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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

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Amino acid uptake by *Rhizobium leguminosarum* is dominated by two ABC transporters, the general amino acid permease (Aap) and the branched-chain amino acid permease (Bra_{Rl}). Characterization of the solute specificity of Bra_{Rl} shows it to be the second general amino acid permease of *R. leguminosarum*. Although Bra_{Rl} has high sequence identity to members of the family of hydrophobic amino acid transporters (HAAT), it transports a broad range of solutes, including acidic and basic polar amino acids (L-glutamate, L-arginine, and L-histidine), in addition to neutral amino acids (L-alanine and L-leucine). While amino and carboxyl groups are required for transport, solutes do not have to be α-amino acids. Consistent with this, Bra_{Rl} is the first ABC transporter to be shown to transport γ-aminobutyric acid (GABA). All previously identified bacterial GABA transporters are secondary carriers of the amino acid-polyamine-organocation (APC) superfamily. Also, transport by Bra_{Rl} does not appear to be stereospecific as D amino acids cause significant inhibition of uptake of L-glutamate and L-leucine. Unlike all other solutes tested, L-alanine uptake is not dependent on solute binding protein $BraC_{Rl}$. Therefore, a second, unidentified solute binding protein may interact with the $BraDEFG_{Rl}$ membrane complex during L-alanine uptake. Overall, the data indicate that Bra_{Rl} is a general amino acid permease of the HAAT family. Furthermore, Bra_{Rl} has the broadest solute specificity of any characterized bacterial amino acid transporter.

The ABC superfamily is a large ubiquitous group of transporters which possess a common minimum structure consisting of four domains: two hydrophobic integral membrane domains and two ATP-binding domains (15, 20). The genome sequences indicate that humans possess 48 ABC transporters (9), while some bacteria have in excess of 150 (13, 57). Bacterial ABC transporters are involved in a number of diverse processes, including multidrug resistance (53), protein secretion (58), quorum sensing (52), and nutrient uptake (16). The properties of a number of these transporters are also being exploited as scientific tools, for example, for vaccine development (14) and as environmental biosensors (4).

A subfamily of ABC transporters that are responsible for the uptake of solutes is found exclusively in prokaryotes (49). The members of this family can be distinguished from other ABC transporters by the presence of a solute binding protein (SBP), in addition to integral membrane domains and ATP-binding domains. This SBP is located in the periplasm of gram-negative bacteria and is attached to the cell membrane in gram-positive bacteria and *Archaea* (20). The SBP ABC transporters are required for the uptake of a variety of small molecules (including amino acids, metal ions, and sugars) and can accumulate solutes against very large concentration gradients (>10,000-fold) (15).

There are two main classes of ABC transporters of amino acids, the polar amino acid transporter (PAAT; transporter classification [T.C.] 3.A.1.3) and the hydrophobic amino acid transporter (HAAT; T.C. 3.A.1.4) families (25, 47). The PAAT family is one of the best-defined subclasses of SBP ABC transporters and includes the first ABC transporter to be sequenced, the histidine permease of *Salmonella enterica* serovar Typhimurium (17, 18). The ATP-binding protein of the His permease was also the first for which a crystal structure became available (26). In contrast, the HAAT family is poorly characterized, with only two recognized subclasses: the branched-chain amino acid transporters of *Pseudomonas aeruginosa* (Bra_{Pa}) and *Escherichia coli* (Liv_{Ec}; T.C. 3.A.1.4.1) (22, 33) and the neutral amino acid permease (Nat) of *Synechococcus* sp. strain PCC6903 (T.C. 3.A.1.4.2) (36). Although the membership of HAAT is increasing as the microbial genome sequencing projects are completed (25), the experimental characterization of these transporters is rarely carried out.

The rhizobia, a group of α-proteobacteria which includes *Rhizobium*, *Mesorhizobium*, *Sinorhizobium, Bradyrhizobium*, and *Azorhizobium* species, form a species-specific symbiotic relationship with leguminous plants in which the plant provides the bacteroid (symbiotic bacteria) a carbon source (C_4-dicarboxylic acid), while the plant receives reduced atmospheric nitrogen from the bacteroid (8, 38). Prior to the establishment of 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 high-affinity ABC transporters present in *Sinorhizobium me-*

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

Strain, plasmid, or cosmid	Description	Source or reference
Strains		
A34	*R. leguminosarum* bv. *viciae* [formerly known as 8401/pRLJI1)]	10
3841	*R. leguminosarum* bv. *viciae* Sm^r derivative of strain 300	30
RU1131	A34 *braE*::Tn*phoA*	24
RU1356	A34 Δ*aapJQM*::ΩSp	24
RU1357	A34 *braE*::Tn*phoA* Δ*aapJQM*::ΩSp	24
RU1470	A34 *braC*::Tn*5-lacZ*	This study
RU1472	A34 *braC*::Tn*5-lacZ* Δ*aapJQM*::ΩSp	This study
RU1386	RU1357(pRK415)	This study
RU1358	RU1357(pIJ1427)	This study
RU1359	RU1357(pBIO206)	This study
RU1413	RU1357(pRU733)	This study
RU1482	RU1357(pRU826)	This study
RU1361	RU1357(pRU191)	This study
RU1360	RU1357(pRU3024)	This study
RU1362	RU1357(pRU3131)	This study
Cosmids		
pIJ1427	pLAFR1 cosmid containing *braDEFGC* from strain A34	7
pBIO206	pIJ1427 *braE*::Tn*phoA*	24
pRU3024	pLAFRI cosmid containing *aapJQMP* from strain 3841	55
pRU3131	pLAFRI cosmid containing *bra-2* from strain 3841	2
pRU3158	pIJ1427 *braC*::Tn*lacZ*	This study
Plasmids		
pLAFR1	Wide-host-range mobilizable P-group cloning vector; Tc^r	12
pBluescript II SK−	Phagemid, pUC19 derivative, f1 origin of replication, ColE1 replicon; Amp^r	Stratagene
pRK415-1	Broad-host-range P-group cloning vector; Tc^r	32
pPHJI1	P-group chaser plasmid	19
pCR-BluntII-TOPO	Cloning vector for PCR products	Invitrogen Life Technologies
pRU191	*aapJQMP* in pRK415-1	55
pRU729	5-kb *Hpa*I/*Eco*RI fragment (*braDEFG*) from pIJ1427 in pBluescript II SK(−)	This study
pRU733	5-kb *Kpn*I fragment from pRU729 (*braDEFG*) in pRK415-1	This study
pRU821	1.5-kb PCR product (primers P273 and P274; *braC*) from pIJ1427 cloned into pCR-Blunt II-TOPO	This study
pRU826	1.5-kb *Eco*RI fragment from pRU821 (*braC*) in pRK415-1	This study

liloti, *Mesorhizobium loti*, and *Agrobacterium tumefaciens* (13, 31, 57).

