Occurrence of Choline and Glycine Betaine Uptake and Metabolism in the Family *Rhizobiaceae* and Their Roles in Osmoprotection

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Received 22 December 1998/Accepted 18 February 1999

The role of glycine betaine and choline in osmoprotection of various *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Agrobacterium*, and *Bradyrhizobium* reference strains which display a large variation in salt tolerance was investigated. When externally provided, both compounds enhanced the growth of *Rhizobium tropici*, *Sinorhizobium meliloti*, *Sinorhizobium fredii*, *Rhizobium galegae*, *Agrobacterium tumefaciens*, *Mesorhizobium loti*, and *Mesorhizobium huakuii*, demonstrating their utilization as osmoprotectants. However, both compounds were inefficient for the most salt-sensitive strains, such as *Rhizobium leguminosarum* (all biovars), *Agrobacterium rhizogenes*, *Rhizobium etli*, and *Bradyrhizobium japonicum*. Except for *B. japonicum*, all strains exhibit transport activity for glycine betaine and choline. When the medium osmolarity was raised, choline uptake activity was inhibited, whereas glycine betaine uptake was either increased in *R. leguminosarum* and *S. meliloti* or, more surprisingly, reduced in *R. tropici*, *S. fredii*, and *M. loti*. The transport of glycine betaine was increased by growing the cells in the presence of the substrate. With the exception of *B. japonicum*, all strains were able to use glycine betaine and choline as sole carbon and nitrogen sources. This catabolic function, reported for only a few soil bacteria, could increase competitiveness of rhizobial species in the rhizosphere. Choline dehydrogenase and betaine-aldehyde dehydrogenase activities were present in the cells of all strains with the exception of *M. huakuii* and *B. japonicum*. The main physiological role of glycine betaine in the family *Rhizobiaceae* seems to be as an energy source, while its contribution to osmoprotection is restricted to certain strains.

The responses of bacteria to growth-inhibiting salt concentrations have been studied extensively in the family *Enterobacteriaceae* (for reviews, see references 9 and 24). To adapt to changes in the osmolarity of their environment, bacteria accumulate compatible solutes that confer protection against the deleterious effects of the low water activity, maintain the appropriate cell volume, and protect intracellular macromolecules (9, 20). They are able to take up a variety of organic compounds which can serve as osmoprotectants when present externally. Glycine betaine, for example, is a very common compatible solute used by gram-negative and gram-positive bacteria, as well as members of the family *Archaea* (4, 9, 35). In addition, many bacteria synthesize glycine betaine by oxidation of choline (4, 19). Choline is a common constituent of the eucaryotic membranes in the form of phosphatidylcholine and therefore should be widespread in different environments, including the soil and the rhizosphere. In *Sinorhizobium meliloti*, a plant root-associated bacterium, choline is oxidized to glycine betaine, and this conversion, in a two-step process with glycine betaine aldehyde as an intermediate, confers osmotolerance to the cells grown at inhibitory osmolarities (33). However, choline itself has no osmoprotectant activity, as indicated by mu-

tants which are defective in their ability to convert choline to glycine betaine (30). Recently, we have also identified a new gene of *S. meliloti* which encodes a choline sulfatase that allows the bacterium to convert choline-*O*-sulfate and, to a lesser extent, phosphorylcholine into choline (28). Although the choline-glycine betaine pathway has been well characterized in *S. meliloti*, one of the most halotolerant species among members of the family *Rhizobiaceae*, the occurrence of this pathway, if any, is poorly known in other members.

Previous studies on the capacity of rhizobial strains to grow in the presence of salts have shown a marked variation in salt tolerance. A number of strains such as *Bradyrhizobium japonicum* are inhibited by NaCl concentrations lower than 100 mM (11), and it would be of great interest to enhance the salt tolerance of this very sensitive species in commercial inocula as well as long-term survival in the soil. In contrast, growth of various strains of *S. meliloti* still occurs at salt concentrations of more than 300 mM (3), and *Rhizobium* spp. isolated from nodules of *Hedysarum*, *Acacia*, *Prosopis*, and *Leucaena* plants can tolerate up to 500 mM NaCl (38).

