carbohydrates represent one of the major structural building blocks of all organisms. In bacterial cells, three energy-dependent sugar uptake mechanisms have been characterized. One that is widely used operates by proton symport (19); this system belongs to the major facilitator superfamily MFS (43) and is used in *Escherichia coli* for the uptake of galactose, xylose, and lactose. A second system, the phosphoenolpyruvate:sugar phosphotransferase system (PTS), is found in many bacteria (44) and is the main transporter for glucose, fructose, mannose, and sucrose in many gram-negative bacteria (41). A third transport mechanism found in all three kingdoms is the periplasmic binding protein-dependent ATP-binding cassette (ABC)-type carrier (4). In bacteria, the ABC superfamily transports a wide range of substrates, including a variety of monosaccharides like arabinose or ribose as well as disaccharides such as maltose and tri- or higher oligosaccharides (43).

Sinorhizobium meliloti is a gram-negative aerobe that exists either as a free-living heterotrophic bacterium in the soil and the rhizosphere or as an endosymbiotic bacteroid within *Medicago* nodules. As a free-living cell, *S. meliloti* utilizes a wide variety of sugars as sole carbon substrates (51), including sucrose, glucose, and fructose, which are among the main photosynthates transported to the root nodules of its plant host, alfalfa (13). In addition, since these carbohydrates can be excreted by the roots, they are available to *S. meliloti* for the colonization of the rhizosphere (52). At the symbiotic stage, it is clearly established that the dicarboxylic acids are the energy source supplied to the nitrogen-fixing bacteroids within the nodule (56, 64), but other compounds might be taken up, albeit at a slower rate, and used as the carbon source for cellular biosynthetic activities. If *S. meliloti* mutants deficient in the C_4-dicarboxylate *dct* transport system are Fix-deficient, they are not affected in the root hair invasion and bacteroid differentiation processes, indicating that other compounds supplied by the plant are available to the bacteria in the infection thread where proliferation still occurs (57). It is thus possible that hexoses are metabolized during the nodulation process, but as mutants of *Rhizobium leguminosarum* lacking enzymes of hexose catabolism are able to form effective nitrogen-fixing nodules, it is generally accepted that these sugars are not essential for symbiosis (18). However, this might depend on the plant, as the various host-dependent symbiotic phenotypes of *Rhizobium* spp. gluconeogenesis-deficient mutants indicate that some plants supply only gluconeogenic substrates in the infection thread, while others supply compounds that can be metabolized by these mutants (12, 29, 36). Furthermore, a fruc-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
S. meliloti		
RCR2011	SU47 (wild-type)	42
Rm5000	SU47 *rif-5*	10
Rm1021	SU47 *str-21*	30
UNA186	Rm5000, *frcC*::Tn*phoA*	This work
E. coli		
DH5α	F^- *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1*Δ(*argF-lacZYA*)	Bethesda Research Laboratories
XL_2Blue	*recA1 endA1 gyrA96 thi-1 hsd17 supE44 relA1 lac* (F′ *proAB lacI*^{q} *Z*Δ*M15* Tn*10*(Tet^r) Amy Cam^r)	Stratagene
S17.1	*E. coli* 294, with RP4	48
A. tumefaciens		
GM19023	C58 cured of pAtC58 and pTiC58	42
At125	GM19023 pRmeSu47b	11
At128	GM19023 pRmeSu47a	11
Plasmids		
pBluescript SK II(−)	Derivative of pUC19 with f1(−), *oriR*, Amp^r	Stratagene
pLAFR1	IncP cosmid cloning vector, Tet^r	14
pMTn*phoA2*	pSUP102::Tn*phoA*, Tet^r Amp^r Neo^r	32
pMR20	RK2-based cloning vector, Tet^r	21
pBSFru	pBluescript, 14-kb *Stul-Eco*RI fragment with *frc* genes of *S. meliloti*	This work
pMRFru	pMR20, 4.4-kb *Kpn*I-*Nru*I fragment with *frcBCAK* genes of *S. meliloti*	This work
pMRF2	pMR20, 4.4-kb *Hin*dIII-*Nru*I fragment with *frcBCAK* genes of *S. meliloti*	This work

tokinase mutant of *R. meliloti* has been reported to be defective in nitrogen fixation (6).

In gram-negative bacteria, the transport of fructose usually occurs by a fructose-specific PTS with the production of fructose-1-phosphate, and it shows several features that are not found in the PTS of other sugars (22). Principally, it has its own phosphate-carrier FPr, whereas all other systems use HPr to transfer phosphate between enzyme I and the sugar-specific enzyme II. In contrast, in *Rhizobium* spp., fructose uptake does not seem to be PTS dependent, as the sugar is not phosphorylated during transport (15, 17). Furthermore, the inability of *R. meliloti* and *R. leguminosarum* mutants lacking fructokinase or phosphoglucose isomerase activity to grow with fructose as sole carbon source indicates that this sugar is accumulated in its nonphosphorylated form. In fact, fructose catabolism occurs by phosphorylation into fructose-6-phosphate and conversion into glucose-6-phosphate, prior to its metabolization via the Entner-Doudoroff pathway known to be present in these microorganisms (8, 15, 17). A periplasmic fructose-binding protein (FBP) has been detected recently in *Agrobacterium radiobacter*, a member of the family *Rhizobiaceae*, and Western blotting with antiserum to FBP has shown the presence of an immunologically similar protein in *S. meliloti* and *R. leguminosarum* (60). However, the structure of the fructose transport system has not been investigated.

