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A Monocarboxylate Permease of *Rhizobium leguminosarum* Is the First Member of a New Subfamily of Transporters

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Amino acid transport by *Rhizobium leguminosarum* is dominated by two ABC transporters, the general amino acid permease (Aap) and the branched-chain amino acid permease (Bra). However, mutation of these transporters does not prevent this organism from utilizing alanine for growth. An *R. leguminosarum* permease (MctP) has been identified which is required for optimal growth on alanine as a sole carbon and nitrogen source. Characterization of MctP confirmed that it transports alanine (K_m = 0.56 mM) and other monocarboxylates such as lactate and pyruvate (K_m = 4.4 and 3.8 μM, respectively). Uptake inhibition studies indicate that propionate, butyrate, α-hydroxybutyrate, and acetate are also transported by MctP, with the apparent affinity for solutes demonstrating a preference for C_3-monocarboxylates. MctP has significant sequence similarity to members of the sodium/solute symporter family. However, sequence comparisons suggest that it is the first characterized permease of a new subfamily of transporters. While transport via MctP was inhibited by CCCP, it was not apparently affected by the concentration of sodium. In contrast, glutamate uptake in *R. leguminosarum* by the *Escherichia coli* GltS system did require sodium, which suggests that MctP may be proton coupled. Uncharacterized members of this new subfamily have been identified in a broad taxonomic range of species, including proteobacteria of the β-subdivision, gram-positive bacteria, and archaea. A two-component sensor-regulator (MctSR), encoded by genes adjacent to *mctP*, is required for activation of *mctP* expression.

Rhizobium leguminosarum is a member of a group of α-subdivision of the proteobacteria (the rhizobia), which form a species-specific symbiotic relationship with leguminous plants in order to fix atmospheric nitrogen. The plant supplies the bacteroid (symbiotic bacteria) with a carbon source (C_4-dicarboxylic acid), while the plant receives reduced nitrogen from the bacteroid. Other rhizobia include *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Bradyrhizobium,* and *Azorhizobium* species (8, 33, 53). Prior to establishing a *Rhizobium*-legume symbiosis, bacteria must thrive in the soil environment, competing with many organisms for nutrients. Transporters of key nutrients, such as amino acids, may give a competitive advantage to rhizobia, allowing them to better colonize roots. This may account for the large number of transporters that have been revealed by the completed genome sequences of *Sinorhizobium meliloti*, *Mesorhizobium loti*, and *Agrobacterium tumefaciens* (13, 26, 59).

Amino acid transport by *R. leguminosarum* is dominated by two permeases of the ABC transporter superfamily, the general amino acid permease (Aap) and the branched-chain amino acid permease (Bra). Aap and Bra are members of the polar amino acid transporter (transport classification [T.C.] number 3.A.1.3) and hydrophobic amino acid transporter (T.C. number 3.A.1.4) families, respectively (21, 44). However, both are atypical as they transport a broad range of amino acids (19, 56). Indeed, Bra has the broadest specificity of any characterized amino acid transporter in bacteria. Bra has a high affinity for L-alanine, L-leucine, L-histidine, and γ-aminobutyric acid (K_m between 78 and 288 nM) and a lower affinity for L-glutamate (56 μM). D-α-Amino acids inhibit uptake of Bra solutes, indicating they are also transported by this permease. Conversely, Aap is specific for L-α-amino acids, with high affinity for L-glutamate, L-alanine, L-leucine, and L-histidine (K_m between 200 nM and 515 nM) (19). Both Aap and Bra are required for optimal growth of free-living *R. leguminosarum* on a range of amino acids as a sole source of carbon and nitrogen. In fact, double *aap bra* mutants cannot utilize glutamate, glutamine, asparagine, proline, arginine, or γ-aminobutyrate (19). However, growth on alanine and histidine is unaffected by the loss of these transporters. Therefore, although alanine and histidine are transported by Aap and Bra, *R. leguminosarum* has other transporters of these amino acids.

The importance of alanine metabolism in *R. leguminosarum* has been highlighted by the demonstration that alanine can comprise as much as 26% of the total nitrogen secreted by bacteroids isolated from pea nodules (1). In the case of soybean bacteroids, it has been reported that alanine is the sole secretion product of nitrogen fixation (58), although this has been challenged (30). Nevertheless, these findings have questioned the established view that ammonium is the sole secretion product of N_2 fixation from the bacteroid. However, it remains to be established how alanine produced by N_2 fixation is secreted from the bacteroid and made available to the plant.

We report here the identification of a novel *R. leguminosarum* permease (MctP), which is required for optimal growth on alanine as a sole carbon and nitrogen source. Characterization of MctP confirmed that it transports alanine and other monocarboxylates such as pyruvate and lactate. However, it is distinct from characterized transporters of monocarboxylates, such as members of the lactate permease family (LctP) (T.C. number 2.A.14) and the proton-linked monocarboxylate porter

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family (T.C. number 2.A.1.13) (14, 44). Although MctP has significant sequence similarity to members of the sodium/solute symporter family (T.C. number 2.A.21) (42, 44), sequence comparisons indicate it is the first characterized permease of a new subfamily of bacterial transporters, with representatives present in gram-negative and gram-positive bacteria and archaea. A two-component sensor-regulator (MctSR), encoded by genes adjacent to *mctP*, is required for *mctP* expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, and culture conditions. The bacterial strains and plasmids used in this study are detailed in Table 1. *R. leguminosarum* strains were grown at 28°C on either tryptone yeast extract (TY) (5), acid minimal salts medium (AMS), or acid minimal salts agar (AMA) (36). The following carbon and nitrogen sources were added to minimal media at the indicated concentrations; 10 mM D-glucose, 10 mM succinate, 20 mM pyruvate, 10 mM ammonium chloride, and 20 mM alanine. The following antibiotics were used at the indicated concentrations (micrograms milliliter^{-1}); streptomycin, 500; kanamycin, 40; tetracycline, 2 (in AMS) and 5 (in TY); gentamicin, 20; and spectinomycin, 100.

Oligonucleotide primers used in this study were as follows: P69, GAGAGAG AACTAGTGGAGGAAGAAAAAATGAGTAAAGGAGAAGAAC; P237, TCACATGCGTTACATCAGCTTCCG; P238, CGCCCTGATCTCTCTCCAG CTTATG; P266, TGTAGAGCTCATCCATGCCATG; P285 TGCAAAATAA TCTTCCTCGTGCGG; P316, GGGGACCACTTTGTACAAGAAAGCTGGG TCTTGGCGCGTTTCCACAGCA; P317, GGGGACAAGTTTGTACAAAAA AGCAGGCTCGGCAGGAGCAACAAGGAGA; P340, AAGCTTCGCCCTG ATCTCTCTCCAGCTTATG; P341, TTCGAACGGCAGGAGCAACAAGGA GA; P342, AAGAGACGGCTCGCGGACATTGTAA; P343, TGGGCGGAG AAGCAGGATCA; P344, TGGAGGCACTCTGCTATCATTTTGC; P345, TCTCCAGTCCCGTTTGGGCA; P353, CGGAAAAGGTTGGGAAAATGA AAAATATAAAATTCAG; P354, CGGAAAAGGTTGGGAAAATGTTTCA TCTCGATACTTTAG; P355, CTGAATTTTATATTTTTCATTTTCCCAACC TTTTCCG; P356, CTAAAGTATCGAGATGAAACATTTTCCCAACCTTTT CCG; P357, CGCGCGTCCCGTAAGTGTAT; P358, TCCCCGGCAATCTTC AATTATT; and P359, AATTATGAATCAATACGCAGGCTTG.

Genetic modification of bacterial strains. All DNA and genetic analyses and Tn*5* mutagenesis were carried out as described previously (57). Transduction was carried out using the lytic phage RL38 as previously described (7). The sequenced region of the *R. leguminosarum* genome that includes the *mctPSR* is shown in Fig. 1, with a representation of fragments located on the plasmids used in this study and the sites of transposon insertion in mutant strains. Plasmids were conjugated into *R. leguminosarum* as described previously (36).

To obtain mutants in *mctSR* and *mctP*, plasmid pRU890 (Table 1) was mutated in vitro by GeneJumper transposon (Km^r) insertion as described by the manufacturer (Invitrogen Life Technologies). Chromosomal insertion mutants were constructed by conjugating the mutated plasmids into *R. leguminosarum* 3841 and selecting for Gm^s Km^r homogenates on TY containing 10% sucrose. All mutants were confirmed by Southern blotting.

Construction of *aapJ* promoter-*gltS*/*gltP* fusion plasmids. In order to ensure that *E. coli gltS* and *gltP* were expressed in *R. leguminosarum*, the *gltS* and *gltP* genes were fused to the *R. leguminosarum aapJ* promoter region by two PCRs using overlapping primers (18). The intergenic region between *aapJ* and the upstream *metC* gene (accession number X82596) was first amplified using primer P357 (which has identity to the *metC* region) and either primer P355 or P356, which have identity to the region immediately upstream of *aapJ* and a 5′ extension with identity to the N-terminal coding region of *E. coli gltP* or *gltS*, respectively. Similarly, *gltP* and *gltS* were amplified using primers specific to sequence downstream of *gltP* or *gltS* (P358 and P359, respectively) and primers specific to the N terminus coding region of *gltP* or *gltS* and the region immediately upstream of *aapJ* (P353 and P354, respectively). The PCR products from these reactions were purified by gel extraction (QIAquick gel extraction kit; QIAGEN Ltd.), mixed (i.e., P357-P355 product with P353-P358 product or P357-P356 product with P354-P359 product), and used as template for a second round of PCR. The second PCRs used primers specific for the *aapJ-metC* intergenic region and a sequence downstream of *gltP* or *gltS* (i.e., P357 with P358 for *aapJ* promoter-*gltP* fusion and P357 with P359 for *aapJ* promoter-*gltS* fusion). The resulting PCR products were cloned by TOPO reaction into pCR2.1 TOPO (Invitrogen Life Technologies), and fusion junctions were confirmed by sequencing, before subcloning into the broad-host-range vector pJP2, using the *Xba*I and *Hin*dIII sites located on the multicloning site of these two vectors. Thus, the resulting plasmids, pRU976 and pRU986, contain *aapJ* promoter-*gltP* and *aapJ* promoter-*gltS* fusions, respectively.

Construction and detection of the *mctP* promoter reporter plasmid. The promoter reporter plasmids used were derived from the pOT family of vectors described previously (2). Another plasmid in this family, pOT2gfp+, has been constructed as part of a separate project, and full details will be described elsewhere. Essentially, the *gfp*$^+$ gene was amplified from pMN402 using primers P69 and P266 and cloned into pCR2.1 TOPO. A *Spe*I/*Xho*I fragment from this was used to replace the same fragment in pOT2, creating pOT2gfp+. *gfp*$^+$ has been compared directly against *lacZ* as a reporter gene and shown to give almost identical results (47). Plasmids pOT2 and pOT2gfp+ were modified for Gateway technology by cloning the Gateway vector conversion cassette (reading frame A; Invitrogen Life Technologies) into the two *Sma*I sites in the multicloning site to create pOT3gfpuv and pOT3gfp+. The orientation of the Gateway cassette was confirmed by DNA sequencing.