Uptake of amino acids by *Rhizobium leguminosarum* has been found to be due in part to the general amino acid permease (Aap; T.C. 3.A.1.3.8). This permease is a member of the PAAT subfamily of ABC transporters but is unusual in that it transports a broad range of amino acids (55). Typically members of PAAT will only transport a single amino acid or a group of structurally related amino acids (25, 54). For example, the ArtPIQMJ system of *E. coli* is very specific for arginine, while the HisJQMP system of *S. enterica* serovar Typhimurium will transport histidine and arginine (17, 56). The glutamate, glutamine, aspartate, and asparagine transporter (BztABCD) of *Rhodobacter capsulatus* (T.C. 3.A.1.3.7) is the only other reported broad-specificity transporter of the PAAT family (59).

Although mutation of *aap* significantly reduces the rate of uptake of most amino acids, a considerable rate is retained (55), indicating the presence of other transporters of these solutes in this organism. While studying the bidirectional transport of solutes by SBP ABC transporters, we identified a second high-affinity transporter of amino acids in *R. leguminosarum* (Bra_{Rl}) (24). The bra_{Rl} operon encodes five products, an SBP ($BraC_{Rl}$), two integral membrane proteins ($BraD_{Rl}$ and $BraE_{Rl}$), and two ATP-binding proteins ($BraF_{Rl}$ and $BraG_{Rl}$). On the basis of sequence similarity, Bra_{Rl} can be classified as a member of the HAAT family and is expected to transport neutral and aliphatic amino acids. For example, the similar LIV-I transporters of *E. coli* and *P. aeruginosa* transport neutral amino acids (i.e., leucine, isoleucine, valine, alanine, threonine, and possibly serine) (1, 23, 33, 41, 43). However, in this study we report that Bra_{Rl} can transport a broad range of solutes, including polar amino acids and γ-aminobutyric acid (GABA), in addition to hydrophobic and neutral amino acids. Therefore, Bra_{Rl} is the second general amino acid permease of *R. leguminosarum*. Indeed, we show that D amino acids significantly inhibit uptake of solutes by Bra_{Rl}, indicating that it may be a global amino acid transporter.

MATERIALS AND METHODS

Bacterial strains, plasmids, 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) (40) with 10 mM D-glucose and 10 mM ammonium chloride or 10 mM L-glutamate as the sole source of carbon and nitrogen. Antibiotics were used at the following concentrations: streptomycin, 500 μg ml^{-1}; kanamycin, 40 μg ml^{-1}; tetracycline, 2 (in AMS) and 5 μg ml^{-1} (in TY); gentamicin, 20 μg ml^{-1}; spectinomycin, 100 μg ml^{-1}.

Identification of HAAT genes in genomic sequences. The *Sinorhizobium meliloti* 1021, *Mesorhizobium loti* MAFF303099, and *Agrobacterium tumefaciens* C58

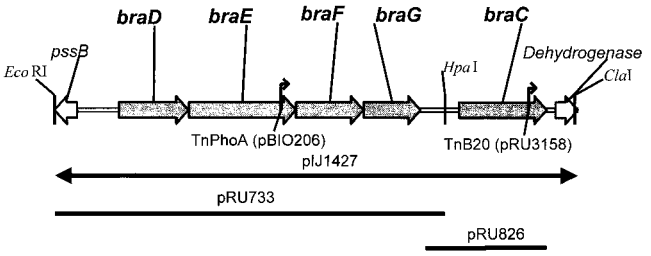

FIG. 1. Map of the *bra* operon. The region of the *R. leguminosarum* genome containing the *bra* operon is represented. Thin arrows, locations of the transposon insertions used to construct mutants used in this study. The orientations of the transposons are indicated by the directions of the arrows, representing the directions of transcription of the *phoA* and *lacZ* genes. The lines beneath the operon indicate the regions located on the cosmids or plasmids used in this study.

genome sequences were searched for HAAT orthologues by BLAST (3) with $braC_{Rl}$, $braF_{Rl}$, and $braG_{Rl}$ as the query sequences. The searches were conducted by using the facility provided at each of the relevant genome annotation databases (http://sequence.toulouse.inra.fr/meliloti.html, http://www.kazusa.or.jp/rhizobase/, and http://cancer.lbi.ic.unicamp.br/agroC58/).

Genetic modification of bacterial strains. Plasmids were conjugated into *R. leguminosarum* as described previously (40). The region of the *bra* operon located on the plasmids and cosmids and the sites of transposon insertion for mutants used in this study are shown in Fig. 1. Cosmid pIJ1427 was mutagenized as described by Simon et al. (50). Chromosomal *braC* mutations were made by conjugation of the mutated cosmid (pRU3158) into either A34 or RU1356. After purification, incompatible plasmid pPHJI1 was conjugated into each strain and the homogenotes were isolated by the technique of Ruvkun and Ausubel (45).

For complementation studies, *braC* was amplified by PCR using primers P273 (TTAGTGATGGTGATGGTGATGTGTCCCATAAAGGGGAGCGA) and P274 (TCCGTGTCATCGTCTTGCTCTTAGG), cloned with a Zero Blunt TOPO PCR cloning kit (Invitrogen Life Technologies), and subcloned as an *Eco*RI fragment into broad-host-range vector pRK415-1 (32) to form pRU826. A 5-kb *Hpa*I/*Eco*RI fragment containing *braDEFG* was cloned from pIJ1427 into pBluescript II SK(−) (Stratagene) and transferred into pRK415-1 as a *Kpn*I fragment to form pRU733.

Transport assays. *R. leguminosarum* uptake assays were performed by the rapid-filtration method as previously described (39). The final concentration of solute was 25 μM (0.125 μCi of ^{14}C), and competing solutes were added to 0.5 mM. The kinetics of solute uptake by *R. leguminosarum* strains were determined by using various ^{14}C-solute concentrations in standard uptake assays. All cultures were grown on liquid minimal salts with glucose and ammonium chloride.