We report here the molecular characterization of a high-affinity fructose-binding ABC transporter in *S. meliloti*. A mutant of this system has lost the ability to transport fructose and cannot use it for growth, demonstrating that fructose assimilation occurs only through this carrier system. In addition, we found that mannose and ribose are also transported by this system, but the mutant retains the capacity to use these compounds as sole carbon source for growth. We have also begun to study the regulation of the fructose operon in free-living cells using the *phoA* reporter gene, and we have shown induction by fructose or mannitol and catabolite repression by succinate.

MATERIALS AND METHODS

Bacteria, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are presented in Table 1. All strains were routinely grown in complex Luria-Bertani (LB) media (27) which was supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ in the case of *S. meliloti* or were grown in defined M9 media (31) containing 0.2% (wt/vol) of the filter-sterilized desired carbon source and supplemented with 1 mM $MgSO_4$, 0.25 mM $CaCl_2$, and 1 μg of D-biotin per ml for the growth of *S. meliloti* strains. When required, antibiotics were added at concentrations previously described (10). Bacterial growth was monitored spectrophotometrically at 600 nm. Indicator plates for alkaline phosphatase activity contained the chromogen 5-bromo-4-chloro-3-indolylphosphate (BCIP) at a concentration of 40 μg/ml.

Genetic techniques. Bacterial matings were performed as previously described (37). A random Tn*phoA* (28) mutagenesis of the *S. meliloti* strain Rm5000 was done using the pMTn*phoA*-2 suicide plasmid (32). The resulting mutants were screened on BCIP indicator plates to identify insertions expressing a functional alkaline phosphatase. The genomic localization of the *frc* genes was determined by probing total DNA from *S. meliloti, Agrobacterium tumefaciens* GMI9023, At125 (GMI9023 pRmeSU47b), and At128 (GMI9023 pRmeSU47a) as described previously (11).

DNA manipulations and plasmid constructions. Standard methods were used for restriction analysis, DNA ligation, and transformation (45). Southern blotting and hybridization were performed according to instructions of the suppliers (Amersham and Promega). To clone the genomic region disrupted by the transposon, chromosomal DNA from strain UNA186 was isolated as described previously (37), digested with *Sac*I, and ligated into pBluescript SK II(−). Transformants were selected on LB medium with kanamycin for plasmids containing the Tn*phoA* transposon (Km^r). The isolated plasmid was designated pG503; it contained an 11-kb *Sac*I fragment. The wild-type *frc* operon was isolated from an *S. meliloti* genomic pLAFR1 library (14) by colony hybridization using a 0.7-kb *frcC* internal probe from pG503. A cosmid that partially overlaps pG503 was identified and designated p29F3. The complete *frc* operon was obtained by ligating the 4.7-kb *Stu*I-*Apa*I fragment from pG503 with the 6-kb *Apa*I-*Eco*RI fragment from p29F3 and cloning the resulting construct into a *Sma*I-*Eco*RI-digested pBluescript SK II(−) plasmid to give pBSFru (Fig. 1). For functional complementation assays, the *Kpn*I-*Nru*I and *Hin*dIII-*Nru*I fragments from

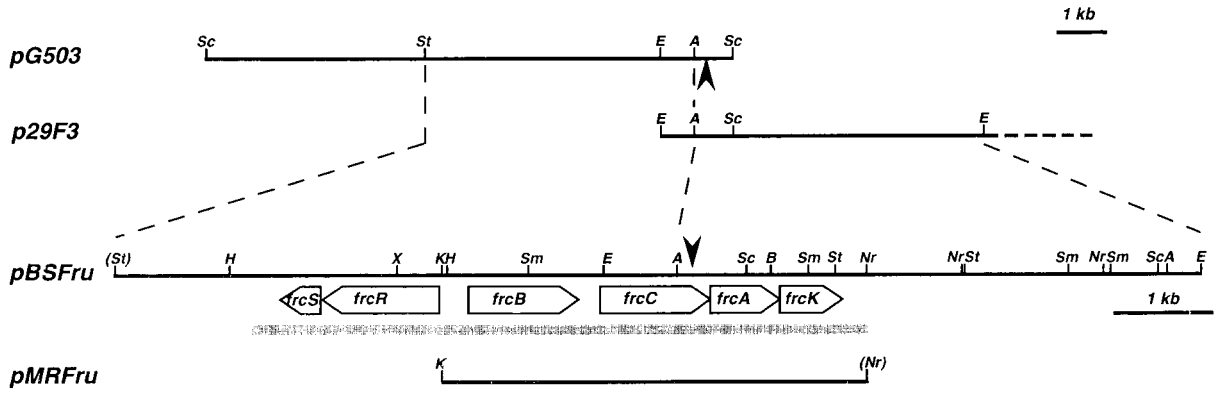

FIG. 1. Physical and genetic map of the pBSFru plasmid containing the *frc* genes of *S. meliloti*. The genes deduced from the nucleotide sequence analysis are represented by open arrows. The construction of pBSFru from plasmids pG503 and p29F3 is shown with dotted lines. The position of the Tn*phoA* insertion is indicated by black arrowheads. The gray bar shows the fragment for which DNA sequence was determined. Restriction sites: A, *Apa*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Nr, *Nru*I; Sc, *Sac*I; Sm, *Sma*I; St, *Stu*I; X, *Xho*I.

pBSFru were cloned into the low-copy plasmid pMR20, resulting in pMRFru and pMRF2, respectively.