The *mctR-mctP* intergenic region, which contains a putative *mctP* promoter, was amplified by PCR using primers P316 and P317. To create a Gateway entry clone (pRU917), the resulting PCR product was cloned into pDONR201 by using the Invitrogen Life Technologies BP cloning mixture (Int and IHF). The *mctP* promoter reporter plasmids (pRU922 and pRU923) were formed from pRU917 and either pOT3gfpuv or pOT3gfp+ by using the Invitrogen Life Technologies LR cloning mixture (Int, IHF, and Xis).

Gfp$^+$ expression in bacterial cultures was quantified in microtiter plates with a GENios plate reader (Tecan), using a 485-nm-wavelength excitation filter and a 510-nm-wavelength emission filter. Cell optical density was measured at 595 nm. The data are expressed as relative fluorescence (fluorescence emission at 510 nm/optical density at 595 nm).

Transport assays. *R. leguminosarum* uptake assays were performed by the rapid filtration method as previously described (35). Uptake assays were performed with a final concentration of 500 μM (0.5 μCi) L-[U-^{14}C]alanine, 500 μM (0.5 μCi) [1-^{14}C] acetate, 5 μM (0.125 μCi) L-[U-^{14}C]lactate, or 5 μM (0.125 μCi) [1-^{14}C] pyruvate. Competing solutes were added to a final concentration of 5 mM for inhibition of alanine or 0.5 mM for inhibition of lactate or pyruvate. The kinetic constants of solute uptake by *R. leguminosarum* strains were determined using various ^{14}C-labeled-solute concentrations in standard uptake assays.

To determine the affect of sodium on solute uptake, transport assays were carried out in sodium-free RMS (10 mM potassium phosphate buffer [pH 7], 1 mM $MgSO_4$). Sodium chloride or lithium chloride was added to a final concentration of 5 mM.

Plant nodulation phenotype. Seeds of *Pisum sativum* cv. avola were surface sterilized and then added to cotton wool-plugged 250-ml conical flasks containing 230 ml of sterile vermiculite, which was wetted with nitrogen-free rooting solution (34). The seeds were inoculated with a bacterial culture, and the flask was wrapped with foil to exclude light from the roots. Plants were incubated at 25°C in a grow room with illumination provided by a Philips Sont-Agro grow light. When the shoot reached the cotton wool plug it was pulled through. Four weeks after inoculation, plants were harvested and acetylene reduction was carried out on whole plants as described previously (51). Sample nodules were removed, surface sterilized in calcium hypochlorite (0.7%), and crushed, and bacteria were streaked on TY. Isolated bacteria were then replica plated and screened for appropriate antibiotic and nutritional markers.

Nucleotide sequence accession number. The sequence of the region of the *R. leguminosarum* genome containing the *mct* operon was submitted to the EMBL database under accession no. AJ421944.

RESULTS

Identification of a novel alanine transporter in *R. leguminosarum*. To identify the pathways of alanine catabolism, an *R. leguminosarum* Tn*5* library (approximately 15,000 mutants) was screened for growth on 20 mM alanine as a sole carbon and nitrogen source. The characterization of one alanine growth mutant with an insertion in *dadR*, which encodes a regulator of alanine catabolic genes (*dadX* and *dadA*), has been reported previously (1). Another mutant of interest was RU1180, as it formed only small colonies on alanine minimal medium, indicating a decreased growth rate. This mutant also displayed slow growth on pyruvate-ammonia minimal medium, but grew as wild type on glucose-ammonia and succinate-am-

TABLE 1. Bacterial strains, cosmids, and plasmids used in this study

Strain, cosmid, or plasmid	Description	Source or reference
Strains		
3841	*Rhizobium leguminosarum bv. viciae* Sm^r derivative of strain 300	24
A34	*Rhizobium leguminosarum bv. viciae* (formerly known as 8401 pR11JI)	10
VF39SM	*Rhizobium leguminosarum bv. viciae* Sm^r	38
RU1357	A34 *braE*::Tn*phoA* Δ*aapJQM*::ΩSp	20
RU1180	3841 *mctP*::Tn*lacZ*	This study
RU1580	pRU895 homogenote in 3841; *mctS*::GeneJumper Km^r transposon	This study
RU1581	pRU897 homogenote in 3841; *mctP*::GeneJumper Km^r transposon	This study
RU1582	pRU898 homogenote in 3841; *mctR*::GeneJumper Km^r transposon	This study
RU1583	pRU899 homogenote in 3841; *mctR*::GeneJumper Km^r transposon	This study
RU1584	pRU900 homogenote in 3841; *mctS*::GeneJumper Km^r transposon	This study
RU1585	pRU901 homogenote in 3841; *mctR*::GeneJumper Km^r transposon	This study
RU1586	pRU902 homogenote in 3841; *mctS*::GeneJumper Km^r transposon	This study
RU1587	pRU903 homogenote in 3841; *mctS*::GeneJumper Km^r transposon	This study
RU1601	3841 pRU923	This study
RU1602	RU1580 pRU923	This study
RU1603	RU1581 pRU923	This study
RU1604	RU1582 pRU923	This study
RU1632	RU1357 pRU976	This study
RU1633	RU1357 pRU986	This study
Cosmid		
pRU3147	pLAFR1 cosmid containing *mct* region from strain 3841	This study
Plasmids		
pCR2.1 TOPO	Cloning vector for PCR products; Km^r, Amp^r	Invitrogen Life Technologies
pCR-Blunt-TOPO	Cloning vector for PCR products; Km^r	Invitrogen Life Technologies
pDONR201	PCR cloning vector for Gateway Technology	Invitrogen Life Technologies
pJQ200SK	pACYC derivative, P15A origin of replication; Gm^r	39
pLAFR1	Wide-host-range mobilizable P-group cloning vector; Tc^r	11
pPHJI1	P-group chaser plasmid	16
pRK415-1	Broad-host-range P-group cloning vector; Tc^r	27
pJP2	pTR102 GUS (mini-RK2 derivative) with artificial MCS; Amp^r Tc^r	37
pMN402	GFP+ expression vector; hygromycin resistance	47
pOT2	Promoter probe vector with promoterless *gfpUV*; Gm^r	2
pOT2GFP+	Promoter probe vector with promoterless *gfp*$^+$; Gm^r	This study
pOT3GFPuv	pOT2 with Gateway vector conversion cassette (reading frame A; Invitrogen Life Technologies) cloned into *Sma*I site	This study
pOT3GFP+	pOT2GFP+ with Gateway vector conversion cassette (reading frame A; Invitrogen Life Technologies) cloned into *Sma*I site	This study
pRU738	2-kb PCR product (primers P237 and P238; *mctP*) from pRU3147 cloned into pCR2.1-TOPO	This study
pRU739	2-kb *Eco*RI fragment from pRU738 in pRK415-1 with *mctP* under the control of the *lac* promoter	This study
pRU747	2-kb *Eco*RI fragment from pRU738 in pRK415-1 with *mctP* not under the control of the lac promoter	This study
pRU785	3.6-kb *Sal*I fragment from pRU3147 in pRK415-1 with *mctRP* not under the control of the lac promoter	This study
pRU788	3.6-kb *Sal*I fragment from pRU3147 in pRK415-1 with *mctRP* under the control of the lac promoter	This study
pRU887	4.8-kb PCR product (primers P238 and P285; *mctSRP*) from pRU3147 cloned into pCR2.1-TOPO	This study
pRU890	pJQ200SK containing a 4.4-kb *Bam*HI fragment from pRU887	This study
pRU895	pRU890 *mctS*::GeneJumper Km^r transposon	This study
pRU897	pRU890 *mctP*::GeneJumper Km^r transposon	This study
pRU898	pRU890 *mctR*::GeneJumper Km^r transposon	This study
pRU899	pRU890 *mctR*::GeneJumper Km^r transposon	This study
pRU900	pRU890 *mctS*::GeneJumper Km^r transposon	This study
pRU901	pRU890 *mctR*::GeneJumper Km^r transposon	This study
pRU902	pRU890 *mctS*::GeneJumper Km^r transposon	This study
pRU903	pRU890 *mctS*::GeneJumper Km^r transposon	This study
pRU917	PCR product (primers P316 and P317) containing the putative *mctP* promoter region from pRU3147 in pDONR201	This study
pRU922	*mctP* promoter region from pRU917 in pOT3GFPuv	This study
pRU923	*mctP* promoter region from pRU917 in pOT3GFP+	This study
pRU935	1.8-kb PCR product (primers P345 and P343; *mctS*) from pRU3147 cloned into pCR-Blunt II-TOPO	This study
pRU936	2.7-kb PCR product (primers P342 and P343; *mctSR*) from pRU3147 cloned into pCR-Blunt II-TOPO	This study

Continued on following page

TABLE 1—*Continued*

Strain, cosmid, or plasmid	Description	Source or reference
pRU941	2.3-kb PCR product (primers P340 and P341; *mctP*) from pRU3147 cloned into pCR2.1-TOPO	This study
pRU942	1.5-kb PCR product (primers P342 and P344; *mctR*) from pRU3147 cloned into pCR2.1-TOPO	This study
pRU947	2.3-kb *Hin*dIII fragment from pRU941 in pRK415-1 with *mctP* under the control of the *lac* promoter	This study
pRU948	2.3-kb *Hin*dIII fragment from pRU941 in pRK415-1 with *mctP* not under the control of the *lac* promoter	This study
pRU950	1.8-kb *Eco*RI fragment from pRU935 in pRK415-1 with *mctS* under the control of the *lac* promoter	This study
pRU951	1.5-kb *Eco*RI fragment from pRU942 in pRK415-1 with *mctR* under the control of the *lac* promoter	This study
pRU952	2.7-kb *Eco*RI fragment from pRU936 in pRK415-1 with *mctSR* under the control of the *lac* promoter	This study
pRU953	1.5-kb *Eco*RI fragment from pRU942 in pRK415-1 with *mctR* not under the control of the *lac* promoter	This study
pRU974	2.7-kb *Eco*RI fragment from pRU936 in pRK415-1 with *mctSR* not under the control of the *lac* promoter	This study
pRU971	1.8-kb PCR product (primers P353, P355, P357 and P358; *aapJ* promoter-*gltP* fusion) cloned into pCR2.1-TOPO	This study
pRU976	1.8-kb *Xba*I/*Hin*dIII fragment from pRU971 (*aapJ* promoter-*gltP* fusion) in pJP2	This study
pRU980	1.8-kb PCR product (primers P354, P356, P357 and P359; *aapJ* promoter-*gltS* fusion) cloned into pCR2.1-TOPO	This study
pRU986	1.8-kb *Xba*I/*Hin*dIII fragment from pRU976 (*aapJ* promoter-*gltS* fusion) in pJP2	This study

monia. To confirm that the alanine growth defect of this mutant is tightly linked to the transposon insertion, the transposon was transduced into wild-type 3841 and eight kanamycin-resistant transductants were tested for growth on alanine or glucose as the sole carbon source. These transductants displayed the same slow growth on alanine observed in the original mutant.