The osmoadaptive responses of rhizobial species have not been thoroughly studied, in comparison to those of the enteric bacteria. Nevertheless, these two groups utilize many of the same compatible solutes and osmoprotectants during osmoadaptation, including potassium, glutamate (5), trehalose (7), ectoine (36), glycine betaine, and proline betaine (3, 13). However, there are certain distinguishing features of rhizobial species that have been noted. These include (i) the inability to use proline as a compatible solute through uptake (26), (ii) the synthesis of *N*-acetyl-glutamylglutamine amide (34), and (iii) the capacity of *S. meliloti* to catabolize betaines (13, 33). Since these osmoregulatory mechanisms have been studied mainly in

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FIG. 1. Dendrogram (constructed by using the unweighted pair group method with arithmetic mean) showing the genetic relationships among 16S ribosomal DNA genotypes identified by PCR-restriction fragment length polymorphic analysis (17) of the *Rhizobiaceae* strains used in this study.

S. meliloti, they should not be generalized to other rhizobial species. In addition, we must emphasize that except in the case of *S. meliloti*, most of the studies have been conducted with isolates poorly represented in most culture collections.

Recently, the taxonomy of the legume root-nodulating bacteria has undergone major revisions and improvements and still is in a state of transition (10, 14). Based largely on sequencing of the 16S and 23S rRNA genes, five genera have been recognized: *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Azorhizobium*, and *Bradyrhizobium*. Thus, to examine the salt tolerance of various *Rhizobiaceae* strains, we have tested some of the collection strains used by Laguerre *et al.* (17) together with *Agrobacterium tumefaciens*, *B. japonicum*, and *S. meliloti* as type and (or) reference strains. The objective of our study was also to establish whether the selected bacteria could use choline and glycine betaine as osmoprotectants and (or) sole carbon or nitrogen sources. Therefore, we have analyzed choline and glycine betaine uptake capacities, measured choline dehydrogenase and betaine aldehyde dehydrogenase activities encoded, respectively, by *betA* and *betB* in *S. meliloti* (30), and used *betA* and *betB* fragments from this bacterium to probe for DNA from the other strains.

MATERIALS AND METHODS

Bacterial strains and media. The strains used in this study are listed in Fig. 1. They represent reference and type strains of species currently accepted as members of the family *Rhizobiaceae*, namely, *Rhizobium*, *Agrobacterium*, *Sinorhizobium*, *Mesorhizobium*, and *Bradyrhizobium*. The dendrogram was constructed from the distance matrix by using the unweighted pair group method with arithmetic mean (17). It shows the genetic relationships among 16S ribosomal DNA genotypes identified by PCR-restriction fragment length polymorphic analysis by Laguerre *et al.* (17). Tryptone-yeast extract (TY) medium was used as complex medium for growth of all strains (2). Defined minimal medium, carbon- and nitrogen-free S medium (33) supplemented with 0.1% (vol/vol) lactic acid and 0.1% (wt/vol) aspartic acid (LAS medium), was used for analysis of the osmotolerance phenotype of all strains, except for *B. japonicum* which was grown in S medium with 0.2% (wt/vol) mannitol, 0.2% (wt/vol) glutamate, and vitamins as described previously (1). Choline and glycine betaine were prepared as 0.5 M solutions and sterilized by filtration before incorporation into defined medium. Their final concentrations were 1 mM for osmoprotection assays in LAS medium and 10 mM when used as carbon and nitrogen sources in S medium.

Growth rate determination. TY medium precultures of the bacteria in early stationary phase were pelleted and resuspended in the same volume of LAS or S medium. Ten microliters was used to inoculate 2 ml of the desired medium. The cells were grown aerobically (agitation at 250 rpm) at 30°C. Bacterial growth was monitored spectrophotometrically at 600 nm. The generation time was determined from the log phase of the growth curve.