DNA sequencing and analysis. The complete DNA sequence of both strands of the *frc* coding region (6,276-bp fragment) was determined after subcloning by using the fluorescent ABI dye-labeled deoxyterminator method of Genome Express (Grenoble, France). DNA and deduced protein sequences were analyzed using Wisconsin Genetics Computing Group (GCG) programs (5) and BLAST protocols (1, 16).

Sugar transport assays. The radioactive sugars [U-^{14}C]fructose (10.2 GBq/mmol), [U-^{14}C]glucose (10.9 GBq/mmol), and [U-^{14}C]sucrose (23.2 GBq/mmol) were purchased from Amersham Corp. Cells grown in M9 medium were harvested at an optical density at 600 nm (OD_{600}) of 0.5 to 0.7, washed twice in carbon-free M9, and resuspended in this medium at a final OD of 0.5. All assays were carried out in duplicate on two independent cultures at 30°C with 1 ml of cell suspension and radioactive substrate (100,000 dpm) at final concentrations of 0.5 to 500 μM for 1 to 10 min. Uptake was determined by rapid filtration through glass microfiber filters (Whatman GF/F) which were rinsed with 3 ml of M9 medium. The radioactivity remaining on the filters was determined with a liquid scintillation spectrometer (model LS6000SC; Beckman Instruments, Villepinte, France). For competition experiments, cold fructose, mannitol, mannose, ribose, xylose, galactose, and glucose were added at a final concentration of 2.5 mM into a 50 μM [U-^{14}C]fructose solution. Competitions were run on a 1-min incubation before filtration.

Alkaline phosphatase assays. Rm5000 and UNA186 strains were grown in M9 medium with the desired carbon source to an OD_{600} of between 0.2 and 0.5 (12 to 20 h). The cells (1 ml aliquots) were collected by centrifugation and resuspended in 0.9 ml of 1 M Tris-HCl (pH 8) buffer. After 10 min of preincubation at 30°C, the reaction was started by the addition of 0.1 ml of a 40-mg/ml *p*-nitrophenyl-phosphate solution in the same buffer. After 15 to 30 min of incubation at 30°C, the reaction was stopped with 0.1 ml of 1 M KH_2PO_4 solution. The OD_{420} and OD_{600} were measured and the alkaline phosphatase activity, given in arbitrary units, was determined as described previously (28, 64).

Immunoblotting. Total cell proteins or periplasmic proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Hybond protein; 0.2-μm pore size) by electroblotting. Immunoblotting was performed using a 1/5000 dilution of a polyclonal serum raised against the FBP of *A. radiobacter* (60). The immunoblots were developed with a Renaissance kit (Dupont/NEN) as instructed by the manufacturer.

Periplasmic protein extractions and binding assays. Cells were grown in LB medium to an OD_{600} of 1.5, collected by centrifugation (10,000 × *g*, 10 min, 20°C), and resuspended in 10 mM Tris-HCl, pH 7.5. Periplasmic proteins were released by cold osmotic shock according to the method of Neu and Heppel (33) and concentrated by ultrafiltration on Microsep 10K membranes (Pall Filtron; Poly Labo, Strasbourg, France). For binding activities, 100 μg of periplasmic proteins were incubated with 0.3 nmol of [U-^{14}C]fructose (3,333 Bq) at 4°C for 24 h and separated by nondenaturing PAGE as described previously (25). The gel was quickly dried and autoradiographed at room temperature using X-Omat S Kodak film during 1 week. Competition assays were done by adding 30 nmol (100-fold excess) of the desired unlabeled sugar to the binding mix.

Nodulation and nitrogen fixation assays. The symbiotic phenotype of the *S. meliloti* mutant strain UNA186 was determined on alfalfa (*Medicago sativa* L., cv. Allegro) seedlings. Plants were grown at 25°C in sterile tubes (three plantlets per tube) containing 20 ml of nitrogen-free nutrient (39) with 0.8% agarose prepared as a slope and inoculated twice, 5 and 10 days after germination, with the appropriate *S. meliloti* strains Rm5000 or UNA186. The number of nodules were determined after 5, 6, and 7 weeks after the second inoculation. Nitrogen fixation activity was determined by C_2H_2 reduction by using a gas chromatograph (ATI-Unicam model 610) equipped with a column of Porapak T as described previously (54). Phenotypes of bacteria recovered from the nodules were checked on the appropriate media. Bacteroids were isolated from harvested nodules, and proteins were extracted following the procedures described previously (11).

Nucleotide sequence accession number. The nucleotide sequence of the *frc* gene has been deposited in the GenBank database and assigned accession number AF196574.

RESULTS

Isolation of an *S. meliloti* Tn*phoA* mutant deficient in fructose transport. In previous work, the survival of *S. meliloti* subjected to desiccation was shown to be improved by the addition of mono- and disaccharides to the cell suspensions prior to air drying (24). In order to study the physiology of this response, we decided to isolate mutants deficient in the uptake of some of these compounds. A random Tn*phoA* mutagenesis was performed on Rm5000, a Phodeficient rifampin-resistant derivative of *S. meliloti* SU47. This transposon contains a reporter gene, *phoA*, which expresses an active alkaline phosphatase when it is fused in frame with a gene encoding a protein localized in the cell envelope (28). A total of 200 mutants displaying phosphatase activity were further screened for growth with different sugars as sole carbon source. One strain, named UNA186, was unable to grow on fructose as sole carbon source, whereas its growth could not be distinguished from that of the parental strain Rm5000 when mannitol or sugars such as sucrose, trehalose, or maltose were present.