Mutant RU1180 was characterized by cloning the genome fragment containing the transposon and sequencing the region flanking the transposon insertion. BLAST (3) searches identified similarity to sodium/solute symporters, including the sodium/proline symporter (ecPutP) and sodium/pantothenate symporter (ecPanF) from *E. coli* (22, 31, 43). Mutant RU1180 was complemented for growth on alanine by cosmid pRU3147, and direct sequencing of this cosmid confirmed it contained the region of DNA corresponding to the transposon insertion site of RU1180. Further sequence of this region was obtained (Fig. 1) and submitted to the EMBL database under accession number AJ421944. The sequence confirmed that the gene inactivated in RU1180 encodes a putative permease with significant identity to members of the sodium/solute symporter family (T.C. number 2.A.21 [44]).

To confirm the role of this permease in alanine uptake, the transport properties of the wild type, RU1180 and RU1180 pRU3147 were compared (Fig. 2). Alanine uptake rates were lower in RU1180 than in wild-type 3841 and enhanced by the cosmid pRU3147. Therefore, this permease is an alanine transporter required for the utilization of alanine as a sole carbon and nitrogen source. This permease was named MctP (for monocarboxylate transport permease protein) as it also transports other monocarboxylates, such as lactate and pyruvate (see below).

Topology prediction software (TopPred 2, HMMTOP, and TMHMM [29, 52, 54]) indicates MctP is a membrane protein with 13 transmembrane helices, with the N terminus located in the periplasm. This prediction supports the relationship of MctP and the sodium/solute symporters as it is in accordance with the experimentally determined topology of the sodium/proline transporter of *E. coli* (25).

Complementation of *mctP* mutants. To confirm that the phenotype observed in the *mctP* mutant (RU1180) can be complemented in *trans* by *mctP*, a number of plasmids were constructed and conjugated into RU1180, and growth on minimal media containing alanine or pyruvate and ammonia was determined. A PCR product containing the complete *mctP* gene and part of the upstream noncoding region was cloned into a broad-host-range vector in both orientations (pRU739 and pRU747). Plasmid pRU739, which contained the PCR product in the same orientation as the *lac* promoter (p_{lac}), did restore growth on minimal media containing alanine or pyruvate-ammonia (Table 2) to the rates observed in the wild-type strain. Therefore, the decreased apparent growth rates observed in RU1180 are caused by mutation of *mctP*. However, pRU747, which contains the same insert in the opposite orientation, failed to complement the *mctP* mutation (Table 2). Thus, the region of DNA cloned in pRU747 cannot include an active promoter. Therefore, it is probable that the portion of the *mctR-mctP* intergenic region that is not located on the cloned PCR product is required for promoter activity. A convenient *Sal*I restriction site is present in the *mctS* gene. This was utilized to clone a *Sal*I fragment containing *mctR*, *mctP*, and the intergenic region between these genes. Growth on alanine or pyruvate and ammonia was enhanced in RU1180 by the presence of plasmids containing this *Sal*I fragment regardless of orientation (pRU785 and pRU788; Table 2). Therefore, this fragment contains an active *mctP* promoter. As the *mctS* and *mctR* genes overlap (Fig. 1) and are probably cotranscribed, it is unlikely that there is a promoter immediately upstream of *mctR* on the cloned *Sal*I fragment. The presence of an *mctP* promoter in the *mctR-mctP* intergenic region was

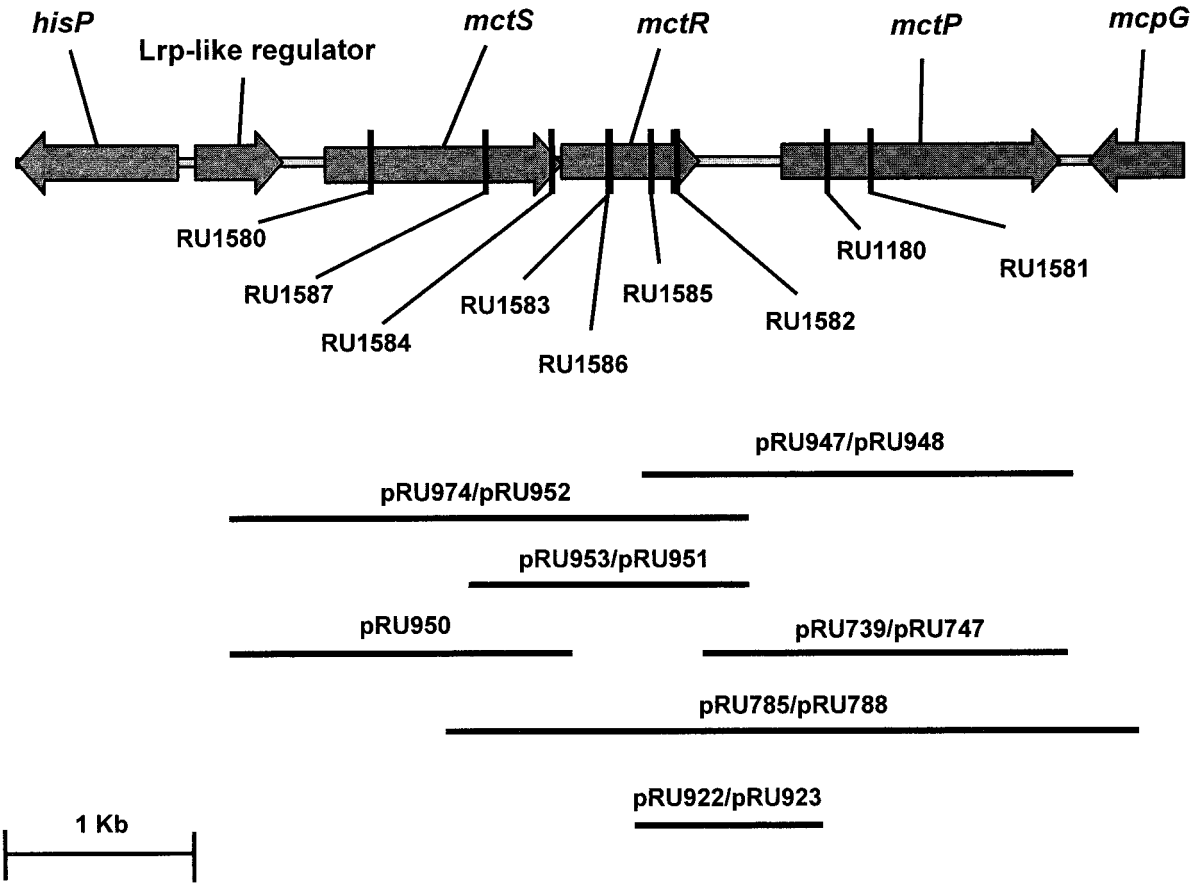

FIG. 1. Map of the *mct* region. The region of the *R. leguminosarum* genome containing the *mct* permease is represented here (the sequence has been submitted to the EMBL database under accession number AJ421944). Vertical lines represent the locations of the insertions used to construct mutants used in this study. The lines beneath the map indicate the region located on the plasmids used in this study.

confirmed by the complementation of the alanine and pyruvate-ammonia growth phenotype of RU1180 by a PCR product containing this intergenic region, *mctP*, and only a small portion of *mctR*, cloned in both orientations in pRK415 (pRU947 and pRU948) (Table 2).

Distribution of MctP orthologues. BLAST (3) searches of the sequence databases (including the unfinished microbial genome sequences) indicates that putative MctP orthologues are present in a broad range of microorganisms, including gram-negative β-proteobacteria (*Burkholderia cepacia*), gram-positive eubacteria (*Bacillus subtilis*, *Bacillus cereus*, and *Streptomyces coelicolor* A3) and archaea (*Ferroplasma acidarmanus*, *Sulfolobus solfataricus*, *Sulfolobus tokodaii*, *Thermoplasma volcanium*, and *Thermoplasma acidophilum*). However, the completed genome sequences of the closely related α-proteobacteria (i.e., *S. meliloti*, *M. loti*, *A. tumefaciens*, and *Brucella melitensis*) did not reveal any genes with significant similarity to *R. leguminosarum mctP*.

Amino acid sequence alignments indicate that the orthologues of MctP form a distinct group from the sodium/proline, sodium/pantothenate, and sodium/phenylacetate symporters (Fig. 3). This new subfamily of permeases is also distinct from other transporters of related MctP solutes, such as the monocarboxylate porter family (e.g., hsMCT1 to -6), the alanine/

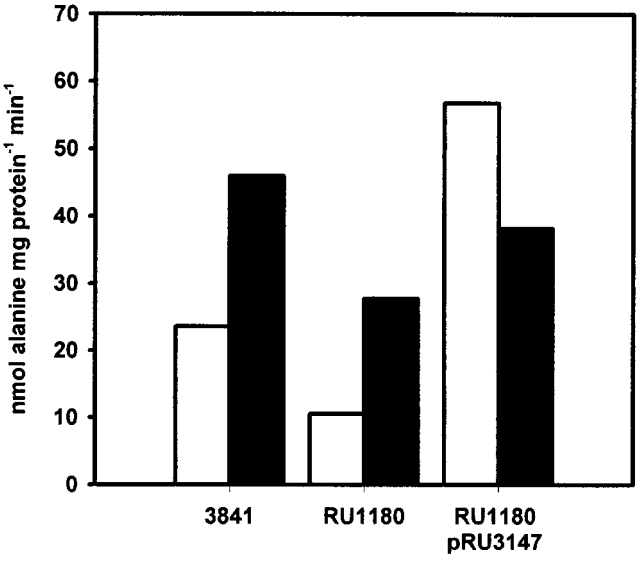

FIG. 2. Uptake of alanine by strains of *R. leguminosarum*. Uptake of 500 μM (0.125 μCi) L-[U-^{14}C] alanine was assayed by the rapid filtration method for different *R. leguminosarum* strains grown in minimal medium (AMS) with added glucose (10 mM) plus ammonium chloride (10 mM) (white bars) or alanine (20 mM) (black bars). Data shown are the means of three independent experiments.

TABLE 2. Growth of mutant strains containing various plasmids on minimal medium (AMA) containing either alanine or pyruvate and ammonia as a sole C and N source

Plasmid	Growth of strain[b]			
	RU1180 (*mctP::Tn5*)	RU1581 (*mctP::GJ*)	RU1580 (*mctS::GJ*)	RU1582 (*mctR::GJ*)
pRK415	±	±	+	+
pRU785 (*mctRP*)	+++	ND	ND	ND
pRU788 (*mctRP*)[a]	+++	ND	ND	ND
pRU739 (*mctP*)[a]	+++	ND	ND	ND
pRU747 (*mctP*)	+	ND	ND	ND
pRU947 (*mctP*)	+++	+++	ND	ND
pRU948 (*mctP*)[a]	++	+++	ND	ND
pRU950 (*mctS*)[a]	ND	ND	±	ND
pRU951 (*mctR*)[a]	ND	ND	ND	+++
pRU953 (*mctR*)	ND	ND	ND	+
pRU952 (*mctSR*)[a]	ND	ND	+++	+++
pRU974 (*mctSR*)	ND	ND	+++	+++

[a] The *mct* gene is under the control of the *lac* promoter, which is constitutive in *R. leguminosarum.* Thus, no IPTG was required to induce the expression of the cloned genes.

