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Characterization of Three Choline Transport Activities in *Rhizobium meliloti*: Modulation by Choline and Osmotic Stress

JEAN-ALAIN POCARD,[1]† THÉOPHILE BERNARD,[1] LINDA TOMBRAS SMITH,[2] AND DANIEL LE RUDULIER[1]‡*

Laboratoire de Microbiologie et Physiologie des Symbioses, U.A. Centre National de la Recherche Scientifique 256, Université de Rennes I, Campus de Beaulieu, 35042 Rennes Cédex, France,[1] and Plant Growth Laboratory, University of California, Davis, California 95616[2]

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Choline has both a nutritional and osmoregulatory role in *Rhizobium meliloti* (T. Bernard, J. A. Pocard, B. Perroud, and D. Le Rudulier, Arch. Microbiol. 143:359–364, 1986). In view of this fact, choline transport was studied in *R. meliloti* 102F34 to determine how the rate of choline uptake is modulated. The effects of the cultural conditions on the kinetics of transport are presented. A high-affinity activity and a low-affinity activity were found in cells grown in minimal medium. The addition of 0.3 M NaCl or other osmolytes to the medium resulted in a marked decrease in the high-affinity activity, whereas the low-affinity activity remained fairly constant. Furthermore, results from osmotic upshock and downshock experiments indicate that the response of the cell to high osmolarity is rapid; hence, the mechanism of regulation by salt likely does not involve gene induction. A second high-affinity transport activity was induced by choline itself. Like the constitutive low-affinity transport activity, this activity was not greatly altered when the cells were grown in media of elevated osmotic strength. We conclude that although all three kinetically distinct transport systems are efficient at low osmolarity, only the induced high- and low-affinity activities are important for osmoregulation. The characteristics of the three transport activities from *R. meliloti* are compared with those from other bacterial species that use choline for growth and/or osmoregulation.

A great variety of aerobic and anaerobic bacteria have the ability to use choline as a carbon, nitrogen, and/or energy source (6, 9, 21). This ability is not surprising in view of the fact that significant amounts of choline are readily liberated to the environment from plant and animal residues (9, 25). However, choline can have an additional role in bacterial survival, particularly in osmotic stress adaptation. Many bacteria respond to inhibitory levels of osmolarity in the environment by accumulating small organic compounds in the cytosol. These compounds, termed osmoprotectants, function by increasing the internal osmotic strength, thus allowing the cell to avoid cytoplasmic dehydration (2, 26). A potent osmoprotectant is glycine betaine, the oxidized product of choline (8, 11–14). Hence, choline functions in osmotic stress adaptation as a precursor to glycine betaine (10, 24).

In the enteric bacteria *Salmonella typhimurium* and *Escherichia coli*, choline and glycine betaine have only an osmoregulatory role. These compounds cannot serve as a carbon or nitrogen source (3, 13, 18); thus, it appears that the transport of these compounds into enteric bacteria is modulated only by the osmotic strength of the medium (4, 14, 18, 24). On the other hand, in the root nodule bacterium *Rhizobium meliloti*, betaine and choline can support growth, and they are also effective osmoprotectants (1, 12). Therefore, choline transport might be modulated by both high osmotic strength and the carbon and nitrogen composition of the medium. This type of control would be significantly different from that in *E. coli*.

In this report, we provide evidence that choline transport is catalyzed by three kinetically distinct separate systems in *R. meliloti*: a high- and a low-affinity activity that are constitutively expressed, and a second high-affinity activity that is induced by choline. We also present evidence that the mechanisms by which choline transport is controlled in *R. meliloti* and *E. coli* differ in many aspects.

MATERIALS AND METHODS

Materials. All reagents used were analytical grade or the best grade available. [*methyl*-^{14}C]choline (2.15 MBq/μmol) was purchased from Amersham Corp. (Amersham, England).

Bacterial strain and media. All experiments were carried out with *R. meliloti* 102F34 (kindly provided by R. C. Valentine). The strain was maintained on solid mannitol-salts-yeast extract (MSY) medium (17). The minimal medium (MM) contained 0.1% (wt/vol) D,L-sodium lactate, 0.13% sodium aspartate, 20 μg of biotin per liter, and the same salts as in MSY (19). The osmotic pressure was measured by freezing point depression with an H. Roebling microosmometer (Bioblock Scientific, Illkirch, France).

Cultures were grown aerobically at 30°C with shaking at 150 rpm. Inocula were grown overnight in MSY medium and used at a final concentration of 3%. Bacterial growth was monitored spectrophotometrically at 420 nm, and the protein concentration of the bacterial culture was determined by the method of Lowry et al. (15).

Transport assays. Choline transport was measured with [*methyl*-^{14}C]choline by a method similar to that of Perroud and Le Rudulier (18). Cultures were grown in MM containing various supplements, as indicated. When the medium was supplemented with choline, the choline was added at 7

* Corresponding author.
† Present address: Michigan State University-Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, MI 48824-1312.
‡ Present address: U.A. Centre National de la Recherche Scientifique 1114, Faculté des Sciences et des Techniques, Université de Nice, Parc Valrose, 06034 Nice Cédex, France.