The physiological characterization of the mutant in regard to fructose uptake required us first to analyze the parameters of transport of this compound in the wild-type strain. Thus, Rm5000 was grown in M9 medium with mannitol or fructose as sole carbon source, and the uptake was measured using fruc-

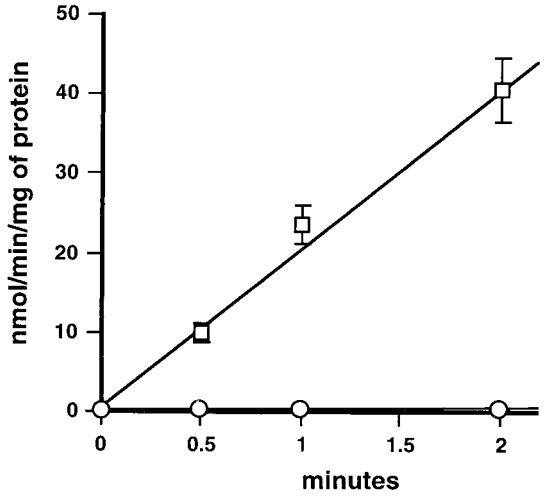

FIG. 2. Fructose uptake activity in *S. meliloti* Rm5000 wild-type (□) and UNA186 Rm::Tn*phoA* mutant cells. The cells were grown in minimal medium M9 with mannitol as carbon source, harvested at an OD_{600} of 0.5, and assayed for uptake of [U-^{14}C]fructose at a final concentration of 10 μM. Values are the mean from duplicates of four independent cultures, with standard errors indicated by bars.

tose concentrations ranging from 0.5 to 500 μM. The apparent K_m of fructose uptake by cells grown in M9 plus mannitol was found to be 6 ± 1 μM with a V_{max} of 32 ± 5 nmol/min/mg of protein, indicating the presence of a high-affinity fructose transport system. Growth in presence of 0.2% fructose did not significantly modify the K_m but resulted in a doubling of the V_{max} (77 ± 6 nmol/min/mg of protein), indicating that the substrate induces its own transport. A time course analysis of fructose uptake in the mutant, performed with a substrate concentration of 10 μM, clearly demonstrated that UNA186 could no longer transport this sugar (Fig. 2). Similar results were obtained for substrate concentrations up to 100 μM. In contrast, transport assays using glucose or sucrose showed that the uptake of these sugars was not altered in UNA186 compared to Rm5000, in agreement with the growth phenotype (data not shown). These results clearly suggested that fructose is transported by a unique high-affinity system in *S. meliloti* which has been inactivated in the mutant strain UNA186.

Cloning and sequence analysis of the fructose transport genes. As an attempt to complement the mutant using a cosmid library of *S. meliloti* (14) was unsuccessful, the mutated region was cloned from the chromosome in order to identify the inactivated gene. Genomic DNA from UNA186 was digested with several restriction enzymes that do not cut the transposon (*Apa*I, *Eco*RV, *Kpn*I, *Pvu*I, *Sac*I, and *Stu*I) and analyzed by Southern blotting using a Tn*phoA*-specific probe. For each digest, one band was found, indicating that only one transposition event had occurred in UNA186. The fragment sizes ranged from 14 to 22 kb, including the 7.7 kb representing the Tn*phoA* fragment (data not shown). The *Sac*I fragment (11 and 7.7 kb) was subsequently cloned, and a restriction analysis of the resulting pG503 plasmid located the transposon at less than 1 kb from the end of the fragment (Fig. 1). The DNA sequence flanking the insertion was determined and shown to belong to a gene encoding a transmembrane protein homologous to permeases from the ABC transporter family. The insertion of the *phoA* reporter gene led to a translational fusion protein. The complete DNA sequence of a 6,276-bp region was determined from the plasmid pBSFru, which was constructed from pG503 and the cosmid p29F3, as neither plasmid contained the entire transport system (see Materials and Methods). Six open reading frames (ORFs) preceded by conserved ribosome-binding sites were identified as forming two putative transcriptional units, composed respectively of two (*frcRS*) and four (*frcBCAK*) genes (Fig. 1). These units are transcribed divergently and separated by 351 bp. Transcriptional coupling is very likely in the case of *frcR* and *frcS*, which are separated by 4 bp only, and for *frcC*, *frcA*, and *frcK*, which are overlapping by one and two nucleotides, while *frcB* and *frcC* are separated by an intergenic region of 290 bp. A GenBank search of this intergenic region did not detect any repeated element that can form a complex secondary structure characteristic of the mosaic element frequently found in *S. meliloti* (35, 37, 61).