[b] There was no discernible difference between growth of strains on minimal medium with alanine (20 mM) or pyruvate (20 mM) and ammonium chloride (10 mM). Symbols: ±, negligible growth; +, positive for growth; +++, strongly positive for growth. ND, not determined.

glycine/cation symporter family (e.g., ahDagA), the lactate permease family (e.g., ecLctP), and the dicarboxylate/amino acid/cation symporter family (e.g., rlDctA) (Fig. 3).

MctSR is a two-component system with putative orthologues in α-proteobacteria. Immediately upstream of *mctP* are two genes (*mctS* and *mctR* [for monocarboxylate transporter sensor and regulator, respectively) with homology to the sensor and response-regulator components of bacterial two-component systems (17). MctS has similarity to histidine protein kinases, including the conserved histidine (His266), asparagine (Asn326), and aspartate (Asp353) residues, which are characteristic of this family of sensor proteins (49). MctS also has one putative transmembrane helix (amino acid 160 to 182), with the N terminus probably located in the periplasm and, constitutes a hypothetical sensory domain. MctR has similarity to bacterial regulators and contains a helix-turn-helix signature of the LuxR family (Prosite accession number PS00622) from amino acid 164 to 191. These helix-turn-helix signatures are involved in DNA binding (12, 15). MctR also possesses the conserved amino acids (Asp13, Asp59, and Lys109) associated with regulators of the two-component systems, with Asp59 being the probable site of phosphorylation (49).

BLAST searches of the completed microbial genome sequences indicated that *A. tumefaciens* and *B. melitensis* contain putative MctSR orthologues (accession numbers AAL52763, AAL52764, AAL45956, and AAL45955). These putative orthologues are more than 60% identical to *R. leguminosarum* MctS and MctR. This is the same level of identity as the *A. tumefaciens* orthologue has to the *B. melitensis* orthologue. However, in *A. tumefaciens* and *B. melitensis*, no *mctP* orthologue could be identified. Rather, in both these species, the two-component response regulators are adjacent to transporters of the tripartite ATP-independent periplasmic transporter family (T.C. number 2.A.56) (28, 44). The relevance of the MctSR orthologues in these organisms and any possible role for the tripartite ATP-independent periplasmic transporters in the uptake of monocarboxylates requires further investigation.

MctS differs from other sensors of two-component systems, including the putative *A. tumefaciens* and *B. melitensis* orthologues, as it has only one putative transmembrane domain. Other sensors have an additional membrane-spanning domain at the extreme N terminus (48). A closer comparison of the *R. leguminosarum mctS* sequence with that of the putative *A. tumefaciens* and *B. melitensis* orthologues reveals significant amino acid similarity upstream of the putative ATG start codon of *mctS*, but no alternative start codon is present between this ATG and the first upstream stop codon. This possible truncation of *mctS* in *R. leguminosarum* could be the result of a single base (adenine) insertion, 136 nucleotides upstream of the ATG start codon. Repeated sequencing of different clones indicates this sequence is authentic.

Inhibition of alanine uptake by competing solutes. As *R. leguminosarum*3841 has two high-affinity transporters of alanine (i.e., Aap and Bra) (19, 20, 56), characterization of alanine uptake by the MctP was performed using a previously constructed *aap bra* double mutant strain (RU1357). To determine the solute specificity of MctP, a number of solutes were tested for inhibition of alanine uptake (Fig. 4). The known solutes of sodium/solute symporters (i.e., proline and pantothenate) did not inhibit alanine uptake. Therefore, the solute specificity of MctP is distinct from these previously characterized symporters. Uptake of [^{14}C]alanine was only partially inhibited (between 48 and 62% inhibition) by a 10-fold excess of alanine (L- and D-isomers), cysteine, or histidine, while other amino acids tested did not significantly inhibit alanine uptake. In contrast, monocarboxylates (acetate, pyruvate, lactate, propionate, butyrate, and α-hydroxybutyrate) were strong inhibitors of alanine uptake (between 77 and 97% inhibition), while dicarboxylates (malonate and succinate) and some C_4-monocarboxylates (β-hydroxybutyrate and γ-hydroxybutyrate) did not inhibit alanine uptake. Therefore, inhibition studies indicate that MctP is a monocarboxylic acid transporter.

Uptake of lactate, pyruvate, and acetate by MctP. Although competitive inhibition of solute uptake can indicate which solutes may be transported by a permease, not all competitive inhibitors are transported. Therefore, to confirm that MctP is indeed a transporter of monocarboxylates, uptake of lactate, pyruvate, and acetate was measured in wild-type and *mctP* mutant strains. Mutation of *mctP* (RU1180) reduced the rate of uptake of lactate and pyruvate by 95% relative to wild-type 3841 (Fig. 5A and C). Furthermore, plasmids that complemented the growth phenotype of RU1180, also restored lactate and pyruvate uptake rates in this mutant to at least the rates observed in wild type 3841 (Fig. 5A and C). Therefore, MctP transports lactate and pyruvate, and is the dominant transporter of these solutes in free-living *R. leguminosarum*.

In contrast, mutation of *mctP* did not affect the rate of acetate uptake by free-living *R. leguminosarum*. Both 3841 (wild type) and RU1180 (*mctP*) transport acetate with K_m values of 20.1 ± 10.4 and 16.5 ±3.6 μM and V_{max} values of 41.7 ± 4.3 and 37.8 ± 1.6 nmol of acetate mg of protein^{-1} min^{-1}, respectively (unless otherwise noted, results are presented as means ± standard errors of the means). Therefore, MctP is not the dominant transporter of acetate in free-living *R. leguminosarum*. However, it is possible that MctP can transport acetate,

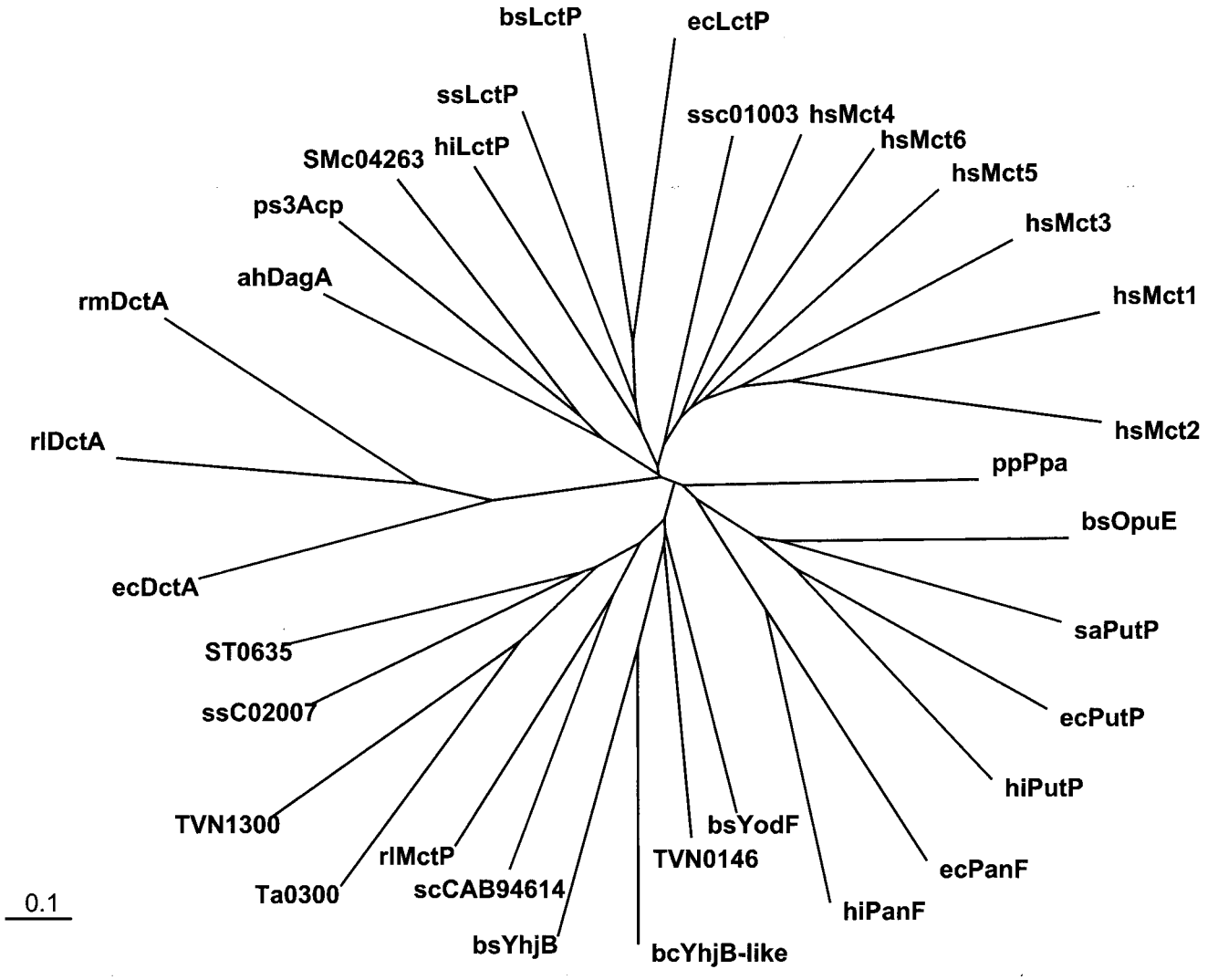

FIG. 3. Phylogenetic tree indicating the relationship between rlMctP and related permeases. A phylogenetic tree was constructed from the amino acid sequences of rlMctP and a number of related transporters using Vector NTI Suite (version 6), which uses the ClustalW algorithm, and Treeview (version 1.6.1). Protein designations and accession numbers are as follows: scCAB94614, *S. coelicolor* A3 putative permease (CAB94614); Ta0300, *T. acidophilum* conserved hypothetical protein (CAC11445); ST0635, *S. tokodaii* conserved hypothetical protein (BAB65633); ssC02007, *S. solfataricus* conserved hypothetical protein c02007 (CAA69485); bsYodF, *B. subtilis* YodF (C69903); TVN0146, *T. volcanium* proline permease (BAB59289); TVN1300, *T. volcanium* Na^+/pantothenate symporter; bsYhjB, *B. subtilis* metabolite permease (B69833); bcYhjB-like, *B. cereus* YhjB-like protein (AF387344); ecPanF, *E. coli* Na^+/pantothenate symporter (P16256); hiPanF, *Haemophilus influenzae* Rd Na^+/pantothenate symporter (P44963); ecPutP, *E. coli* Na^+/proline symporter (P07117); hiPutP, *H. influenzae* Rd Na^+/proline symporter (P45174); bsOpuE, *B. subtilis* osmoregulated Na^+/proline symporter (O06493); ppPpa, *P. aeruginosa* phenylacetic acid transporter (CAA94864); hsMCT1 to -6, human monocarboxylic acid transporters 1 to 6 (P53985, AF049608, O15427, O15374, O15375, and O15403); ssc01003, *S. solfataricus* orf c01003 (CAA69453); rlDctA, *R. leguminosarum* C_4-dicarboxylate transporter (Q01857); rmDctA, *S. meliloti* C_4-dicarboxylate transporter (P20672); ecDctA, *E. coli* C_4-dicarboxylate transporter (P37312); ps3Acp, thermophilic bacterium PS3 alanine carrier protein (D12512); ahDagA, *Alteromonas haloplanktis* D-alanine/glycine permease (M59081); ssLctP, *S. solfataricus* L-lactate permease (CAA69452); bsLctP, *B. subtilis* L-lactate permease (BAA21776); ecLctP, *E. coli* L-lactate permease (AAC76627); hiLctP, *H. influenzae* Rd L-lactate permease (AAC22871).

but the unidentified acetate permease prevents this being detected in whole-cell assays.