Nucleotide sequence accession numbers. The sequences of the regions of the *R. leguminosarum* genome containing the *bra* and *bra2* operons were submitted to EMBL under accession no. AJ272047 and AJ427840, respectively.

RESULTS

Growth of *R. leguminosarum* strains on glutamate as the sole C and N source. In previous studies we showed that strains of *R. leguminosarum* that lack Aap are unable to grow on solid minimal media (AMA) containing L-glutamate (10 mM) as the sole source of carbon and nitrogen (55). Conversely, mutation of *bra* (RU1131) did not affect growth on solid minimal salts with L-glutamate (10 mM) as the sole carbon and nitrogen source. However, during preliminary studies to investigate the regulation of *aap* and *bra*, control cultures of an *aap* mutant (RU1356) were observed to grow on liquid minimal medium (AMS) with L-glutamate (10 mM) as the sole carbon and nitrogen source, although RU1356 has a higher doubling time than the wild-type strain (19.8 ± 1.1 versus 8.7 ± 0.1 h, respectively). Also, the *bra* mutant RU1131 did not grow as well as the wild-type strain on liquid minimal salts with L-glutamate (doubling time, 13.8 ± 0.4 h), and growth on L-glutamate was eliminated in RU1357, an *aap bra* double mutant. The reason for the apparent contradiction between growth on solid and liquid minimal salts media containing L-glutamate is unknown. However, the data indicate that both Aap and Bra_{Rl} are involved in the uptake of L-glutamate in liquid medium. This implies that the solute specificity of Bra_{Rl} is not restricted to hydrophobic or neutral amino acids.

Although Bra_{Rl} does not support growth on solid minimal salts medium with L-glutamate in an *aap* mutant, this may be due to insufficient expression of *bra*. To investigate this, the effect of increased gene copy number on growth was determined. When the cosmid containing the complete *bra* operon (pIJ1427) was present in *aap* mutants (RU1356 and RU1357), growth on L-glutamate was restored, while the same cosmid containing a transposon insertion in *braE* (pBIO206) did not restore growth. Similar restoration of growth was observed in *aap* mutants when plasmid pRU733, which contains *braDEFG* but which lacks *braC*, the SBP gene, was present. Therefore, Bra_{Rl} can complement *aap* mutations for growth on solid minimal salts with L-glutamate, but only when the copy numbers of genes encoding the membrane-associated transport components are enhanced.

Uptake of amino acids by Bra_{Rl}. As Bra_{Rl} allows growth on liquid minimal salts with L-glutamate and, in multicopy, allows growth on solid minimal salts with L-glutamate, it was considered possible that this permease might have a broader specificity than previously characterized members of HAAT. Therefore, the specificity of solute transport by Bra_{Rl} was investigated by using a representative set of L amino acids (glutamate, α-aminoisobutyric acid [AIB], histidine, leucine, alanine, and arginine). Uptake of each of these amino acids in *aap* and *braE* mutants (RU1356 and RU1131, respectively) was decreased, with only negligible uptake in the *aap braE* double mutant (RU1357; Fig. 2A). Cosmids containing either *aap* (pRU3024) or *bra* (pIJ1427) were able to compensate for this loss of amino acid uptake, raising uptake rates for each amino acid above those observed in the wild-type strain, while control cosmids containing mutations in *bra* did not elevate the rate of amino acid transport (Fig. 2B). Strains containing *braC* mutations (RU1470 and RU1472) gave results similar to those for *braE* mutants, except that the rate of transport of L-alanine remained unchanged (Fig. 2). Therefore, transport of L-glutamate, AIB, L-histidine, L-leucine, and L-arginine is dependent on *braC* but L-alanine uptake is not. These data suggest that *R. leguminosarum* has an unidentified SBP, which interacts with the membrane components of Bra_{Rl} during L-alanine uptake.

braC carried on a plasmid (pRU826) restored L-glutamate, L-histidine, L-leucine, L-arginine, and AIB uptake levels in RU1472 (*braC aap*) to those of the unmutated strain (RU1356; Fig. 3B). In plasmid pRU826, *braC* is transcribed divergently from the vector *lac* promoter, suggesting that a promoter for this gene is present in the *braG-braC* intergenic region. Also, complementation studies confirmed that *braDEFG* (pRU733) is sufficient to restore uptake rates in *braE* mutants for each amino acid tested (RU1357; Fig. 3A). If no promoter were present in the *braG-braC* intergenic region, *braDEFG* alone would not be expected to complement *braE*::Tn*5* (Fig. 3), as this mutation would be polar on *braC*. A *braC* promoter would also be consistent with the large (498-bp) intergenic region

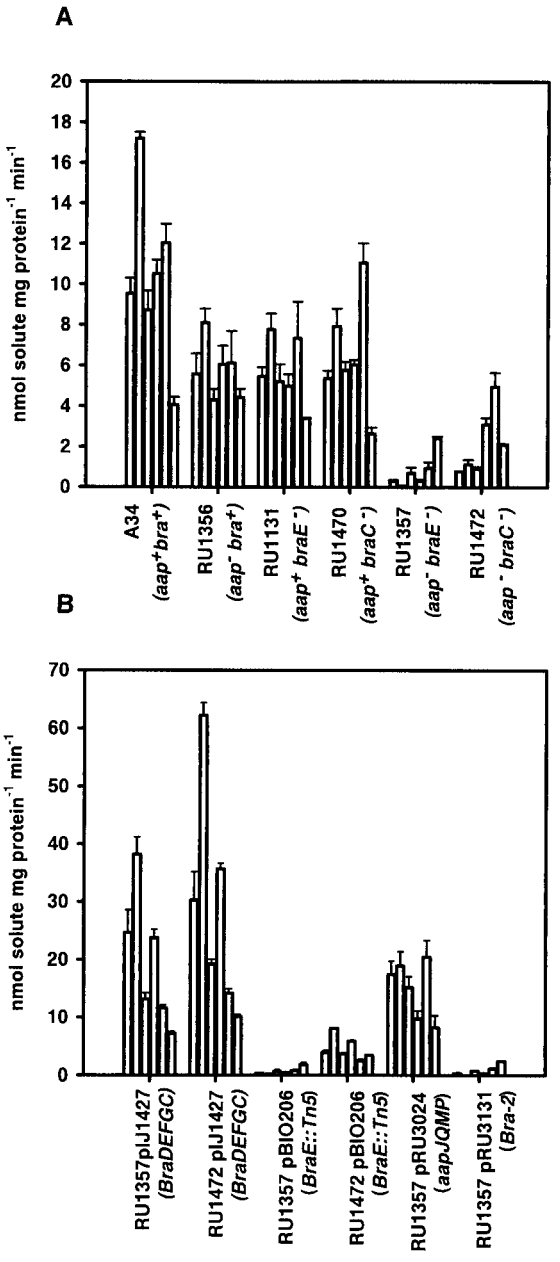

FIG. 2. Uptake of amino acids by mutated strains of *R. leguminosarum*. The uptake rates of L-glutamate, AIB, L-histidine, L-leucine, L-alanine, and L-arginine (respectively represented by the bars, left to right, within each group) were determined for a number of mutant strains of A34 (A) and mutated strains containing cosmids (B).