The amino acid sequence of the six gene products showed significant homologies with proteins in the databases. The *frcR* gene encodes a 409-amino acid (aa) protein which shares 26 and 24% identity with, respectively, XyIR from *Bacillus subtilis* and NagC of *E. coli*, and, thus, is likely to be a transcriptional regulator of the ROK (repressor, ORF, kinase) family (53). As expected for such a repressor, a recognizable DNA-binding domain that contains a helix-turn-helix motif is identified at the N-terminal domain of FrcR. The *frcS* gene product is a small 142-aa protein similar to *E. coli* FucU (37% identity) and RbsD (23% identity), which are involved in the metabolism of fucose and the D-ribose high-affinity transport system (3, 26), respectively, although the exact function of these proteins is still unclear. The proteins encoded by the genes *frcB* (360 aa), *frcC* (341 aa), and *frcA* (260 aa) are, respectively, the fructose-binding periplasmic protein, the integral membrane protein (permease), and the ATP-binding cytoplasmic protein, which form together the fructose transporter. As in almost every reported case (4), the gene encoding the periplasmic solute binding protein is directly upstream of the associated inner membrane permease gene. The best homology was observed with the characterized ribose transport system from *B. subtilis* (GenBank accession no. Z92953), with 33% (FrcC versus RbsC), 39% (FrcB versus RbsB), and 28% (FrcA versus RbsA) identity. Significantly higher homology was found with the putative ribose uptake genes from *Rhodobacter capsulatus*, another α-subdivision proteobacterium, but this system has been identified only by homology (GenBank accession no. AF010496). The FrcB protein exhibits at the N terminus 25 residues with the characteristics of an export signal peptide, consisting of positively charged amino acids followed by a hydrophobic stretch and a QA/AE sequence similar to the cleavage site LA/AD found in gram-negative bacteria (34). The FrcC protein contains an EAA loop ($EAAX_3GX_9I$) at residue 245, a domain conserved in ABC transporter permeases which is probably involved in the interaction with the ATP-binding subunit (46). Hydropathic profiles determined according to the method of Kyte and Doolittle (23) and analysis for predicting transmembrane helices (50) suggest the presence of 10 membrane-spanning segments in FrcC. The FrcA protein contains the ATP-binding and hydrolysis motifs Walker A (GX_2GXGKS) at residue 39 and Walker B

(X_4DEPT) at residue 80, as well as the linker peptide (LSGGQQ/RQ) at residue 161 and the switch region at residue 202, which are all specific features of the ATP-binding subunits of ABC transporters (4, 46). However, FrcA is a small ATPase, as it contains only 260 aa, whereas most ATP-binding proteins of sugar ABC transport systems are much longer, usually 300 to 500 aa (4). The last protein (206 aa) encoded by *frcK* and located downstream of *frcA* presents some homology with kinases like pantothenate kinase (*B. subtilis* CoaA; 31% identity; GenBank accession no. P54556) and uridine/cytidine kinase (*E. coli* Urk; 25% identity; GenBank accession no. AAC75127). An ATP-binding domain was identified at residues 26 (Walker A motif) and 132 (Walker B), and three additional domains are conserved between the different kinases at residues 102 (PVF), 124 (IVLXEG), and 147 (DYSIFID). The role of this protein in fructose uptake or metabolism is not yet established.

Complementation assays and genomic location of the *frc* locus. To confirm that the absence of fructose uptake observed in UNA186 was caused by the Tn*phoA* insertion in the *frcC* gene, the *frcBCAK* operon was cloned into pMR20, a mobilizable plasmid stable in *S. meliloti*. When transferred into the mutant strain, the resulting plasmid (named pMRFru; Fig. 1) restored normal growth in M9 medium with fructose as sole carbon source. A control with the pMR20 vector alone retained the mutant phenotype. Transport assays performed with a 50 μM concentration of substrate confirmed that fructose was transported only in the UNA186 pMRFru strain. Compared to the wild-type Rm5000, a threefold increase in fructose uptake was observed. Either an increased copy number of the operon or the absence of the putative repressor gene *frcR* on the plasmid could account for such increment. These results clearly demonstrated that the fructose uptake phenotype of UNA186 is caused by the inactivation of the *frcBCAK* operon.

Since the *S. meliloti* genome is characterized by three replicons, a 3.4-Mb chromosome and two megaplasmids of 1.4 Mb (pSyma) and 1.7Mb (pSymb), respectively (49), we wanted to localize the *frc* locus. The hybridization method described by Finan et al. (11) on *A. tumefaciens* strains cured of the Ti plasmid and carrying either the *S. meliloti* pSyma or pSymb megaplasmid was performed using a radiolabeled probe corresponding to the *Eco*RI-*Apa*I fragment from the *frcC* gene (Fig. 1). The probe strongly hybridized to a 4.5-kb fragment present in all three strains, probably corresponding to the *frc* locus of *A. tumefaciens*. No additional hybridization signal representing the *frc* locus from *S. meliloti* could be detected, indicating that the *S. meliloti frc* genes are localized on the chromosome (data not shown).

Substrate specificity of the Frc transporter. As shown by the mutant phenotype and uptake assays, the Frc system is clearly a fructose transporter. However, the structural proteins of Frc exhibit significant homology to proteins of the ribose transport system from other bacteria, like *E. coli* and *B. subtilis*, bacteria in which fructose is transported by a PTS. The possibility that the *S. meliloti* Frc system is also able to transport other compounds in addition to fructose has led to the analysis of its substrate specificity. This was done by using competition assays with the wild-type Rm5000 strain. The uptake of [U-^{14}C]fructose was inhibited by the addition of cold mannose and ribose with, respectively, 91 and 86% inhibition when present in 50-fold excess (Table 2). Mannitol, glucose, galactose, xylose, and sucrose, at the same concentration ratio, had only minor effects on fructose uptake in the competition assays. These results suggest that Frc is also a high-affinity transport system for ribose and mannose. Interestingly, the mutant UNA186 is still able to grow with these two compounds as sole carbon source, which means that in *S. meliloti* ribose and mannose can be transported efficiently by a second carrier, in contrast to fructose.

TABLE 2. Effect of various competitors on fructose uptake

Competitor	Mean % inhibition of uptake[a]
None	0
D-Mannitol	5
D-Glucose	20
D-Galactose	20
D-Sucrose	24
D-Xylose	12
D-Ribose	86
D-Mannose	91
D-Fructose	97

[a] The results are expressed as percent inhibition of fructose uptake and are means of four measurements from two independent experiments, with a variation of less than 5%. Uptake was realized with [U-^{14}C]fructose at 50 μM and 50-fold excess of unlabeled competitors. The control value was 26 nmol of fructose transported/min/mg of protein.