The apparent affinity of MctP for lactate and pyruvate is higher than that for alanine, as alanine did not inhibit uptake of either of these solutes (Fig. 5B and D). Indeed, other than pyruvate, no solute tested inhibited the uptake of [^{14}C]pyruvate (Fig. 5D). Therefore, of the solutes tested, MctP has the greatest apparent affinity for pyruvate. Further competition assays indicated that uptake of lactate is inhibited by pyruvate and propionate (95 and 78% inhibition, respectively) but only partially by butyrate (38% inhibition). Other C_4-monocarboxylates (including α-hydroxybutyrate), C_2-monocarboxylates (acetate), and dicarboxylates (C_3 and C_4) did not inhibit lactate uptake (Fig. 5B). Therefore, MctP has a preference for C_3-monocarboxylates, with the following apparent relative affinity order (greatest to least): pyruvate, lactate, propionate, butyrate, α-hydroxybutyrate, alanine (acetate).

Kinetics of solute uptake by MctP. As *R. leguminosarum* 3841 has two high-affinity transporters of alanine (Aap and Bra), the kinetics of alanine uptake by MctP were determined

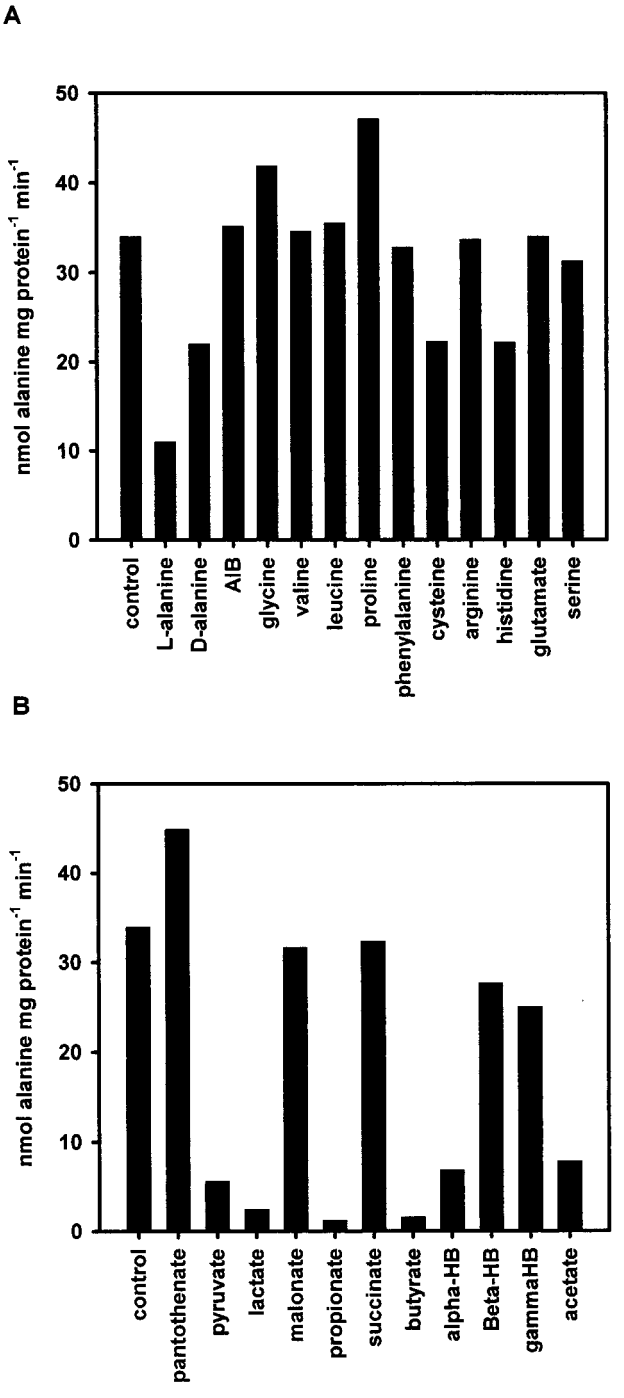

FIG. 4. Inhibition of alanine uptake by other solutes. Uptake of 500 μM (0.125 μCi) L-[U-^{14}C]alanine by RU1357, grown on minimal medium (AMS) with alanine (20 mM), was assayed by the rapid filtration method. Competing solutes were added to a final concentration of 5 mM. Data shown are the means of at least three independent experiments. Abbreviations: AIB, 2-aminoisobutyric acid; HB, hydroxybutyrate.

using the *aap bra* mutant RU1357. Preliminary experiments using cells grown in minimal media containing glucose and ammonia gave low alanine uptake rates at the concentrations tested. Therefore, RU1357 was grown on alanine minimal medium, as this increased the rate of transport (data not shown). Although the rate of alanine uptake was also increased in strain RU1180 by growth on alanine (Fig. 2), this can be attributed to up-regulation of *aap* or *bra* expression on the basis of specificity and K_m values (data not shown). The kinetic constants obtained indicate that MctP has a low affinity for alanine (K_m = 0.56 ± 0.16 mM), but the V_{max} for alanine uptake is high (122 ± 15 nmol of alanine mg of protein^{-1} min^{-1}).

At high lactate concentrations, an unidentified lactate permease, which was induced by growth on alanine, became apparent (data not shown). To overcome this, apparent kinetic constants were determined by subtracting uptake rates obtained from RU1180 from those for wild-type 3841, with each strain grown on glucose and ammonia minimal media. MctP has an apparent K_m for lactate uptake of 4.4 ± 0.7 μM (V_{max} = 22.4 ± 1.01 nmol of lactate mg of protein^{-1} min^{-1}), while the lactate permease retained in the *mctP* mutant, RU1180, has a K_m of 38.1 ± 7.3 μM lactate (V_{max} = 8.5 ± 0.7 nmol of lactate mg of protein^{-1} min^{-1}).

As the characterization of pyruvate uptake had indicated that the uptake of pyruvate is entirely dependent on MctP when *R. leguminosarum* 3841 is grown on glucose and ammonia, this strain and growth conditions were used for kinetic analysis of pyruvate uptake. The K_m for pyruvate uptake by MctP is 3.8 ± 1.1 μM, and the V_{max} is 9.8 ± 1.0 nmol of pyruvate mg of protein^{-1} min^{-1}.

The K_m values obtained for lactate and pyruvate are more than 100-fold lower than that for alanine. Therefore, MctP is a high-affinity transporter of lactate and pyruvate but a low-affinity transporter of alanine. Although the V_{max} values obtained for lactate and pyruvate are significantly lower than that for alanine, this reflects the different growth conditions used (i.e., glucose and ammonia rather than alanine).

Sodium concentration does not affect solute uptake by MctP. As MctP has significant similarity to sodium/solute symporters, the affect of sodium on transport by MctP was investigated. Lactate and succinate were selected as representative solutes transported by MctP and DctA (a proton-coupled dicarboxylate transporter [6, 23, 50]), respectively, and the uptake of these solutes by strain 3841 was determined in sodium-free transport buffer (RMS) with and without the addition of sodium or lithium. The addition of sodium or lithium had no affect on the uptake of either lactate or succinate (Fig. 6).

However, there is, to our knowledge, no characterized sodium-dependent transporter in *Rhizobium* spp. which can be used as a control. Therefore, two glutamate transporters of *E. coli*, GltS (a sodium-coupled permease [9]) and GltP (a proton-coupled permease [9, 55]), were used as controls to confirm that *R. leguminosarum* is able to use sodium ions as a source of energy for transport. Also, it is notoriously difficult to remove sodium from assay buffers. Therefore, GltS was also a control of the experimental conditions. GltS and GltP were expressed in *R. leguminosarum* RU1357 (*aap bra*) under the control of the *aapJ* promoter, and glutamate uptake was determined with or without the addition of sodium. Uptake of glutamate by GltS was increased fivefold by the addition of sodium, whereas, the rate of glutamate uptake by GltP was not altered by sodium concentration (Fig. 6). Hence, the maximum rate of glutamate uptake by GltS is dependent on the addition of sodium, so the level of any contaminating sodium in the