TABLE 1. Kinetic parameters of choline transport in *R. meliloti* 102F34[a]

Activity	Medium	K_m (μM)	V_{max}[b] (nmol/min per mg of protein)	
			Calculated	Observed
High affinity	MM	6.7	50	4.3
	MM–0.3 M NaCl	0.5	0.9	
Low affinity	MM	100	50	40.5
	MM–0.3 M NaCl	150	50	
Choline activated, high affinity	MM	0.40	26	21.8
	MM–0.3 M NaCl	0.45	22.7	

[a] For choline-activated cultures, 7 mM choline was added to the medium. Cells were harvested and assayed for transport as described in the text. High- and low-affinity activities were measured over a concentration range of 0.12 to 50 μM and 0.1 to 4 mM, respectively. K_m and V_{max} values were calculated from double-reciprocal plots (Fig. 1).

[b] Due to the substrate inhibition which occurred with cultures grown without NaCl added to the medium, the observed maximum rates were lower than the V_{max} values obtained from double reciprocal plots.

mM because maximal stimulation of the enzymes involved in the choline degradative pathway occurs at this concentration in this strain (22). Cells were harvested when the culture reached 0.2 to 1.0 optical density unit. When grown in the presence of choline or one of its analogs, cells were washed once in unsupplemented growth medium to remove these compounds before assaying for choline transport. The choline concentration in the assay mixture varied from 0.12 μM to 4 mM (2.15 MBq/μmol to 2.6 kBq/μmol); the final volume was 0.5 ml. Unless otherwise indicated, reactions were run for 10 min and then terminated by rapid filtration through HAWP cellulose-nitrate filters (0.45-μm pore size; Millipore Corp.). 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 filters were solubilized in scintillation vials containing 6 ml of ACS liquid (Amersham Corp.), and radioactivities were determined in a Packard liquid scintillation spectrometer (18).

Initial transport rates are expressed as nanomoles of choline taken up per minute per milligram of protein. In all assays less than 15% of the radioactive substrate was used, and the transport rates were linear with time and protein concentration. In the case of high-affinity activities (constitutive and induced) and low-affinity activity, initial rates of uptake were calculated by the amount of radioactive choline accumulated by the cells in 1 and 10 min, respectively. All data presented in this report are mean values of two to five experiments, each run in duplicate.

Inhibition of induction by chloramphenicol. An experiment was carried out with the protein synthesis inhibitor chloramphenicol to determine whether choline acts as a genetic inducer of its own transport. A bacterial culture was grown in MM plus 0.3 M NaCl until it reached 0.6 optical density unit. The culture was then divided into three portions. One portion remained untreated to measure the level of constitutive transport during the course of the experiment. At time zero, the other two portions were supplemented with 7 mM choline; 1.5 h later chloramphenicol (100 μg/ml) was added to one of the choline-treated cultures. At the indicated time points, samples were removed and assayed for choline transport with 10 μM substrate (reaction time, 5 min) and for cell viability on solid MSY medium. The number of viable cells in all three cultures remained similar.

Osmotic upshock and downshock experiments. Cultures were grown overnight in MM supplemented, when indicated, with 0.3 M NaCl and/or 7 mM choline. Samples of 10 ml were harvested, rinsed once with choline-free medium of identical osmotic strength, and subjected to an osmotic upshock or downshock by suspending the pellet in 10 ml of MM appropriately supplemented. Samples were periodically withdrawn, and transport rates were determined after a 2-min reaction with 10 μM labeled choline.

RESULTS

Kinetics of choline transport. We used a kinetic approach to investigate the possibility of multiple transport systems for choline in *R. meliloti* 102F34. When cells were grown in MM, two separate transport activities were distinguished by their relative affinities for choline: a high-affinity and a low-affinity transport activity with K_ms of 6.7 and 100 μM, respectively (Table 1). The maximum velocity for both activities was 50 nmol/min per mg of protein. However, both activities also showed significant levels of substrate inhibition (Fig. 1A and B), so that the observed V_{max} was only 4.3 and 40.5 for the high- and low-affinity activities, respectively. The significance of the substrate inhibition is discussed below.

Since choline stimulates the enzymes involved in its degradative pathway in *R. meliloti* 102F34 (22), it was of interest to determine whether choline could also modulate its own uptake. To investigate this question, kinetic parameters were determined from cultures grown in MM with choline added to the medium. Under these conditions a third kinetically distinct choline transport activity was observed (Table 1): the K_m and V_{max} were 0.4 μM and 26 nmol/min per mg of protein, respectively, about 16.8- and 2-fold lower than for the high-affinity transport activity from cells grown without choline. Furthermore, this new activity was also inhibited by choline (Fig. 1C) but only at very high concentrations, so that the observed maximum velocity was nearly the same as the extrapolated value. These results suggest the possibility that choline added to the medium may induce a new high-affinity transport system. Results from the experiments described below provide additional evidence for a third choline transport activity. No significant variation in the V_{max} of the low-affinity activity could be detected at low osmolarity when choline was added to the medium.