FrcB is a periplasmic FBP. The product of the *frcB* gene is homologous to high-affinity substrate-binding periplasmic proteins, a characteristic of ABC transporters. To demonstrate the presence of a fructose-binding activity in *S. meliloti*, periplasmic protein fractions prepared from Rm5000 were incubated with [U-^{14}C]fructose and separated by nondenaturing PAGE as described in Materials and Methods. The autoradiogram revealed a single radioactive band which totally disappeared when a 100-fold excess of unlabeled fructose was added (Fig. 3A, lanes 1 and 2), showing the presence of only one specific FBP in the periplasmic fluid. Since mannose and ribose are also transported by the Frc system, they were tested for binding. In the presence of a 100-fold excess of unlabeled mannose or ribose, a strong inhibition of the [U-^{14}C]fructose binding activity was observed (Fig. 3A, lanes 3 and 4), while the same excess of sucrose, glucose, galactose, or xylose had no effect (Fig. 3A, lanes 5 to 8), indicating that the specificity of the binding is rather narrow. These results are consistent with the previous uptake competition assays and confirm that *S. meliloti* uses the Frc system for fructose, mannose, and ribose uptake.

The fructose binding activity was still present in the mutant UNA186, as the Tn*pho*A insertion is located downstream of the *frcB* gene (Fig. 1). In order to see if this activity is related to the Frc system, we expressed the *S. meliloti frc* locus in *E. coli*, which transports fructose via the PTS and thus does not show any periplasmic fructose-binding activity. The pMRF2 plasmid, where the *frcBCAK* operon is expressed from the pMR20 *lac* promoter, produced in *E. coli* a strong binding signal at the same position as in Rm5000 (Fig. 3B, lane 3) that was totally absent in the control strain S17.1 pMR20 (Fig. 3B, lane 2). These data demonstrated that FrcB is the high-affinity FBP of *S. meliloti* and that it is correctly addressed in a functional form in *E. coli*.

Interestingly, a polyclonal antibody against an uncharacterized FBP from *A. radiobacter* has previously been shown to

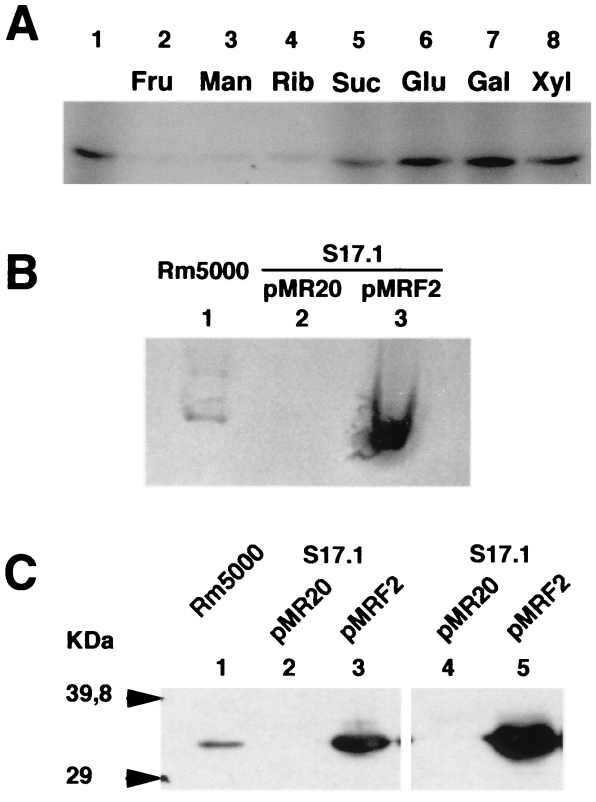

FIG. 3. Fructose binding activity. (A) Autoradiography of periplasmic proteins (100 μg) from *S. meliloti* Rm5000 wild-type strain subjected to 10% nondenaturing PAGE. Proteins from cells grown in LB medium were incubated with [U-^{14}C]fructose (3,333 Bq, 0.3 nmol) in the absence (track 1) or the presence (tracks 2 to 8) of unlabeled competitors (100-fold excess). Results are for fructose, mannose, ribose, sucrose, glucose, galactose, and xylose, respectively, from tracks 2 to 8. (B) Detection of the FBP in periplasmic extracts from *S. meliloti* Rm5000 (track 1) and in *E. coli* strain S17.1 carrying pMR20 (track 2) or pMRF2 which contains the *S. meliloti frcBCAK* operon (track 3). (C) Immunodetection of the FBP in *S. meliloti* Rm5000 (track 1) and in *E. coli* strain S.17.1 (tracks 2 to 5) with antiserum to FBP from *A. radiobacter*. Periplasmic proteins were used in tracks 1, 4, and 5, and total proteins were used in tracks 2 and 3.