Transport assays. Cells were grown in LAS medium to late log phase and washed twice in the same medium. When grown in the presence of glycine betaine, the cells were washed once in unsupplemented growth medium to remove this compound before being assayed for transport. The cells were resuspended in LAS medium at a concentration of 0.1 mg of protein/ml and incubated in the presence of 1.7 kBq of either [*methyl*-¹⁴C]choline or [*methyl*-¹⁴C]glycine betaine. [*methyl*-¹⁴C]choline (2.15 GBq/mmol) was purchased from Amersham. [*methyl*-¹⁴C]glycine betaine was prepared enzymatically from [*methyl*-¹⁴C]choline by using choline oxidase from *Alcaligenes* spp. (Sigma Chemical Co). The substrate concentration in the assay mixture was 1 μM. Reactions were run at 30°C with agitation for 1 to 10 min and then terminated by rapid filtration through GF/F glass microfiber filters (Whatman) as described previously (29). The filters were rinsed once with 5 ml of the corresponding growth medium, which was maintained at 30°C. Under these rinsing conditions, no leakage of intracellular labeled substrate was observed. The radioactivity of the filters was determined in a Beckman liquid scintillation spectrometer, and the initial transport rates were expressed as nanomoles of substrate taken up per minute per milligram of protein.

Protein concentration was determined by the method of Lowry *et al.* (23) and with bovine serum albumin as a standard. All data are mean values of results from three independent experiments, each run in duplicate.

Enzyme assays. The bacteria were grown in LAS medium supplemented with 7 mM choline to late log phase (33). Choline dehydrogenase (CDH; EC 1.1.99.1) activity was determined by measuring production of [*methyl*-¹⁴C]glycine betaine aldehyde from [*methyl*-¹⁴C]choline as described by Landfald and Strøm (19). The cells were permeabilized by toluene treatment (0.5% [vol/vol]) for 10 min at 30°C with agitation at 330 rpm. The concentration of substrate in the assay mixture was 10 mM, and approximately 0.8 mg of protein was used. Betaine-aldehyde dehydrogenase (BADH; EC 1.2.1.8) activity was determined by measuring the NADH production at 340 nm as described previously (33). The cells were disrupted by two passages through a French press at 8,500 lb/in², and the debris was removed by centrifugation at 12,000 × g for 20 min at 4°C. Approximately 0.1 to 0.2 mg of protein from a cell extract was used per assay.

Molecular biology techniques. Standard methods were used for plasmid DNA isolation, restriction analysis, agarose gel electrophoresis, and Southern blotting (32). Genomic DNA from rhizobia was extracted as described previously (28). DNA probes were labeled by using the Prime-a-Gene random priming system (Promega) and [α -³²P]dCTP (111 Tbq/mmol, Amersham). Two *EcoRI* restriction fragments for the *betA* and *betB* genes of *S. meliloti* were used, respectively (30). The Southern blot analysis was conducted under high-stringency conditions on *XhoI*- or *EcoRI*-digested genomic DNA. Hybridizations were done at 65°C

TABLE 1. Effect of salt stress on growth of rhizobial species in LAS minimal medium and utilization of choline and glycine betaine as sole carbon and nitrogen sources