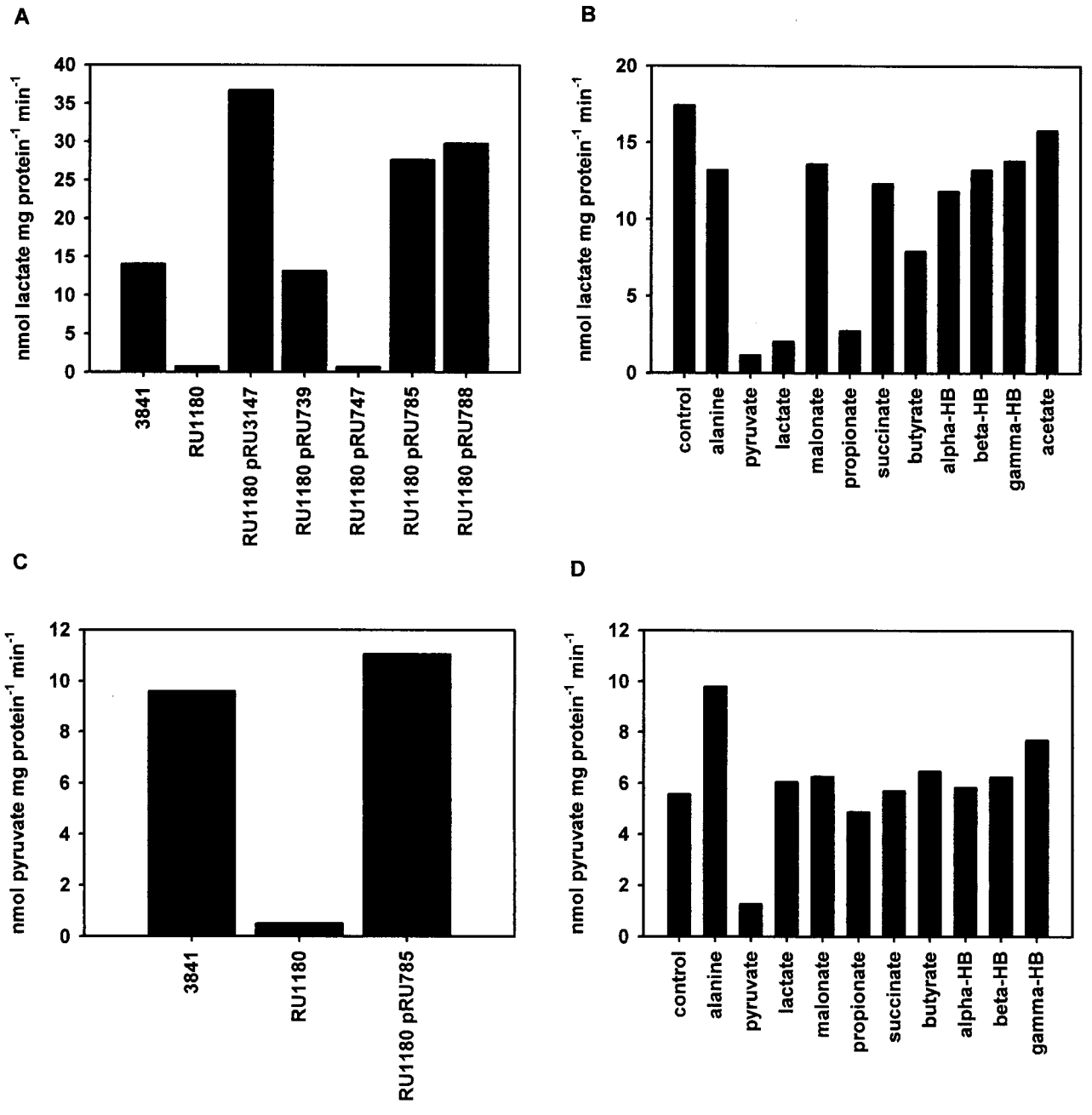

FIG. 5. Uptake of lactate and pyruvate by strains of *R. leguminosarum*. Uptake of 5 μM (0.125 μCi) L-[U-^{14}C]lactate (A) and [1-^{14}C]pyruvate (C) was assayed by the rapid filtration method for different *R. leguminosarum* strains grown in minimal medium (AMS) with added glucose (10 mM) plus ammonium chloride (10 mM). The apparent specificity of lactate (B) and pyruvate (D) uptake by strain 3841 was determined by the addition of competing solutes to a final concentration of 0.5 mM. Data shown are the means of at least three independent experiments. HB, hydroxybutyrate.

sodium-free assay buffer is not sufficient to mask the sodium dependence of this permease.

Therefore, although known sodium-coupled transporters can function in *R. leguminosarum*, uptake by MctP is not apparently influenced by sodium concentration. However, a caveat is that if MctP were to use sodium with much higher affinity than GltS, our experiments may not have detected sodium dependence. The transport of solutes by MctP does require metabolic energy, as lactate uptake was inhibited 97% by 5 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP). While this is consistent with proton dependence, sodium-dependent transporters (for example, GltS) are also inhibited by CCCP (9). At present we conclude there is no evidence for sodium as opposed to proton coupling, but absolute proof is difficult to acquire in studies with whole cells.

Symbiotic phenotype of *mctP* mutants. Strain RU1180 formed normal red nodules on peas, and the plants were healthy and green. Plants inoculated with the wild type versus

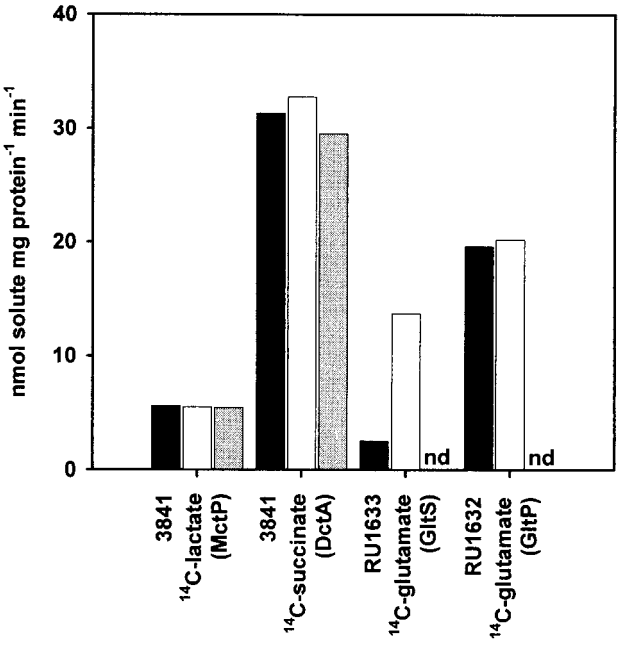

FIG. 6. Affect of sodium concentration on solute uptake. Uptake of 5 μM (0.125 μCi) L-[U-^{14}C]lactate or 25 μM (0.125 μCi) [2,3-^{14}C]succinate or 25 μM (0.125 μCi) L-[U-^{14}C]glutamic acid was assayed by the rapid filtration method for *R. leguminosarum* strains 3841, RU1632 (RU1357 pRU976),or RU1633 (RU1357 pRU986) grown in minimal medium (AMS) with added succinate (10 mM) plus ammonium chloride (10 mM). Uptake assays were performed in sodium-free RMS with no additions (black columns), with added NaCl (5 mM) (white columns), or with added LiCl (5 mM) (grey columns). Data shown are the means of at least three independent experiments. nd, not determined.

strain RU1180 strain reduced acetylene at rates of 2 ± 0.6 μmol h^{-1} plant^{-1} and 1.5 ± 0.1 μmol h^{-1} plant^{-1} (means ± standard errors of the means), respectively, all of which indicates a Fix$^+$ phenotype for the mutant.

The MctSR two-component system regulates *mctP* expression. The proximity of the *mctSR* genes to *mctP* suggests that the two-component response system may regulate expression of *mctP*. To investigate this, a 4.4-kb DNA fragment containing *mctSRP* was mutated in vitro by GeneJumper insertion. Eight plasmids with transposon insertions in *mctS* (three plasmids), *mctR* (four plasmids), or *mctP* (one plasmid) were identified by restriction mapping, and the location of the transposon insertion was confirmed by DNA sequencing. The mutated plasmids were then used to obtain chromosomal insertion mutants by homologous recombination. The position of the GeneJumper transposon insertion in each of the resulting strains is shown on Fig. 1. The *mctS*::GeneJumper, *mctR*::GeneJumper, and *mctP*::GeneJumper mutants each displayed the same low growth rate on alanine or pyruvate and ammonia minimal media as observed for the original *mctP* transposon mutant, RU1180. Therefore, in addition to *mctP*, *mctS* and *mctR* are required for optimal growth on alanine or pyruvate and ammonia.

As with RU1180, the *mctP*::GeneJumper mutant (RU1581) can be complemented for growth by plasmids containing *mctP* and the *mctR-mctP* intergenic region (Table 2). The *mctS*::GeneJumper and *mctR*::GeneJumper mutants (RU1580 and RU1582, respectively) can be complemented by plasmids which contain *mctSR* (pRU952 and pRU974) (Table 2). However, RU1580 cannot be complemented by *mctS* alone, and complementation of *mctR* mutants requires the gene to be expressed with p_{lac} (Table 2). This can be explained by *mctS* and *mctR* being transcriptionally linked, with insertion mutations of *mctS* having a polar affect on *mctR* expression and no promoter being present immediately upstream of *mctR*.

The *mctR-mctP* intergenic region, which contains the putative *mctP* promoter (p_{mctP}), was amplified by PCR and cloned by Gateway technology into the transcription reporter vector pOT3gfp+ (pRU923). Promoter activity (p_{mctP}) could not be detected in *mctS* or *mctR* mutant strains but was detected in wild-type and *mctP* mutant strains (Fig. 7A). Therefore, the MctSR two-component sensor-regulator is an essential activator of *mctP* expression and *mctP* is not involved in self-regulation.

To determine the affect of solutes on *mctP* expression, wild-type 3841 containing the p_{mctP} reporter plasmid was grown under different growth conditions. Approximately twofold-lower p_{mctP} activity was recorded when succinate, rather than glucose, was present as a carbon source. However, there was no increase in *mctP* expression in response to alanine, pyruvate, or lactate in the growth media (Fig. 7B). Therefore, although MctSR is an activator of *mctP*, no environmental signal for this regulator has yet been identified. Indeed, *mctP* is expressed at high levels in both nutrient-rich medium (TY) and minimal medium (AMA) containing only glucose and ammonia as carbon and nitrogen sources, indicating that *mctP* expression is constitutive. The *mctP* reporter vector, pRU922, was conjugated into two other wild-type strains of *R. leguminosarum* (A34 and VF39), which are distinct from 3841. As in 3841, the fluorescence derived from the *mctP-gfp* fusion was high on TY and minimal media with glucose and ammonia, indicating the constitutive expression of *mctP* is not strain dependent.

DISCUSSION

The data presented here indicate that the MctP of *R. leguminosarum* is a novel monocarboxylate transporter. This permease has a low affinity for alanine (K_m = 560 μM) but a higher affinity for lactate and pyruvate (K_m = 4.4 μM and 3.8 μM, respectively). Uptake inhibition studies indicate that acetate, propionate, butyrate, and α-hydroxybutyrate are also solutes transported by MctP, with the apparent affinity indicating a preference for C_3-monocarboxylates. Dicarboxylates (malonate and succinate) did not inhibit uptake by MctP. Therefore, the specificity of MctP is distinct from those of previously characterized bacterial carboxylate transporters. Typically, bacterial carboxylate transporters transport a narrow range of related compounds. For example, the characterized members of lactate permease family (i.e., two *E. coli* transporters, GlcA [YghK] and LctP [LldP]) are specific for 2-hydroxymonocarboxylates (L-lactate, D-lactate and glycolate) (32). However, the data suggest MctP transports a broad range of monocarboxylates, including 2-hydroxymonocarboxylates (lactate and α-hydroxybutyrate), 2-aminomonocarboxylates (alanine), and 2-ketomonocarboxylates (pyruvate), in addition to