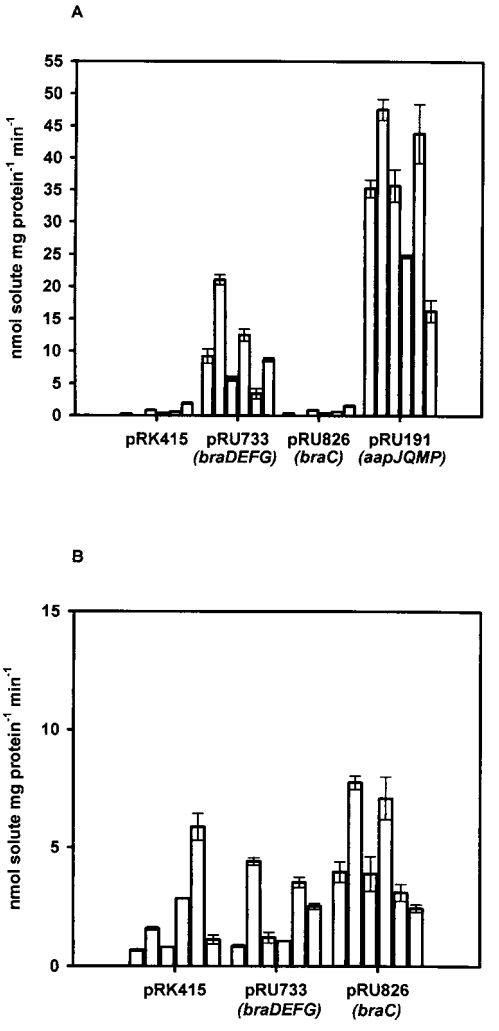

FIG. 3. Complementation of *aap* and *bra* transport mutants. The uptake rates of L-glutamate, AIB, L-histidine, L-leucine, L-alanine, and L-arginine (respectively represented by the bars, left to right, within each group) were determined for RU1357- (*aap braE* mutant; A) and RU1472 (*aap braC* mutant; B)-derived strains containing plasmids carrying a number of transport component genes.

between *braG* and *braC*, while the genes encoding the other transport components are probably cotranscribed from a promoter upstream of *braD*, as they are separated by only between 2 and 7 nucleotides.

The specificity of Bra_{Rl} was further investigated by uptake competition experiments. L-Leucine and L-glutamate were selected, as they represent high- and low-affinity solutes of Bra_{Rl} (see below). Therefore, the relative affinities of competing solutes (added at a 20-fold excess concentration) could be determined. Strain RU1356 was used in these studies as the presence of Aap in the wild-type strain would confound the results. Uptake of L-[^{14}C]leucine was inhibited by the addition of all L amino acids tested, except L-glutamate, L-aspartate, L-glutamine, L-asparagine, and L-arginine (Fig. 4A). However, the uptake of L-[^{14}C]glutamate was inhibited by all L amino acids, including those that did not inhibit L-leucine uptake (Fig. 4B). This difference between the inhibition of L-leucine uptake and the inhibition of L-glutamate uptake most probably reflects a lower affinity of Bra_{Rl} for L-glutamate, L-aspartate, L-glutamine, L-asparagine, and L-arginine. Therefore, Bra_{Rl} has at least as broad a solute specificity as Aap and is able to transport polar amino acids as well as aliphatic amino acids.

Uptake of GABA by Bra_{Rl}. Nodules and *Rhizobium* bacteroids contain high concentrations of GABA (44, 51), and this solute can be used as the sole carbon and nitrogen source by rhizobia in vitro (11, 29). However, no transporter of GABA