Species	Growth in LAS medium with:					Growth rate in S medium with ^c :	
	No salt, rate ^a	0.1 M NaCl		0.2 M NaCl		Cho	GB
		Rate	Yield ^b	Rate	Yield		
<i>R. leguminosarum</i> bv. <i>viciae</i>	3.8	NG ^d		NG		7.8	7.5
<i>R. leguminosarum</i> bv. <i>trifolii</i>	3.6	NG		NG		6.8	5.6
<i>R. leguminosarum</i> bv. <i>phaseoli</i>	3.0	NG		NG		7.3	9.8
<i>R. tropici</i> IIA	10.8	6.7	15	NG		ND ^e	ND
<i>R. tropici</i> IIB	9.4	6.4	100	4.4	30	5.0	5.1
<i>A. rhizogenes</i>	8.2	NG		NG		6.4	4.1
<i>S. meliloti</i>	15.3	15.3	100	9.9	100	9.5	9.9
<i>S. fredii</i>	15.4	9.9	100	5.9	65	9.8	8.9
<i>R. etli</i>	6.4	NG		NG		7.4	9.6
<i>R. galegae</i>	3.5	2.5	15	NG		4.2	5.4
<i>A. tumefaciens</i>	7.7	8.3	100	8.9	100	7.2	13.2
<i>M. loti</i>	3.9	2.9	20	NG		4.0	4.3
<i>M. huakuii</i>	3.9	2.8	35	2.7	24	10.1	8.5
<i>B. japonicum</i>	3.1	NG		NG		NG	NG

^a The rate is expressed as the number of generations per hour (10^2) determined during the logarithmic phase. For every growth experiment, data are the averages of results of at least three different repeats with a standard deviation of less than 10%.

^b The yield is the cell density (optical density at 600 nm) reached at the stationary phase. A value of 100% corresponds to the optical density obtained with cells grown in the absence of salt.

^c Choline (Cho) or glycine betaine (GB) was added to carbon- and nitrogen-free S medium at a concentration of 10 mM.

^d NG, no growth.

^e ND, not determined.

for 14 h (32). The membranes were exposed to X-ray film (X-Omat; Kodak) at -70°C for 12 to 24 h.

RESULTS

Salt stress tolerance and osmoprotection by glycine betaine and choline. To characterize the intrinsic osmotolerance of the different rhizobial species listed in Fig. 1, we examined growth rate and yield in LAS medium with NaCl concentrations ranging from 0.1 to 0.3 M (Table 1). Although some species like *Rhizobium leguminosarum* and *Rhizobium galegae* grew poorly in this medium, it was still sufficient to monitor their response to osmotic stress. While some species such as *R. leguminosarum* (all biovars), *Rhizobium etli*, *Agrobacterium rhizogenes*, and *B. japonicum* were very sensitive to salt, with growth inhibited completely at only 0.1 M, the other species were able to withstand this concentration, showing either a severe reduction in yield of 65 to 80% (*Rhizobium tropici* IIA, *R. galegae*, *Mesorhizobium loti*, and *Mesorhizobium huakuii*) or only a slight modification in growth rate (*R. tropici* IIB, *S. meliloti*, *Sinorhizobium fredii*, and *A. tumefaciens*). In the presence of 0.2 M NaCl, growth parameters were strongly affected in the case of *R. tropici* IIB, *S. fredii*, and *M. huakuii*, and only two species (*S. meliloti* and *A. tumefaciens*) were still able to grow, albeit slowly, at a concentration of 0.3 M NaCl (data not shown). In all cases, the first effect of increased salt concentration was the reduction in the growth rate, followed by a reduction in the yield.

The osmoprotective effect of choline and glycine betaine was tested at a salt concentration of 0.1 M NaCl in the case of the most salt-sensitive strains and for the others at a concentration which reduced the growth significantly (Table 2). The most osmosensitive species, such as *R. leguminosarum* (all biovars), *A. rhizogenes*, *R. etli*, and *B. japonicum*, were not protected by the addition of glycine betaine (1 mM) in minimal medium containing added NaCl. In contrast, glycine betaine stimulated the growth of all other rhizobial species and, in the case of *R. tropici* IIB, *S. meliloti*, *R. galegae*, and *M. loti*, the yield was

particularly enhanced. It is noteworthy that in the presence of glycine betaine, two species, *S. meliloti* and *A. tumefaciens*, could still grow very well in the presence of 0.5 M NaCl, with yields of 100 and 46%, respectively. Thus, it appeared that the highest growth stimulation was obtained in the most salt-tolerant strains. Similar results were obtained when choline was added (Table 2).