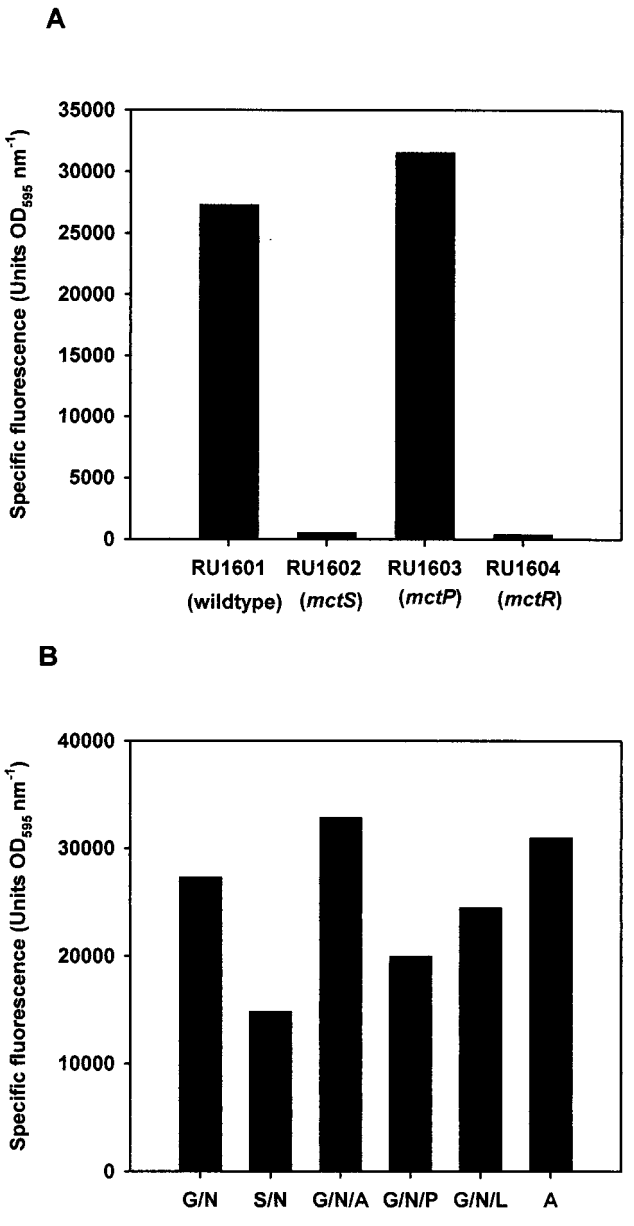

FIG. 7. Regulation of the *mctP* promoter. Specific fluorescence (fluorescence [excitation at 485 nm; emission at 510 nm] optical density at 595 nm^{-1}) was used as a measure of expression of the *mctP* promoter-green fluorescent protein fusion in different strains grown in AMS minimal medium with 10 mM glucose and 10 mM ammonium chloride (A) or in RU1601 (3841 pRU923) grown on different carbon and nitrogen sources (B). Data shown are the means of at least three independent experiments. Abbreviations: G, 10 mM glucose; N, 10 mM ammonium chloride; S, 10 mM succinate; A, 20 mM alanine; P, 20 mM pyruvate; L, 20 mM lactate.

monocarboxylates with no side group (propionate and butyrate).

Amino acid sequence comparisons also indicate MctP is distinct from other transporters of alanine or monocarboxylates, such as members of the D-alanine/glycine/cation symporter family (T.C. number 2.A.25) (42, 44), the LctP family (T.C. number 2.A.14) (32, 44), and the proton-linked monocarboxylate porter family (T.C. number 2.A.1.13) (14, 44) (Fig. 3). However, MctP does have significant similarity to the sodium/solute symporter family (42). In accordance with this, in silico predictions indicate MctP is a protein with 13 transmembrane helices, the same number demonstrated experimentally for *E. coli* PutP (sodium/proline symporter), the best-characterized member of this family (25). Other bacterial members of this family include the sodium/pantothenate symporter from *E. coli*, the sodium/phenylacetate symporter from *Pseudomonas putida*, and the sodium/glucose symporter from *Vibrio parahaemolyticus* (22, 45, 46). Therefore, the solutes transported by permeases of this family are diverse.

Although similar to members of the sodium/solute symporter family, the putative MctP orthologues form a distinct group from the previously characterized sodium/solute symporters (Fig. 3). Also, we were unable to demonstrate any dependence on sodium for transport of solutes by MctP. However, as it is difficult to ensure that transport buffers are truly sodium free, it is possible that small levels of sodium present in the assay buffers were sufficient to allow transport to proceed if MctP has a high affinity for sodium. Uptake was inhibited by CCCP (5 to 10 μM), indicating that transport by MctP requires metabolic energy and may be proton linked, as it is with the monocarboxylate porter family (14). Although the previously characterized members of the sodium/solute symporter family use sodium under physiological conditions, it has been demonstrated that the human Na^+/glucose transporter can utilize H^+, although the affinity for glucose was 10-fold lower than when Na^+ was used (40). Therefore, it is not surprising that MctP may act as a proton symporter, although this requires confirmation.

Putative MctP orthologues have been identified in a wide taxonomic range of prokaryotes (Fig. 3), and two species (*B. subtilis* and *T. volcanium*) have two putative MctP-like paralogues. It is noteworthy that a gene encoding a putative MctP orthologue in *B. cereus* (YhjB-like protein) is adjacent to the alanine spore germination operon, *gerL* (4). It is possible that this permease is required for the uptake of alanine during outgrowth from endospores and therefore has a similar specificity to MctP.

Two-component systems are a well-established mechanism for signal transduction in bacteria (17). The sensor component senses an environmental signal and, through a phosphorylation reaction, transmits this signal to the response regulator. Many of the response regulators of this family act by binding to a promoter and activate or repress gene expression (17). Specific two-component sensor regulators are known to activate expression of dicarboxylate and tricarboxylate transporter genes (23). For example, the DctBD two-component system activates expression of *dctA* in *R. leguminosarum* (41), and CitST activates *citM* expression in *B. subtilis* (60). The data presented indicate the MctSR two-component system of *R. leguminosarum* is required for activation of the monocarboxylate transporter gene *mctP*.

Typically, solutes transported by a permease act as inducers for the two-component regulators of the permease. However, we were unable to identify any solute that induced expression of *mctP*; indeed, it is constitutive. The putative orthologues of MctS identified in *A. tumefaciens* and *B. melitensis* have two transmembrane domains toward the N terminus, whereas *R. leguminosarum* MctS has only one. This appears to have re-

sulted from a single nucleotide insertion upstream of the apparent start codon, leading to a truncation of the protein and loss of the N terminus transmembrane domain. The consistency of repeated sequencing reactions indicates the authenticity of this apparent nucleotide insertion. It is possible that the loss of the N-terminal transmembrane region has resulted in the sensor being inactivated in such a way as to constitutively activate the regulator, which in turn activates expression of MctP. The expression of the p_{mctP}-*gfp* fusion (pRU922) was the same in *R. leguminosarum* strains A34 and VF39. This suggests that *R. leguminosarum* has adapted the regulation of *mctP* to allow constitutive expression and that the putative truncation observed in 3841 is not a specific mutation of this strain.

MctP is not required for *R. leguminosarum* to form symbiotic nodules and fix atmospheric nitrogen, which is consistent with the lack of putative orthologues in the genomes of sequenced rhizobia (i.e., *S. meliloti* and *M. loti*) (13, 26). However, MctP is required for optimal growth of free-living *R. leguminosarum* on alanine or pyruvate and ammonia. Furthermore, the characterization of MctP has resulted in the identification of a new subfamily of C_3-monocarboxylate transporters in bacteria.