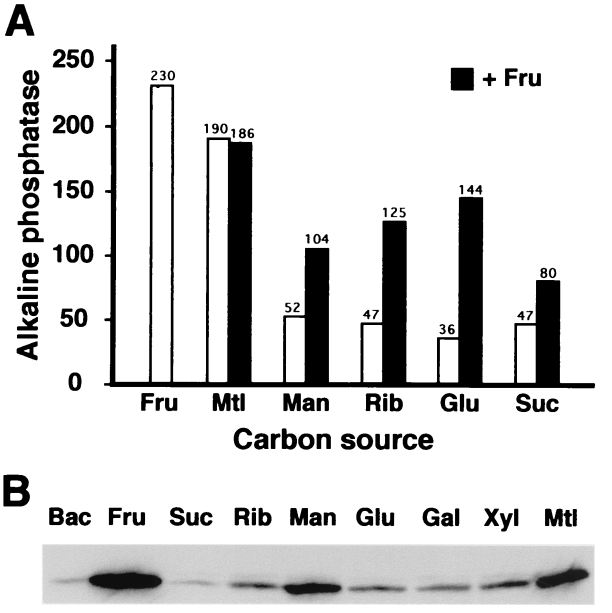

FIG. 4. Analysis of *frcBC* expression. (A) Alkaline phosphatase activity in *S. meliloti* strain UNA186 complemented with pMRFru (Fig. 1) and grown in the presence of various carbon sources (fructose, mannitol, mannose, ribose, glucose, succinate [open bars]) or in the presence of the same carbon sources in combination with fructose (black bars). (B) Immunodetection of FBP (as in Fig. 3C) in total protein extracts from bacteroids isolated from nodules induced by *S. meliloti* Rm5000 on alfalfa (Bac) or from Rm5000 strain grown in fructose, succinate, ribose, mannose, glucose, galactose, xylose, or mannitol.

recognize a protein in *S. meliloti* total extracts (60). This anti-FBP antibody was able to detect by immunoblotting a 35-kDa protein not only in periplasmic protein extracts from Rm5000 (Fig. 3C, lane 1) but also in total and periplasmic extracts from *E. coli* strain S17.1 pMRF2 (lanes 3 and 5, respectively), while no signal was observed with extracts from the S17.1 pMR20 strain (lanes 2 and 4). These data confirmed that the anti-FBP antibody recognizes specifically the FrcB protein, and in addition suggested that the Frc system is probably conserved among members of the family *Rhizobiaceae*.

Regulation of *frcBC* expression by carbon sources. The [U-^{14}C]fructose transport assays have shown that fructose uptake is apparently induced by the presence of the substrate in the growth medium. The expression of the *frcC* gene fused to the *phoA* reporter gene of the Tn*phoA* in the mutant strain UNA186 could be analyzed by measuring the level of alkaline phosphatase activity in cells grown with various carbon sources. However, since this strain is unable to use fructose for growth, we used the UNA186 strain complemented with pMRFru. In the absence of fructose and when compared to the control (cells grown with mannitol), a fourfold repression was observed with mannose, ribose, glucose, and succinate (Fig. 4A). Except in the presence of mannitol, the addition of fructose to the previous carbon sources induced *frcBC* gene expression by 2-fold in the presence of mannose, by about 3-fold in the case of ribose and glucose, and by only 1.7-fold with succinate.

These results were completed by a determination of the FrcB protein level in total protein extracts of *S. meliloti* Rm5000 grown in the presence of different carbon sources. Sodium dodecyl sulfate-PAGE and immunodetection using the *A. radiobacter* anti-FBP antibody showed that the use of fructose as sole carbon source resulted in approximately a twofold increase in FrcB levels compared to cells grown with mannitol and mannose (Fig. 4B). Glucose, galactose, xylose, and ribose were much less efficient inducers, and the presence of succinate as carbon source strongly reduced the FrcB level, suggesting catabolite repression by this compound.

Symbiotic phenotype on alfalfa plants. The symbiotic capacity of the UNA186 mutant was tested on *M. sativa* host plant. Seedlings were inoculated with the mutant strain and Rm5000 wild-type strain as a control, and the nodulation efficiency was monitored during 7 weeks. No difference could be observed in the kinetic of nodulation between UNA186 and Rm5000 strains, and after 7 weeks the weight of nodules obtained with both strains was comparable (approximately 150 μg [fresh

weight] for plants). The acetylene reduction activities observed for UNA 186-nodulated plants were not significantly different from what was measured with Rm5000-nodulated plants, indicating that the nitrogen fixation was not altered (data not shown). Thus, the nodulating and the fixing phenotypes of the UNA186 strain are Nod^+ and Fix^+, indicating that fructose uptake is not required for an efficient symbiosis. However, we wanted to know if the *frc* locus is expressed in the nodules. Bacteroids were isolated from nodules (5 weeks old) induced by the Rm5000 strain, and the FrcB protein level was estimated by immunoblotting. As shown in Fig. 4B, this level was similar to the amount observed in succinate-grown free-living cells, indicating a low expression of the *frc* locus in mature nitrogen-fixing nodules.