Glycine betaine and choline transport activities. To test whether the absence of osmoprotection by glycine betaine in some rhizobial species was correlated with the absence of transport activity, we measured the initial glycine betaine uptake in cells grown in the absence or in the presence of salt. Uptake of glycine betaine was readily detectable at a low substrate concentration (1 μM) in all tested strains, except *B. japonicum* (Table 3). Under low-osmolarity growth conditions, transport was much more effective in *S. meliloti* (1.53 nmol/min/mg of protein) than in other strains (0.46 to 0.03 nmol/min/mg of protein). Under high-osmolarity growth conditions, glycine betaine uptake was either significantly increased (*R. leguminosarum* biovars *viciae* and *trifolii* and *S. meliloti*, for example) or only moderately stimulated (*R. etli*, *M. huakuii*, and *A. tumefaciens*, for example). Surprisingly, the uptake was slightly reduced in *R. tropici*, *R. galegae*, and *M. loti*, whereas a 10-fold reduction was observed in *S. fredii*.

To determine whether the presence of glycine betaine in the growth medium could increase the uptake activity, the cells were grown in LAS medium containing 5 mM glycine betaine. Uptake was strongly enhanced in all strains (2.3- to 30-fold stimulation), except *B. japonicum*. Thus, it appears likely that glycine betaine transport process is more or less efficient in all the tested strains except *B. japonicum* and can be increased by the availability of the substrate in the environment.

In parallel, choline transport was investigated in cells grown at a low or elevated osmolarity. Again, with the exception of *B. japonicum*, all strains exhibited a constitutive uptake activity which was always higher than the glycine betaine uptake activity. In close agreement with the previously reported data for *S.*

TABLE 2. Effect of glycine betaine and choline (1 mM) on growth of rhizobial species in LAS minimal medium with added NaCl

Species	NaCl added (M)	Osmoprotection by:					
		Control		Glycine betaine		Choline	
		Rate ^a	Yield ^b	Rate	Yield	Rate	Yield
<i>R. leguminosarum</i> bv. <i>viciae</i>	0.1	NG ^c		NG		NG	
<i>R. leguminosarum</i> bv. <i>trifolii</i>	0.1	NG		NG		NG	
<i>R. leguminosarum</i> bv. <i>phaseoli</i>	0.1	NG		NG		NG	
<i>R. tropici</i> IIA	0.1	6.7	15	9.3	46	8.5	60
<i>R. tropici</i> IIB	0.2	4.4	30	4.8	100	5.0	100
<i>A. rhizogenes</i>	0.1	NG		NG		NG	
<i>S. meliloti</i>	0.5	3.1		7.8	100	6.2	100
<i>S. fredii</i>	0.2	5.9	65	7.9	45	8.2	58
<i>R. etli</i>	0.1	NG		NG		NG	
<i>R. galegae</i>	0.1	2.5	15	3.8	100	4.3	100
<i>A. tumefaciens</i>	0.5	2.9		3.3	46	3.4	53
<i>M. loti</i>	0.1	2.9	20	5.7	100	4.8	100
<i>M. huakuii</i>	0.2	2.7	35	3.3	24	3.7	48
<i>B. japonicum</i>	0.1	NG		NG		NG	

^{a,b,c} See Table 1, footnotes a, b, and d, respectively.

meliloti (29), we found that the addition of salt to the growth medium resulted in a marked decrease (1.2- to 14-fold) in choline uptake activity, except in the case of *M. huakuii*, which showed a 2.4-fold stimulation.