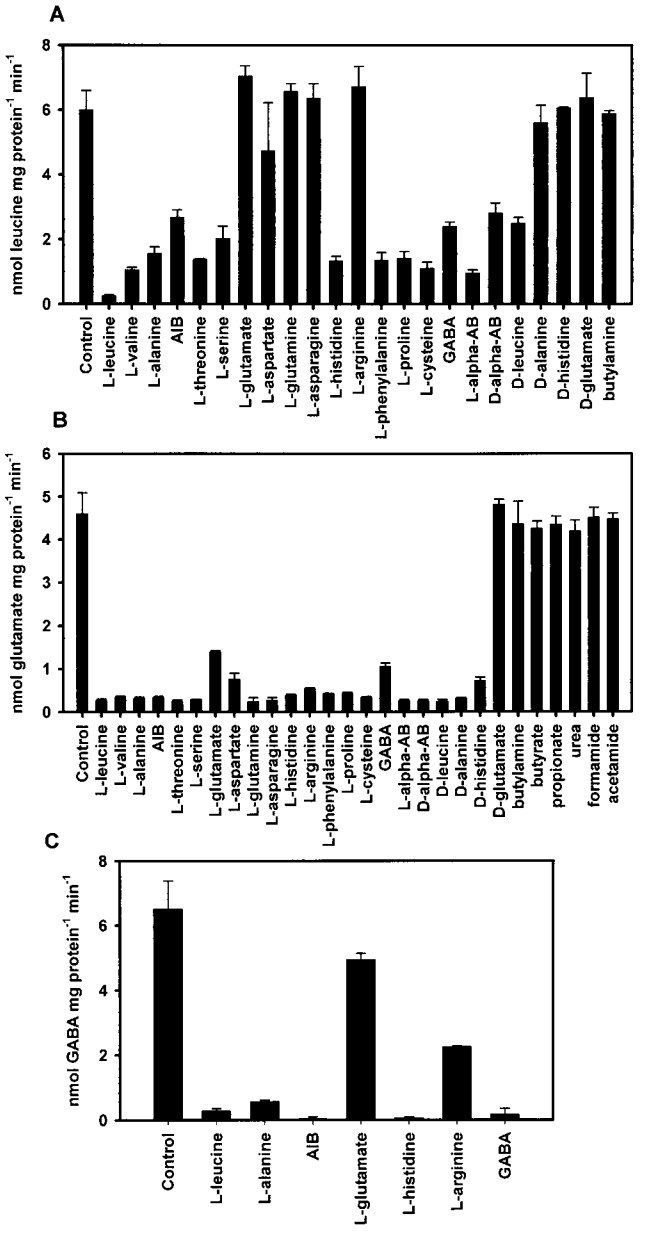

FIG. 4. Inhibition of leucine, glutamate, and GABA uptake by other solutes. Uptake of 25 μM (0.125 μCi) L-[^{14}C]leucine (A), L-[^{14}C]glutamate (B), and [^{3}H]GABA (C) was assayed by the rapid-filtration method. Competing solutes were added to a final concentration of 0.5 mM. (L-alpha-AB, L-α-aminobutyrate; D-alpha-AB, D-α-aminobutyrate.

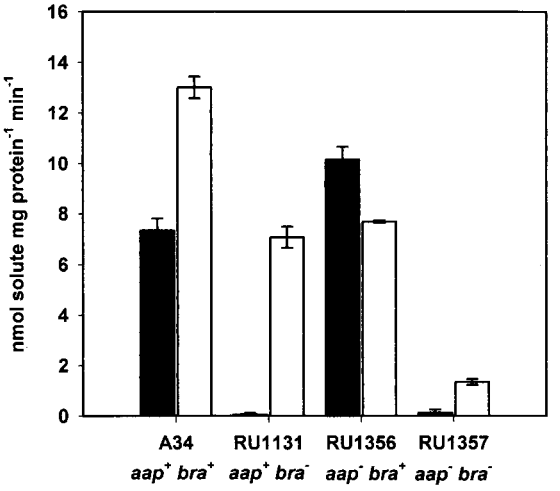

FIG. 5. Uptake of GABA by mutated strains of *R. leguminosarum*. The rates of uptake of GABA (black bars) and L-glutamate (white bars) were determined for a number of mutant strains of A34.

has been reported in rhizobia. Therefore, the possible role of Aap or Bra in GABA uptake was investigated. *R. leguminosarum* (A34) transported [^{3}H]GABA at a rate comparable to that for amino acids tested (compare Fig. 5 and 2A). GABA uptake in RU1131 (*bra* mutant) was undetectable, and mutation of *aap* (RU1356) had no effect, indicating that Bra_{Rl}, but not Aap, transports GABA. In uptake competition assays, GABA inhibited the uptake of L-leucine and L-glutamate by Bra_{Rl} (Fig. 4A and B). Similarly, solutes that inhibit uptake of L-leucine and L-glutamate also inhibit the uptake of GABA (Fig. 4C), confirming that Bra_{Rl} transports GABA and thus that the reduced uptake of GABA is not due to a secondary effect of a mutation of Bra_{Rl} on a separate GABA transporter.

Constraints on the solute specificity of Bra_{Rl}. To determine what constraints there are on solute structure for transport by Bra_{Rl}, the effect of a range of solutes on L-glutamate and L-leucine uptake was determined (Fig. 4). Solutes of Bra_{Rl} must possess both amino and carboxyl groups, as butyrate, propionate, and butylamine did not inhibit L-glutamate uptake. The position of the amino group in relation to the carboxyl group is not critical, as Bra_{Rl} can transport GABA, a γ-amino acid. Therefore, the specificity of Bra_{Rl} is not restricted to α-amino acids, but the apparent affinity is highest for L-α-amino acids, as L-α-aminobutyrate is a better inhibitor of L-glutamate and L-leucine uptake than GABA.

The stereospecificity of characterized HAAT transporters has not been fully determined, and available data are ambiguous. Preliminary characterization of Liv_{Ec} indicated that it could transport D-leucine but with a higher K_m and lower V_{max} than those for the L isomer (41). However, no data were presented in this initial report to substantiate these claims. In contrast, characterization of the binding properties of $BraC_{Pa}$ indicated that a 100-fold excess of D-leucine did not inhibit the binding of L-leucine (21). Therefore, to determine the stereospecificity of Bra_{Rl}, representative D amino acids (D-leucine, D-glutamate, D-alanine, D-histidine, and D-α-aminobutyrate) were used in competition uptake assays. Uptake of L-glutamate was inhibited by D-leucine, D-alanine, D-histidine, and D-α-aminobutyrate, but not by D-glutamate. L-Leucine uptake was inhibited by D-leucine and D-α-aminobutyrate (Fig. 4). Therefore, Bra_{Rl} has a significant affinity for D amino acids but the affinity is lower than that for the corresponding L isomer. Furthermore, the apparent affinity for most D amino acids tested is greater than that for physiologically relevant solutes such as L-glutamate.

An SBP ABC transporter of urea and short-chain amides with similarity to branched-chain amino acid transporters of the HAAT family has been identified in *Methylophilus methy-*

TABLE 2. Kinetics of solute uptake by the Aap and Bra of *R. leguminosarum*[a]

Solute	RU1356 (*aap bra*$^+$)		RU1131 (*aap*$^+$ *bra*)	
	K_m	V_{max} (nmol of solute mg of protein^{-1} min^{-1})	K_m (nM)	V_{max} (nmol of solute mg of protein^{-1} min^{-1})
L-Leucine	205 ± 82 nM	7.15 ± 1.05	515 ± 144	3.17 ± 0.24
L-Alanine	173 ± 37 nM	3.51 ± 0.26	509 ± 206	6.22 ± 0.70
AIB	97 ± 56 nM	5.42 ± 1.16	807 ± 383	3.94 ± 0.59
L-Histidine	78 ± 29 nM	2.78 ± 0.26	200 ± 110	4.17 ± 0.48
L-Glutamate	56 ± 11μM	17.09 ± 1.58	200 ± 66	4.81 ± 0.52
GABA[b]	288 ± 121 nM	6.17 ± 0.82	na[c]	na

[a] Cells were grown on glucose-ammonia minimal medium. Kinetics were derived from the mean rates determined for at least two independent cultures. The standard errors were determined from the non-linear regression curve.
[b] GABA kinetics were determined for the wild-type strain (A34; *aap*$^+$ *bra*$^+$), as Aap has no effect on uptake of this solute.
[c] na, not applicable, as Aap does not transport GABA.

lotrophus (35). However, the solutes of this transporter (urea, acetamide, and formamide) had no effect on uptake of L-leucine or L-glutamate by Bra_{Rl} (Fig. 4). Therefore, the solute specificity of Bra_{Rl} is distinct from that of the urea and amide transporter.