DISCUSSION

Until now, very little was known about fructose uptake in *S. meliloti*. Basically, this transport was characterized as an active nonphosphorylating process (15), and more recently a periplasmic protein which is immunologically similar to an FBP from *A. radiobacter* had been detected (60). In this study, we have characterized the *S. meliloti frc* locus, which encodes a high-affinity transport system for fructose, mannose, and ribose. This system displays many of the characteristics of the binding protein-dependent ABC transporter, which involves a soluble periplasmic substrate-binding protein (FrcB), one integral membrane protein (FrcC), and one energy-transducing polypeptide having an ABC (FrcA). These three proteins display the invariably conserved motifs found in bacterial ABC transporters, and to our knowledge the Frc system described here is the first fructose-specific ABC transport system characterized in gram-negative bacteria. Within the ABC superfamily, of the 18 currently recognized families 2 are specific for the uptake of simple carbohydrates (43). The carbohydrate uptake transporter-1 family (CUT1) includes a variety of systems involved in the uptake of di-, tri-, and higher oligosaccharides and polyols, while the members of the transporter-2 family (CUT2) transport only monosaccharides. Within the family *Rhizobiaceae*, the lactose uptake system of *A. radiobacter* and the sucrose, maltose, and trehalose transporter of *S. meliloti*, which is encoded by the *agl* locus, belong to the CUT1 family (59, 61). Protein sequence comparisons revealed that the *S. meliloti* fructose ABC transporter is closely related to the Rbs ribose transporters from *B. subtilis* and *E. coli*, which are members of the CUT2 carbohydrate transporter family (43). The putative Rbs ribose transporter of another member of the α-subgroup of proteobacteria, *R. capsulatus*, also shares a high homology with the *S. meliloti* Frc transporter (58). However, it should be noted that *R. capsulatus* has a fructose-specific PTS permease, unlike *S. meliloti* (63). The close relationship between the *S. meliloti* fructose ABC transporter with the ribose transporter from other bacteria suggests a common origin. This hypothesis is strengthened by (i) the apparent ability of the *S. meliloti* Frc system to transport ribose, as shown by uptake and binding competition experiments (Table 2 and Fig. 3A) and (ii) the presence in the *frc* locus of *frcS*, a gene encoding a homolog to RbsD, a protein of unclear function located in the ribose uptake operon of *B. subtilis* and *E. coli*, in addition to the three classical components of an ABC transport system (3, 62). Note that FrcS also shows high homology with FucU, a protein which does not belong to a transport system and is found in the fucose metabolic operon of *E. coli* (26). The function of these proteins is still unknown, but the relative locations of *frcS* in *S. meliloti* and *fucU* in *E. coli*, which are close to the putative transcriptional regulators *frcR* and *fucR* of their respective systems, suggest that they might some how be involved in the regulation of these systems. Transcription of the genes encoding the components of ABC transporter is usually regulated by repressors (7) and, in some cases, by activators (4). The gene encoding such a regulatory protein is either included in the operon as the first gene, or it is located on the complementary strand upstream of it (4). The putative regulator of the *S. meliloti* fructose transporter, FrcR, is of the second type, as its gene is located upstream of the structural genes *frcBCAK* and it is transcribed divergently (Fig. 1). The FrcR protein belongs to the ROK family, which includes transcriptional repressors for operons involved in sugar metabolism (53).

ABC transporter genes are often expressed together with genes involved in the metabolism of the transported compound (38). The presence of *frcK* encoding a putative kinase downstream of *frcA* suggests that FrcK may be involved in fructose metabolism. It is noteworthy that the ribose transporter of both *E. coli* and *B. subtilis* is also encoded by an operon which similarly contains the ribokinase gene, *rbsK* (4, 62). However, the FrcK protein from *S. meliloti* shows no similarities to known ribokinases or fructokinases. As the fructokinase of *R. leguminosarum* has been characterized and shown to be homologous to the ribokinases (9), it is unlikely that FrcK is the fructokinase for *S. meliloti*, and its role remains unclear. The possibility that FrcK is a second ATP-binding protein for the transporter is highly unlikely, as it lacks the signature sequences of the linker peptide and the switch region typical of such proteins (47).

Usually bacteria depend on more than one transport systems to allow access of cytoplasmic enzymes to an exogenous carbon source. Based on the absence of growth of the *S. meliloti* Tn*phoA* mutant deficient in the Frc transporter in presence of fructose as the sole carbon source, it is probably the unique uptake system for fructose in *S. meliloti*. Thus, no other system, such as the phosphoenolpyruvate:sugar PTS or the major facilitator system (MFS) for fructose seems to be present in this bacterium. Although the *S. meliloti* Frc system is also involved in mannose and ribose uptake, as shown by competition experiments (Table 2), analysis of its expression indicates that it is primarily targeted toward fructose. Studies using either the translational *frcC*::*phoA* fusion or the fructose-binding periplasmic protein show that the system is induced by the presence of fructose. Ribose is clearly not an inducer. The case of mannose is more complex, as it has an inducing effect on the FBP level compared to other sugars (Fig. 4B), but no increase is observed in alkaline phosphatase activity (Fig. 4A). This discrepancy may result from the different genetic backgrounds used in the two assays, as the first parameter was determined in the wild-type strain while *phoA* expression was measured in the mutant strain. Mannitol, which is not transported by this system, as indicated by competition experiments, was also a good inducer for the Frc system. This is the result of an indirect effect explained by mannitol metabolism into fructose, due to

mannitol dehydrogenase activity. The induction of the Frc system by fructose was not significantly affected by the presence of a second carbon source, except when succinate, and to a lesser extent mannose, were present. In *S. meliloti*, it has previously been suggested that succinate catabolism may have an important regulatory role in carbon utilization (2, 55), and a succinate-repressive effect on mannose and lactose utilization has been shown (2, 20). In contrast to *E. coli*, the genetics of carbon utilization in *S. meliloti* is poorly understood, and the finding that fructose is transported solely by an ABC transport system raises some questions about the regulation of carbon metabolism in this bacterium. In both gram-negative and gram-positive bacteria, proteins of the PTS are involved in the regulation of carbohydrate utilization and control directly the activation or the repression of several catabolic operons in response to inducer availability (40, 44). If no PTS-like system for fructose exists in *S. meliloti* as is strongly suggested by our results, carbon metabolism must be regulated by a different mechanism which is yet unknown and, obviously, requires further studies.

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