Identification of the choline-glycine betaine pathway. As demonstrated in this work, choline can be transported and used as an effective osmoprotectant by most of the rhizobia studied here. To determine whether the utilization of choline as a protective compound involves its conversion into glycine betaine, as it has been found in *Escherichia coli* and other bacteria, including *S. meliloti* (18, 33), we have measured CDH and BADH activities in the different strains. Both enzyme activities were detected in all strains, except for *M. huakuii* and *B. japonicum* (Table 4). The specific CDH activity ranged from 7 (*R. leguminosarum* bv. *trifolii*) to 39 (*R. tropici* IIB) nmol/min/mg of protein, while the specific BADH activity ranged from 8 (*R. etli*) to 134 (*R. leguminosarum* bv. *phaseoli*) nmol/min/mg of protein. With the exception of *R. etli*, which showed

low levels of activity for both enzymes, in most other strains the BADH activity was significantly higher (2.4 to 13-fold) than the CDH activity. This is consistent with an efficient *in vivo* transformation of the glycine betaine aldehyde intermediate. These results indicate that all tested members of the family *Rhizobiaceae*, besides *M. huakuii* and *B. japonicum*, can oxidize choline into glycine betaine.

In *E. coli* and *S. meliloti*, CDH and BADH are encoded, respectively, by the *betA* and the *betB* genes (18, 30). By Southern analysis of genomic DNAs from the different rhizobial species using *betA*- and *betB*-specific probes from *S. meliloti*, the presence of a single homologous gene was observed in all strains, except for *M. huakuii* and *B. japonicum* (data not shown). Such results confirmed the enzymatic data.

Glycine betaine and choline as sole carbon and/or nitrogen sources. Unlike *E. coli* and *Bacillus subtilis* (4, 20), *S. meliloti* can catabolize glycine betaine and use this compound and also choline as sole sources of carbon and nitrogen for growth. To

TABLE 3. Glycine betaine and choline uptake activities^a

Species	Uptake (nmol/min/mg of protein) ^b				
	Glycine betaine			Choline	
	-NaCl	+NaCl	+GB	-NaCl	+NaCl
<i>R. leguminosarum</i> bv. <i>viciae</i>	0.03	0.55 (50)	0.92	0.27	0.06
<i>R. leguminosarum</i> bv. <i>trifolii</i>	0.16	0.50 (50)	1.34	0.95	0.23
<i>R. leguminosarum</i> bv. <i>phaseoli</i>	0.10	NG ^c (50)	ND ^d	0.60	NG
<i>R. tropici</i> IIA	0.24	0.14 (100)	2.19	0.32	0.27
<i>R. tropici</i> IIB	0.33	0.24 (150)	1.67	1.58	0.90
<i>A. rhizogenes</i>	0.06	NG (50)	ND	0.42	NG
<i>S. meliloti</i>	1.53	3.17 (150)	3.57	2.75	0.21
<i>S. fredii</i>	0.46	0.04 (150)	3.67	0.76	0.10
<i>R. etli</i>	0.06	0.09 (50)	1.80	0.42	0.03
<i>R. galegae</i>	0.08	0.02 (150)	1.88	0.34	0.21
<i>A. tumefaciens</i>	0.13	0.22 (150)	1.96	3.45	1.27
<i>M. loti</i>	0.12	0.02 (100)	ND	0.22	0.09
<i>M. huakuii</i>	0.28	0.52 (100)	ND	1.27	3.03
<i>B. japonicum</i>	<0.01	NG (50)	<0.01	<0.01	NG

^a Cells were grown to late log phase in LAS medium without added NaCl (-NaCl), with the salt added (+NaCl) (at the concentration [millimolar] given in parentheses), or without salt in the presence of 5 mM glycine betaine (+GB).

^b Transport activities were measured at a concentration of 1 μ M. The values obtained with triplicate independent cultures never varied more than 10%.

^c NG, no growth.

^d ND, not determined.

TABLE 4. CDH and BADH activities from different rhizobia^a

Species	Enzyme activity (nmol/min/ mg of proteins) ^b	
	CDH	BADH
<i>R. leguminosarum</i> bv. viciae	33	103
<i>R. leguminosarum</i> bv. trifolii	7	93
<i>R. leguminosarum</i> bv. phaseoli	16	134
<i>R. tropici</i> IIA	28	28
<i>R. tropici</i> IIB	39	41
<i>A. rhizogenes</i>	12	83
<i>S. meliloti</i>	33	126
<i>S. fredii</i>	12	92
<i>R. etli</i>	11	8
<i>R. galegae</i>	8	41
<i>A. tumefaciens</i>	26	26
<i>M. loti</i>	22	53
<i>M. huakuii</i>	<1	<1
<i>B. japonicum</i>	<1	<1

^a Cells were grown in LAS medium supplemented with 7 mM choline.