Kinetics of solute uptake. The kinetic constants of uptake confirm that Bra_{Rl} is a high-affinity transporter of amino acids for which K_m values are between 78 nM and 56 μM (Table 2). The K_m for L-histidine uptake (78 nM) is lower than that for L-leucine uptake (205 nM), and the V_{max} for L-glutamate uptake (17 nmol mg of protein^{-1} min^{-1}) is the highest of those for the solutes tested. Therefore, these solutes are transported by Bra_{Rl} under physiologically relevant conditions. The K_m for L-glutamate uptake (56 μM) confirms that Bra_{Rl} has a much lower affinity for this solute than for others tested, explaining its inability to inhibit uptake of L-leucine and GABA (Fig. 4A and C). The K_m values for L-leucine, L-alanine, AIB, and L-histidine are each significantly lower for Bra_{Rl} than for Aap (Table 2).

Growth of *aap* and *bra* mutants on amino acids as the sole carbon and nitrogen source. Since the physiological relevance of Bra_{Rl} to L-glutamate metabolism was first revealed by growth studies, we examined the phenotypes for the growth of *aap*, *bra*, and *aap bra* mutants on amino acids as the sole carbon and nitrogen sources in liquid culture. Although single *aap* and *bra* mutants were able to grow on L-glutamate, L-glutamine, L-asparagine, L-proline, L-serine, L-arginine, and L-citrulline, the rate of growth was lower than that of the wild-type strain. Mutation of both *aap* and *bra* abolished growth on these solutes (Table 3). Therefore, both Bra_{Rl} and Aap have an important physiological role in growth on a broad range of amino acids. Also, the lack of growth of *bra* mutants on GABA confirmed that Bra_{Rl} is the main route of GABA uptake in free-living *R. leguminosarum*. Growth on L-alanine and L-histidine was unaffected by mutation of *aap* and *bra* (Table 3), indicating that *R. leguminosarum* has other transporters of these solutes.

Preliminary characterization of a second HAAT permease from *R. leguminosarum*. During the course of these studies, the coding sequence for a second HAAT-like permease of *R. leguminosarum* ($Bra2_{Rl}$) was identified on cosmid pRU3131. The partial sequence of this operon (accession no. AJ427840) indicated that it encoded at least one SBP (Bra2C), two integral membrane proteins (Bra2DE), and two nucleotide-binding proteins (Bra2FG), each with significant homology to the corresponding proteins of other HAAT members (e.g., see $Bra2F_{Rl}$ in Fig. 6). However, mutation of this transport operon did not affect uptake of L-leucine or L-alanine (data not shown). Also, overexpression in RU1357 (*aap bra* mutant) did not result in increased uptake of any amino acid tested (Fig. 2). Therefore, *R. leguminosarum* has at least two HAAT paralogues with different transport specificities or expression profiles.

Comparison of Bra_{Rl} and related sequences. The completion of microbial genome sequences has revealed that some species contain multiple HAAT paralogues. For example, a search of the *Deinococcus radiodurans* and *Archaeoglobus fulgidus* sequences revealed four HAAT paralogues in each (25). Therefore, the incidence of HAAT proteins in rhizobia was investigated. A search of the complete genome sequences of *S. meliloti* and *M. loti* indicated that each has at least five complete operons resembling HAAT operons and three or four "orphan" BraC-like binding proteins (allocated gene no. mll1986, mlr7182, mlr7721, mlr9716, Sma0576, SMc00078, and Smc00513; see http://sequence.toulouse.inra.fr/meliloti.html and http://www.kazusa.or.jp/rhizobase/). A similar search of the *Agrobacterium tumefaciens* genome sequence revealed

TABLE 3. Growth of *R. leguminosarum* strains following 7 days of incubation at 28°C on AMS liquid media containing various solutes[a]

Solute (concn [mM])	Growth[b] of strain:			
	A34	RU1131	RU1356	RU1357
L-Glutamate (10)	+++++	++++	+++	−
L-Glutamine (10)	+++++	++	++	−
L-Aspartate (10)	−	−	−	−
L-Asparagine (10)	+	+	±	−
L-Alanine (20)	+++++	+++++	+++++	+++++
GABA (10)	++++	−	++	−
L-Proline (10)	+++++	++++	++++	−
L-Serine (10)	++	−	−	−
Glycine (20) + CaCl (2)	−	−	−	−
L-Histidine (10)	+++++	+++++	+++++	+++++
L-Arginine (10)	++	+	±	−
L-Ornithine (10)	−	−	−	−
L-Citrulline (10)	+	+	±	−

[a] 10-ml cultures were inoculated with approximately 5×10^8 CFU.
[b] +++++, at least 7×10^8 CFU/ml by 48 h postinoculation; ++++, at least 7×10^8 CFU/ml by 72 h postinoculation; +++, at least 3.5×10^8 CFU/ml by 72 h postinoculation; ++, at least 4.5×10^8 CFU/ml by 140 h postinoculation; +, at least 2×10^8 CFU/ml by 140 h postinoculation; ±, at least 1×10^8 CFU/ml by 140 h postinoculation; −, $<1 \times 10^8$ CFU/ml by 140 h postinoculation.