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REFERENCES

1. **Allaway, D., E. Lodwig, L. A. Crompton, M. Wood, T. R. Parsons, T. Wheeler, and P. S. Poole.** 2000. Identification of alanine dehydrogenase and its role in mixed secretion of ammonium and alanine by pea bacteroids. Mol. Microbiol. **36:**508–515.
2. **Allaway, D., N. A. Schofield, M. E. Leonard, L. Gilardoni, T. M. Finan, and P. S. Poole.** 2001. Use of differential fluorescence induction and optical trapping to isolate environmentally induced genes. Environ. Microbiol. **3:**397–406.
3. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. H. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:** 3389–3402.
4. **Barlass, P. J., C. W. Houston, M. O. Clements, and A. Moir.** 2002. Germination of *Bacillus cereus* spores in response to L-alanine and to inosine: the roles of *gerL* and *gerQ* operons. Microbiology **148:**2089–2095.
5. **Beringer, J. E.** 1974. R factor transfer in *Rhizobium leguminosarum.* J. Gen. Microbiol. **84:**188–198.
6. **Bhandari, B., and D. J. D. Nicholas.** 1985. Proton motive force in washed cells of *Rhizobium japonicum* and bacteroids from *Glycine max.* J. Bacteriol. **164:**1383–1385.
7. **Buchanan-Wollaston, V.** 1979. Generalized transduction in *Rhizobium leguminosarum.* J. Gen. Microbiol. **112:**135–142.
8. **Day, D. A., P. S. Poole, S. D. Tyerman, and L. Rosendahl.** 2001. Ammonia and amino acid transport across symbiotic membranes in nitrogen-fixing legume nodules. Cell. Mol. Life Sci. **58:**61–71.
9. **Deguchi, Y., I. Yamato, and Y. Anraku.** 1989. Molecular cloning of *gltS* and *gltP*, which encode glutamate carriers of *Escherichia coli* B. J. Bacteriol. **171:**1314–1319.
10. **Downie, J. A., Q. S. Ma, C. D. Knight, G. Hombrecher, and A. W. B. Johnston.** 1983. Cloning of the symbiotic region of *Rhizobium leguminosarum*: the nodulation genes are between the nitrogenase genes and a *nifA*-like gene. EMBO J. **2:**947–952.
11. **Freidman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. M. Ausubel.** 1982. Construction of the broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene **18:**289–296.
12. **Fuqua, W. C., S. C. Winans, and E. P. Greenberg.** 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J. Bacteriol. **176:**269–275.
13. **Galibert, F., T. M. Finan, S. R. Long, A. Puhler, P. Abola, F. Ampe, F. Barloy-Hubler, M. J. Barnett, A. Becker, P. Boistard, G. Bothe, M. Boutry, L. Bowser, J. Buhrmester, E. Cadieu, D. Capela, P. Chain, A. Cowie, R. W. Davis, S. Dreano, N. A. Federspiel, R. F. Fisher, S. Gloux, T. Godrie, A. Goffeau, B. Golding, J. Gouzy, M. Gurjal, I. Hernandez-Lucas, A. Hong, L. Huizar, R. W. Hyman, T. Jones, D. Kahn, M. L. Kahn, S. Kalman, D. H. Keating, E. Kiss, C. Komp, V. Lalaure, D. Masuy, C. Palm, M. C. Peck, T. M. Pohl, D. Portetelle, B. Purnelle, U. Ramsperger, R. Surzycki, P. Thebault, M. Vandenbol, F. J. Vorholter, S. Weidner, D. H. Wells, K. Wong, K. C. Yeh, and J. Batut.** 2001. The composite genome of the legume symbiont *Sinorhizobium meliloti*. Science **293:**668–672.
14. **Halestrap, A. P., and N. T. Price.** 1999. The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. Biochem. J. **343:**281–299.
15. **Henikoff, S., J. C. Wallace, and J. P. Brown.** 1990. Finding protein similarities with nucleotide-sequence databases. Methods Enzymol. **183:**111–132.
16. **Hirsch, P. R., and J. E. Beringer.** 1984. A physical map of pPH1JI and pJB4JI. Plasmid **12:**139–141.
17. **Hoch, J. A., and T. J. Silhavy (ed.).** 1995. Two-component signal transduction. American Society for Microbiology, Washington, D.C.
18. **Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease.** 1989. Engineering hybrid genes without the use of restriction enzymes: gene-splicing by overlap extension. Gene **77:**61–68.
19. **Hosie, A. H. F., D. Allaway, H. A. Dunsby, C. S. Galloway, and P. S. Poole.** 2002. *Rhizobium leguminosarum* has a second general amino acid permease with unusually broad substrate specificity and high similarity to branched-chain amino acid transporters (Bra/LIV) of the ABC family. J. Bacteriol. **184:**4071–4080.
20. **Hosie, A. H. F., D. Allaway, M. A. Jones, D. L. Walshaw, A. W. B. Johnston, and P. S. Poole.** 2001. Solute-binding protein-dependent ABC transporters are responsible for solute efflux in addition to solute uptake. Mol. Microbiol. **40:**1449–1459.
21. **Hosie, A. H. F., and P. S. Poole.** 2001. Bacterial ABC transporters of amino acids. Res. Microbiol. **152:**259–270.
22. **Jackowski, S., and J. H. Alix.** 1990. Cloning, sequence, and expression of the pantothenate permease (*panF*) gene of *Escherichia coli.* J. Bacteriol. **172:** 3842–3848.
23. **Janausch, I. G., E. Zientz, Q. H. Tran, A. Kroger, and G. Unden.** 2002. C4-dicarboxylate carriers and sensors in bacteria. Biochim. Biophys. Acta **1553:**39–56.
24. **Johnston, A. W. B., and J. E. Beringer.** 1975. Identification of the *Rhizobium* strains in pea root nodules using genetic markers. J. Gen. Microbiol. **87:**343–350.
25. **Jung, H., R. Rubenhagen, S. Tebbe, K. Leifker, N. Tholema, M. Quick, and R. Schmid.** 1998. Topology of the Na+/proline transporter of *Escherichia coli*. J. Biol. Chem. **273:**26400–26407.
26. **Kaneko, T., Y. Nakamura, S. Sato, E. Asamizu, T. Kato, S. Sasamoto, A. Watanabe, K. Idesawa, A. Ishikawa, K. Kawashima, T. Kimura, Y. Kishida, C. Kiyokawa, M. Kohara, M. Matsumoto, A. Matsuno, Y. Mochizuki, S. Nakayama, N. Nakazaki, S. Shimpo, M. Sugimoto, C. Takeuchi, M. Yamada, and S. Tabata.** 2000. Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti.* DNA Res. **7:**331–338.
27. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene **70:**191–197.
28. **Kelly, D. J., and G. H. Thomas.** 2001. The tripartite ATP-independent periplasmic (TRAP) transporters of bacteria and archaea. FEMS Microbiol. Rev. **25:**405–424.
29. **Krogh, A., B. Larsson, G. von Heijne, and E. L. L. Sonnhammer.** 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol. **305:**567–580.
30. **Li, Y., R. Parsons, D. A. Day, and F. J. Bergersen.** 2002. Reassessment of major products of N_2 fixation by bacteroids from soybean root nodules. Microbiology **148:**1959–1966.
31. **Nakao, T., I. Yamato, and Y. Anraku.** 1987. Nucleotide-sequence of *putP*, the proline carrier gene of *Escherichia coli* K12. Mol. Gen. Genet. **208:**70–75.
32. **Nunez, M. F., M. T. Pellicer, J. Badia, J. Aguilar, and L. Baldoma.** 2001. The gene *yghK* linked to the *glc* operon of *Escherichia coli* encodes a permease for glycolate that is structurally and functionally similar to L-lactate permease. Microbiology **147:**1069–1077.
33. **Poole, P. S., and D. A. Allaway.** 2000. Carbon and nitrogen metabolism in *Rhizobium.* Adv. Microb. Physiol. **43:**117–163.
34. **Poole, P. S., A. Blyth, C. J. Reid, and K. Walters.** 1994. *myo*-inositol catabolism and catabolite regulation in *Rhizobium leguminosarum* bv *viciae.* Microbiology **140:**2787–2795.
35. **Poole, P. S., M. Franklin, A. R. Glenn, and M. J. Dilworth.** 1985. The transport of L-glutamate by *Rhizobium leguminosarum* involves a common amino acid carrier. J. Gen. Microbiol. **131:**1441–1448.
36. **Poole, P. S., N. A. Schofield, C. J. Reid, E. M. Drew, and D. L. Walshaw.** 1994. Identification of chromosomal genes located downstream of *dctD* that affect the requirement for calcium and the lipopolysaccharide layer of *Rhizobium leguminosarum.* Microbiology **140:**2797–2809.
37. **Prell, J., B. Boesten, P. S. Poole, and U. B. Priefer.** 2002. The *Rhizobium leguminosarum* bv. *viciae* VF39 gamma-aminobutyrate (GABA) aminotransferase gene (*gabT*) is induced by GABA and highly expressed in bacteroids. Microbiology **148:**615–623.
38. **Priefer, U. B.** 1989. Genes involved in lipopolysaccharide production and

symbiosis are clustered on the chromosome of *Rhizobium leguminosarum* biovar *Viciae* VF39. J. Bacteriol. **171:**6161–6168.
39. **Quandt, J., and M. F. Hynes.** 1993. Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria. Gene **127:** 15–21.
40. **Quick, M., D. D. F. Loo, and E. M. Wright.** 2001. Neutralization of a conserved amino acid residue in the human Na^+/glucose transporter (hSGLT1) generates a glucose-gated H^+ channel J. Biol. Chem. **276:**1728–1734.
41. **Reid, C. J., and P. S. Poole.** 1998. Roles of DctA and DctB in signal detection by the dicarboxylic acid transport system of *Rhizobium leguminosarum.* J. Bacteriol. **180:**2660–2669.
42. **Reizer, J., A. Reizer, and M. H. Saier.** 1994. A functional superfamily of sodium/solute symporters. Biochim. Biophys. Acta-Rev. Biomembr. **1197:** 133–166.
43. **Reizer, J., A. Reizer, and M. H. Saier.** 1990. The Na+ pantothenate symporter (*panF*) of *Escherichia coli* is homologous to the Na+ proline symporter (*putP*) of *Escherichia coli* and the Na+ glucose symporters of mammals. Res. Microbiol. **141:**1069–1072.
44. **Saier, M. H.** 2000. A functional-phylogenetic classification system for transmembrane solute transporters. Microbiol. Mol. Biol. Rev. **64:**354–411.
45. **Sarker, R. I., W. Ogawa, T. Shimamoto, and T. Tsuchiya.** 1997. Primary structure and properties of the Na^+/glucose symporter (SglS) of *Vibrio parahaemolyticus*. J. Bacteriol. **179:**1805–1808.
46. **Schleissner, C., E. R. Olivera, M. Fernandezvalverde, and J. M. Luengo.** 1994. Aerobic catabolism of phenylacetic acid in *Pseudomonas putida* U: biochemical characterization of a specific phenylacetic acid transport system and formal demonstration that phenylacetyl coenzyme A is a catabolic intermediate. J. Bacteriol. **176:**7667–7676.
47. **Scholz, Q., A. Thiel, W. Hillen, and M. Niederweis.** 2000. Quantitative analysis of gene expression with an improved green fluorescent protein. Eur. J. Biochem. **267:**1565–1570.
48. **Stock, A. M., V. L. Robinson, and P. N. Goudreau.** 2000. Two-component signal transduction. Annu. Rev. Biochem. **69:**183–215.
49. **Stock, J. B., M. G. Surette, M. Levit, and P. Park.** 1995. Two-component signal transduction systems: structure-function relationship and mechanisms of catalysis, p. 25–51. *In* J. A. Hoch and T. J. Silhavy (ed.), Two-component signal transduction. American Society for Microbiology, Washington, D.C.
50. **Tremblay, P. A., and R. W. Miller.** 1984. Cytoplasmic membrane of *Rhizobium meliloti* bacteroids. II. Functional-differentiation and generation of membrane-potentials. Can. J. Biochem. Cell Biol. **62:**592–600.
51. **Trinick, M. J., M. J. Dilworth, and M. Grounds.** 1976. Factors affecting the reduction of acetylene by root nodules of *Lupinus* species. New Phytol. **77:**359–370.
52. **Tusnady, G. E., and I. Simon.** 1998. Principles governing amino acid composition of integral membrane proteins: application to topology prediction. J. Mol. Biol. **283:**489–506.
53. **Vanrhijn, P., and J. Vanderleyden.** 1995. The rhizobium-plant symbiosis. Microbiol. Rev. **59:**124–142.
54. **von Heijne, G.** 1992. Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. J. Mol. Biol. **225:**487–494.
55. **Wallace, B., Y.-J. Yang, J. Hong, and D. Lum.** 1990. Cloning and sequencing of a gene encoding a glutamate and aspartate carrier of *Escherichia coli* K-12. J. Bacteriol. **172:**3214–3220.
56. **Walshaw, D. L., and P. S. Poole.** 1996. The general L-amino acid permease of *Rhizobium leguminosarum* is an ABC uptake system that influences efflux of solutes. Mol. Microbiol. **21:**1239–1252.
57. **Walshaw, D. L., A. Wilkinson, M. Mundy, M. Smith, and P. S. Poole.** 1997. Regulation of the TCA cycle and the general amino acid permease by overflow metabolism in *Rhizobium leguminosarum.* Microbiology. **143:**2209–2221.
58. **Waters, J. K., B. L. Hughes, L. C. Purcell, K. O. Gerhardt, T. P. Mawhinney, and D. W. Emerich.** 2042. 1998. Alanine, not ammonia, is excreted from N_2-fixing soybean nodule bacteroids. Proc. Natl. Acad. Sci. USA **95:**12038–12041.
59. **Wood, D. W., J. C. Setubal, R. Kaul, D. E. Monks, J. P. Kitajima, V. K. Okura, Y. Zhou, L. Chen, G. E. Wood, N. F. Almeida, L. Woo, Y. C. Chen, I. T. Paulsen, J. A. Eisen, P. D. Karp, D. Bovee, P. Chapman, J. Clendenning, G. Deatherage, W. Gillet, C. Grant, T. Kutyavin, R. Levy, M. J. Li, E. McClelland, A. Palmieri, C. Raymond, G. Rouse, C. Saenphimmachak, Z. N. Wu, P. Romero, D. Gordon, S. P. Zhang, H. Y. Yoo, Y. M. Tao, P. Biddle, M. Jung, W. Krespan, M. Perry, B. Gordon-Kamm, L. Liao, S. Kim, C. Hendrick, Z. Y. Zhao, M. Dolan, F. Chumley, S. V. Tingey, J. F. Tomb, M. P. Gordon, M. V. Olson, and E. W. Nester.** 2001. The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. Science **294:**2317–2323.
60. **Yamamoto, H., M. Murata, and J. Sekiguchi.** 2000. The CitST two-component system regulates the expression of the Mg-citrate transporter in *Bacillus subtilis*. Mol. Microbiol. **37:**898–912.