^b Toluene-treated cells and cells disrupted by passage through a French press were used for CDH and BADH assays, respectively. See Materials and Methods for details. Data are the means of results from duplicate assays with two independent cultures (standard deviations never exceeded 9%).

test whether all the selected rhizobial species could use both compounds as energy sources, growth was monitored in the presence of 10 mM glycine betaine or choline added to the defined carbon- and nitrogen-free S medium. All species, with the exception of *B. japonicum*, were able to grow in such media, and the growth rates in media containing glycine betaine and choline were comparable (Table 1). In the case of *R. tropici*, *A. rhizogenes*, *S. meliloti*, and *S. fredii*, the growth rates were substantially lower than those observed in medium with lactate and aspartate (LAS medium), whereas it was the opposite for the other strains. Both compounds were particularly beneficial in the cases of *R. leguminosarum* (all biovars) and *M. huakuii*, with growth rates approximately twofold higher in the presence of glycine betaine and choline than in LAS medium.

DISCUSSION

Within their natural habitat, rhizobial species are frequently subjected to detrimental effects of increased osmotic stress which affect bacterial growth and root colonization. In the most extensively characterized rhizobial species, *S. meliloti*, several osmoregulatory mechanisms have been defined (5, 28, 33, 34, 36). Among the potent osmoprotectants, glycine betaine can be accumulated in the cytosol either by direct uptake or via synthesis from choline or choline-*O*-sulfate (3, 28, 30). Our results indicate that the potential to transport glycine betaine and choline is widespread among various type and (or) reference species of *Rhizobiaceae* which display a large variation in salt tolerance. In fact, only *B. japonicum*, one of the most salt-sensitive species tested, does not possess a high-affinity uptake system for the transport of these substances.

Within the other species, glycine betaine transport activity is always much higher in cells grown in the presence than in the absence of glycine betaine. Surprisingly, this is the first report showing such an increase in rhizosphere bacteria, and we are currently investigating a possible induction by the substrate. We have also shown that glycine betaine uptake is differently affected by the osmolarity of the growth medium. As previously demonstrated in *S. meliloti* (3), this uptake is significantly stimulated in some rhizobial species such as *R. leguminosarum*, *A. tumefaciens*, and *M. huakuii* when the cells are grown at an

elevated osmolarity. In contrast, it is important to note that this transport is reduced in the presence of salt in other strains like *S. fredii*. To our knowledge, reduction of glycine betaine uptake by increased osmolarity has never been reported before and could not be explained so far. Such results suggest that in these strains, the response towards elevated osmolarity conditions does not involve a rapid uptake of glycine betaine, at least at the substrate concentration used here. In the environment, the amount of glycine betaine available depends on many factors, including the level of organic material, the rate of microbial degradation of dead cells, and the atmospheric conditions. Thus, the level of glycine betaine may exhibit large variations, and it would be of interest to analyze the salt effect on uptake activity measured at higher substrate concentration.