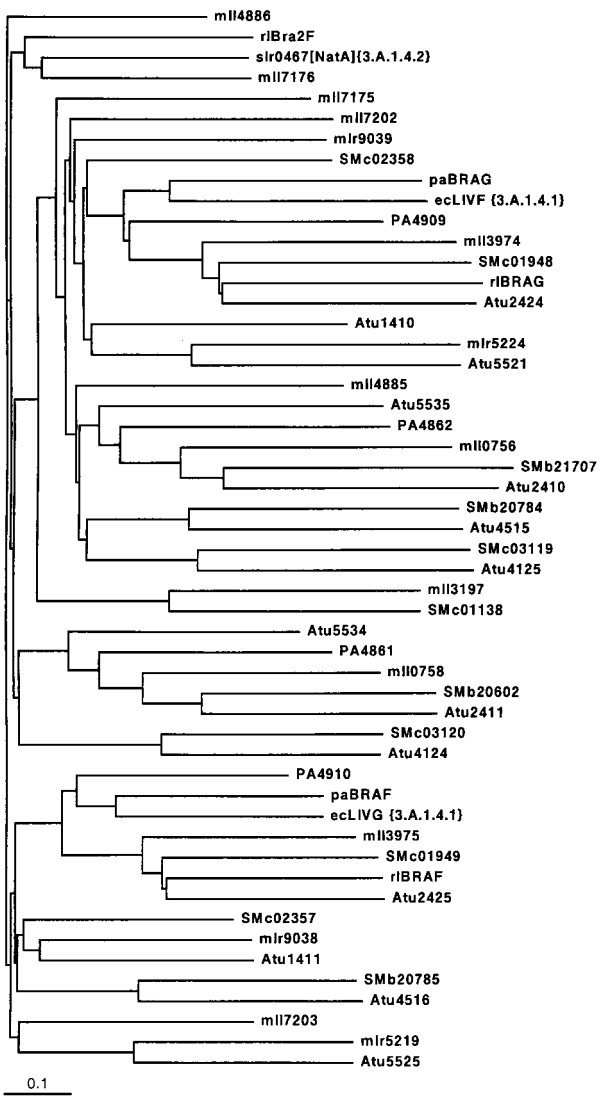

FIG. 6. Phylogenetic tree of members of selected ABC transporters of the HAAT family. Amino acid sequences of the ATP-binding proteins of selected members of the HAAT family were aligned using Vector NTI Suite (version 6), and the resulting phylogenetic tree was drawn by using Treeview (version 1.6.1). In addition to the transporters described here, included in the tree are the permeases for which experimental evidence of function is published and transporters identified from the sequencing projects of *M. loti*, *S. meliloti*, *Agrobacterium tumefaciens*, and *P. aeruginosa*. The sequences are identified by the designated protein name or protein number assigned by the sequencing projects (http://sequence.toulouse.inra.fr/meliloti.html, http://www.kazusa.or.jp/rhizobase/, http://www.pseudomonas.com/, and http://cancer.lbi.ic.unicamp.br/agroC58/). The bacterial species from which the sequences were derived are indicated by a prefix as follows: Atu, *A. tumefaciens*; ec, *E. coli*; mll and mlr, *M. loti*; pa and PA, *P. aeruginosa*; rl, *R. leguminosarum* bv. *viciae*; slr, *Synechocystis* sp.; SMc and SMb, *S. meliloti*. The transporter classification numbers are in parentheses adjacent to the representative members of the transporter subfamily described by M. H. Saier (47).

seven complete operons resembling HAAT operons, with two located on the At plasmid and the remainder distributed on the two chromosomes.

The sequences of $BraF_{Rl}$ and $BraG_{Rl}$ were aligned with the HAAT-like ABC proteins from *S. meliloti*, *M. loti*, *Agrobacterium tumefaciens*, *P. aeruginosa*, and *E. coli* (Fig. 6). The resulting tree indicates that Bra_{Rl} has the highest identity to the Liv_{Ec} and Bra_{Pa} transporters, with orthologues in the sequenced rhizobia and *Agrobacterium tumefaciens* (SMc01948/9, mll3974/5, and Atu2424/5).

DISCUSSION

These data indicate that Bra_{Rl} is the second general amino acid permease present in *R. leguminosarum*. The previously described Aap belongs to one subfamily of ABC transporters, PAAT, while Bra_{Rl} belongs to the other main subfamily of ABC transporters of amino acids, HAAT. Although the affinities of Aap and Bra_{Rl} for solutes differ (Table 2), these two transporters have overlapping solute specificities, with each permease able to transport polar amino acids (e.g., L-glutamate, L-histidine, and L-arginine) and branched-chain amino acids (e.g., L-leucine) (Fig. 2). A significant difference between Aap and Bra_{Rl} is their stereospecificities; Aap is specific for L amino acids (39, 55), whereas Bra_{Rl} also has significant affinity for D amino acids (Fig. 4). As butylamine, butyrate, and propionate do not inhibit uptake of Bra_{Rl} solutes, solutes of Bra_{Rl} must possess amino and carboxyl groups. However, the specificity of Bra_{Rl} is not restricted to α-amino acids as GABA, a γ-amino acid, is transported. Indeed, Bra_{Rl} is the first ABC transporter to be shown to transport GABA. All previously identified bacterial transporters of GABA, which include GabP of *E. coli* (37) and GadC of *Lactococcus lactis* (48), are secondary carriers of the amino acid-polyamine-organocation (APC) superfamily and function as solute/cation symporters or solute/solute antiporters (27).

Both Bra_{Rl} and Aap contribute to the ability of *R. leguminosarum* to grow on amino acids as the sole source of carbon and nitrogen, but growth of *aap bra* double mutants on L-alanine and L-histidine indicates that unidentified transporters of amino acids are present in this species (Table 3). A histidine transporter (HutXWV) of the quaternary amine uptake transporter family has been identified in *S. meliloti* (6). However, this permease is not present in all rhizobia as no apparent orthologue of *hutXWV* is present in the complete genome sequence of *M. loti*. An alternative candidate L-histidine transporter is the uncharacterized homologue of the HisJQMP permease of *S. enterica* serovar Typhimurium (17, 18), which has been identified in *R. leguminosarum* (25).

L-Alanine is clearly transported by Bra_{Rl}, as mutation or overexpression of *braDEFG* alters the rate of uptake of this solute (Fig. 2). However, unlike that of other solutes, uptake of L-alanine is not dependent on $BraC_{Rl}$. Our present understanding of solute transport by SBP ABC transporters indicates that it is unlikely that the membrane components of this permease transport L-alanine without interaction with an SBP (16). Therefore, it is probable that another BraC-like SBP is involved in L-alanine uptake. However, no BraC-like genes are present in the immediate vicinity of the bra_{Rl} operon (Fig. 1). The *S. meliloti* genome contains three orphan *braC* homologues that are not located near other transport components. It is possible that the proteins encoded by these genes interact with HAAT transporters encoded by genes located elsewhere on the genome. Although no such orphan *braC*-like genes have

yet been identified in *R. leguminosarum*, an orphan L-alanine binding protein may interact with $BraDEFG_{Rl}$. The interaction of multiple SBPs with the membrane complex of ABC transporters is well established. For example, in *S. enterica* serovar Typhimurium *hisJ* and *argT* encode a histidine binding protein and an arginine, lysine, and ornithine binding protein, respectively (17), which interact with HisQMP, and in *E. coli livK* and *livJ* encode a leucine-specific binding protein and a leucine, isoleucine, and valine binding protein, respectively, which interact with LivHMGF (33).

The closely related branched-chain amino acid transporters in *E. coli* (Liv_{Ec}; T.C. 3.A.1.4.1) and *P. aeruginosa* (Bra_{Pa}) are the best-characterized members of the HAAT family. Although these transporters are referred to as branched-chain amino acid or LIV transporters, their specificity is broader than these names suggest. One of the two periplasmic binding proteins of Liv_{Ec} (LivJ) binds L-threonine, L-alanine, and L-serine in addition to L-leucine, L-isoleucine, and L-valine, although the affinity for the former three solutes is lower than that for the latter three (41). Indeed, an investigation of L-alanine transport in *E. coli* confirmed that it is transported by Liv_{Ec} (43). Also, the membrane complex of Bra_{Pa} has been solubilized and reconstituted into proteoliposomes, and the transport of L-alanine and L-threonine by this permease was confirmed. However, the affinities of $BraC_{Pa}$ for L-alanine and L-threonine (K_d, 3 to 5 μM) are much lower than those for L-leucine, L-isoleucine, and L-valine (K_d, 0.3 to 0.5 μM), indicating a preference for the transport of branched-chain amino acids (23). The reported specificity of Bra_{Pa} is not as broad as that reported here for Bra_{Rl}, as no significant uptake of L-glutamate or L-proline could be detected in proteoliposomes containing Bra_{Pa}. Also, uptake of L-[^{14}C]leucine was not inhibited by L-histidine, L-glutamate, L-glutamine, or L-proline (23). However, as the Bra_{Pa} uptake assays were carried out with a final solute concentration of only 10 μM and as the competition experiments were performed with only a high-affinity solute (i.e., L-leucine), Bra_{Pa} may have lower, but significant, affinity for other amino acids.

An investigation of amino acid uptake in cyanobacteria identified two open reading frames (ORFs) of *Synechocystis* sp. strain PCC 6803 (*slr0467* and *slr0559*; T.C. 3.A.1.4.2) which, when mutated, produced a decreased rate of amino acid uptake. The product of ORF *slr0467* (renamed *natA*) has 40% identity to $BraF_{Pa}$, and the product of *slr0559* (*natB*) has 26% identity to $BraC_{Pa}$. Mutation of these genes resulted in an almost total impairment of uptake of a broad range of neutral amino acids (i.e., L-alanine, glycine, L-leucine, L-phenylalanine, L-proline, and L-serine), in addition to a 70% decrease in L-aspartate uptake and a 30% decrease in L-histidine uptake. Mutation of *natA* also decreased L-glutamate uptake by 30 to 50% (36). Since the characterization of this transporter relied exclusively on mutation analysis, further characterization is required to confirm the solute specificity, as mutation of *natA* and *natB* may have a secondary effect on the expression of other transporters. Nevertheless, the data suggest that the Nat permease of *Synechocystis* spp. is a broad-specificity amino acid transporter of the HAAT family. However, a comparison of the *natA* and the *R. leguminosarum braF* and *braG* sequences indicates that the Nat permease is more distantly related to Liv_{Ec} and Bra_{Pa} (Fig. 6). Mutation of a second ORF of *Synechocystis* sp. strain PCC 6803 with high sequence identity to ABC binding proteins of the HAAT family (*sll0374*) had no effect on the uptake of the 12 amino acids tested (36). Therefore, *Synechocystis* sp. strain PCC 6803 contains at least one member of the HAAT family with an apparent broad specificity for amino acids and another that is not involved in the uptake of amino acids under the conditions tested.

A high-affinity SBP ABC transporter of short-chain amides and urea has been identified in *Methylophilus methylotrophus* (35). The partial sequence of the operon encoding this transporter revealed three genes, *fmdDEF*, which encode an SBP and two integral membrane proteins, each with significant similarity to the corresponding proteins of Liv_{Ec} and Bra_{Pa}. So, although FmdDEF is involved in the uptake of urea and short-chain amides, it is a member of the HAAT family of ABC transporters. Thus, the solute specificities of transporters of the HAAT family extends beyond amino acids and includes short-chain amides and urea. However, the solute specificities of Bra_{Rl} and Fmd do not overlap as urea, acetamide, and formamide do not inhibit solute uptake by Bra_{Rl} (Fig. 4B). Therefore, the urea and amide transporters are a distinct subclass of the HAAT family.

The complete genome sequences of *S. meliloti*, *M. loti*, and *Agrobacterium tumefaciens* have revealed that these organisms contain a number of HAAT paralogues, and this work reports two *R. leguminosarum* HAAT permeases. It has been noted that *S. meliloti* has a high degree of paralogy, with many ancient gene duplications giving rise to a rich array of transport and regulatory proteins (13). This raises the question of the functions of the different paralogues. Do the HAAT paralogues differ in solute specificity and/or expression profile? The expression of one HAAT operon of *S. meliloti* (encoding SMb20602 to SMb20605 and SMb21707) is induced during nitrogen deprivation (34), and in *Pseudomonas fluorescens*, a HAAT operon is induced by growth in the plant rhizosphere (42). Therefore, some HAAT paralogues clearly function under specific physiological conditions. However, the data presented here caution against assigning solute specificity on the basis of homology to previously characterized transporters. Further research is required to gain a full understanding of the role of this ABC transporter family in bacterial physiology.

A general amino acid permease (Gap1) in *Saccharomyces cerevisiae* has been described (28). However, this is a secondary carrier of the amino acid/auxin permease family and is unique to eukaryotes (46). The general amino acid permeases of *R. leguminosarum*, Aap and Bra_{Rl}, are the only characterized broad-host-range amino acid transporters of the ABC superfamily. Indeed, the characterization of Bra_{Rl} has indicated that it has the broadest specificity of any characterized bacterial transporter of amino acids.

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