The results offer additional evidence that the marked variation in intrinsic salt tolerance of the tested rhizobial species is not correlated with the presence of a glycine betaine uptake system. A number of strains are growth inhibited by less than 100 mM NaCl (Table 1), but growth at salt concentrations greater than 300 mM is observed with *S. meliloti* and *A. tumefaciens*. When glycine betaine is present in medium with added salt, the growth rates and yields of *R. tropici* IIA and IIB, *S. meliloti*, *S. fredii*, *R. galegae*, *A. tumefaciens*, and *M. loti* are strongly improved. Only for the most osmosensitive strains (*R. leguminosarum* [all biovars], *A. rhizogenes*, *R. etli*, and *B. japonicum*), the growth inhibition by salt stress could not be rescued by the addition of glycine betaine (Table 2), although some species like *R. leguminosarum* showed stimulated glycine betaine uptake under osmotic stress (Table 3). An explanation for such a contradictory result might be that with the extremely low intrinsic osmotolerance of these strains, a salt concentration of 100 mM is already too high, and the effectiveness of glycine betaine accumulation cannot be observed. It is also possible that no accumulation occurs due to an efficient catabolism. In fact, as previously observed for *S. meliloti* (3), our data demonstrate that all rhizobial species, except *B. japonicum* again, are able to use glycine betaine as the sole carbon and nitrogen source for growth. In *S. meliloti*, glycine betaine is progressively demethylated through dimethylglycine and sarcosine to glycine (33). The demethylations are partially inhibited if osmolarity of the growth medium is increased, which thus permits cells to accumulate glycine betaine as a compatible solute. We currently cannot tell whether a similar pathway is operating in all rhizobial species, but we can emphasize that, unlike with other soil organisms such as *B. subtilis*, glycine betaine is a good energy substrate for the growth of rhizobial species but that the osmoprotective property seems restricted to fewer strains. Among the soil bacteria, the capacity to use glycine betaine as a growth substrate has been reported only for a few species, including *Azospirillum lipoferum* (15) and *Pseudomonas aeruginosa* (22). It is tempting to speculate that the ability to catabolize glycine betaine could increase competitiveness in the rhizosphere.

In this report, we also present evidence that all rhizobial strains, except *B. japonicum*, possess a choline uptake activity and can use choline as a carbon, nitrogen, and energy source for growth. As was already known for *S. meliloti* (29), choline uptake is constitutively expressed and is inhibited by elevated osmolarity in rhizobial strains, except for *M. huakuii*, in which the opposite effect was observed. Choline can also function as an osmoprotectant but only in strains where glycine betaine is found to be effective (Table 2). The rationale is that choline functions as the precursor of glycine betaine, as has already been demonstrated for many bacteria, including *S. meliloti* (33) and other soil bacteria such as *B. subtilis* (4). Consistent with this expectation, we found CDH and BADH activities in all

strains, except *B. japonicum* and, more surprisingly, *M. huakuii*, which, unlike the former, can use both glycine betaine and choline as building blocks for cellular components or osmoprotective molecules. Considering the absence of CDH and BADH activities in both *M. huakuii* and *B. japonicum*, at least under our experimental conditions, it is plausible to consider that the choline-glycine betaine enzymatic pathway does not operate in these two bacteria. In *M. huakuii*, choline itself could be used as an osmoprotectant and directly degraded. In most aerobic bacteria, decomposition of choline proceeds via glycine betaine, *N,N*-dimethylglycine, sarcosine, and glycine. However, a relatively large number of choline-utilizing coryneform bacteria are able to grow with *N,N*-dimethylethanolamine and with *N*-monomethylethanolamine as the sole C and N sources (16). This suggests that choline may also be degraded by demethylation, as demonstrated in anaerobic bacteria such as *Eubacterium limosum*, which produces *N,N*-dimethylethanolamine from choline (27). Furthermore, in other anaerobic bacteria like clostridia (6) and sulfate-reducing bacteria (12), choline is readily fermented to acetate and trimethylamine, which is subsequently demethylated. In this respect, an in-depth study of choline metabolism in *M. huakuii* should contribute to an elucidation of the mechanism by which choline is used for both functions, in osmoprotection and as an energy source.

ACKNOWLEDGMENTS

This work was funded by the European Communities BIOTECH Program, as part of the Project of Technological Priority 1993–1997 (BIO2CT930400; D.L.R.), and the Centre National de la Recherche Scientifique. E.B. received a doctoral fellowship from the Ministère de l'Enseignement Supérieur et de la Recherche.

We are grateful to N. Amarger and G. Laguerre for providing bacterial strains.

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