Effect of salt stress on the kinetics of choline transport. Because choline has a role in osmotic stress protection in *R. meliloti*, uptake activity was also determined with cells grown at high osmolarity. When cells were grown with 0.3 M NaCl, the K_m and V_{max} for the high-affinity activity decreased 13.4- and 56-fold, respectively (Table 1). Thus, an increase in substrate affinity was correlated with a decrease

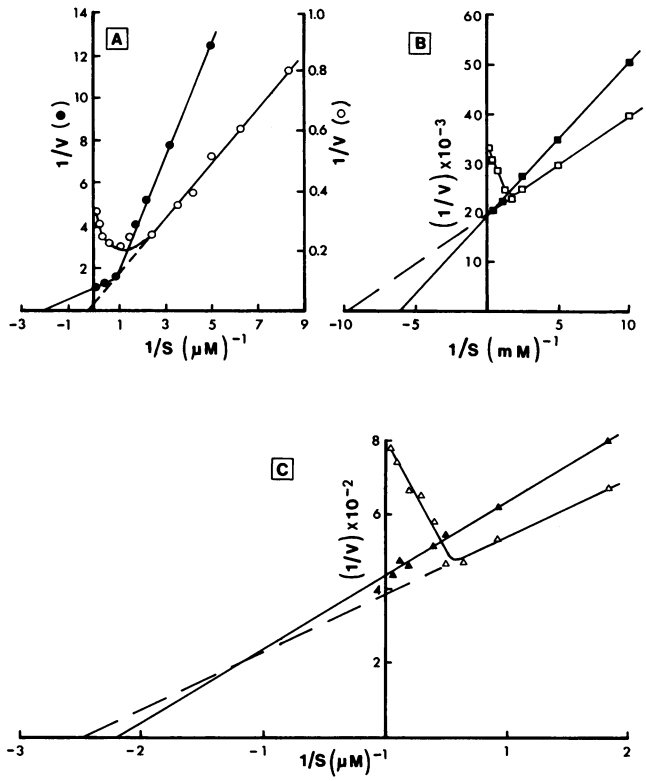

FIG. 1. Double-reciprocal plots of choline transport activities in *R. meliloti* 102F34. Growth and assay conditions are described in Table 1. (A) High-affinity transport activity; (B) low-affinity transport activity; (C) high-affinity transport activity from cells grown in MM plus 7 mM choline. Protein concentrations in the assay mixture were approximately 20 (A), 50 (B), and 80 (C) μg per ml of cell suspension. Symbols: ○, □, and △, MM; ●, ■, and ▲, MM plus 0.3 M NaCl.

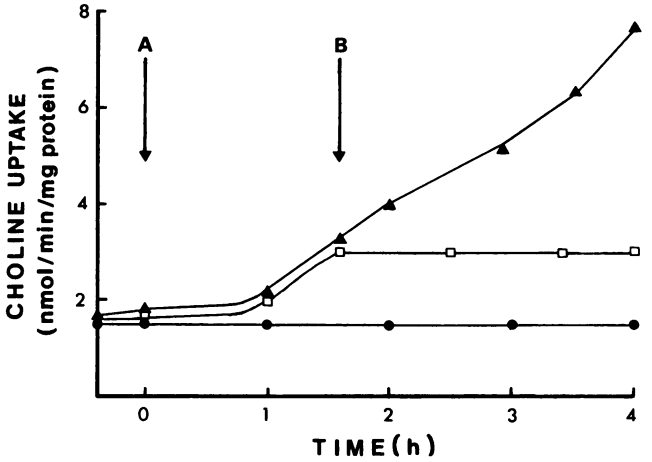

FIG. 2. Effect of chloramphenicol on choline-activated high-affinity transport in *R. meliloti* 102F34. A culture was grown overnight in MM plus 0.3 M NaCl. At time zero (arrow A), the culture was divided into three portions, two of which were incubated with 7 mM choline. Chloramphenicol was added 1.5 h later (arrow B). Samples were periodically removed, rinsed once with MM containing 0.3 M NaCl, and assayed for uptake activity in the presence of 10 μM [*methyl*-^{14}C]choline; reaction time was 5 min. Symbols: ●, no addition; ▲, choline added; □, choline and chloramphenicol added.

in velocity. Moreover, the shape of the double-reciprocal plot changed. The curve was slightly concave upward, and the substrate inhibition previously observed had disappeared (Fig. 1A).

The effect of NaCl in the medium on the choline-activated high-affinity uptake system also served to distinguish this activity from the previous high-affinity system. With salt-stressed cells (0.3 M NaCl), the substrate inhibition completely disappeared (Fig. 1C), whereas the K_m and V_{max} values remained about the same (Table 1). Thus, the value for V_{max} was 25-fold higher than that from cells grown at high osmolarity without choline. This increase in rate may be helpful for osmoprotection.

The kinetic parameters determined for the low-affinity activity were not significantly modified by high osmotic strength in the medium (Table 1). However, as in the high-affinity system, the substrate inhibition completely disappeared when the culture was salt stressed (Fig. 1B). The addition of choline to the medium of high osmolarity increased by 50% the V_{max} for this low-affinity activity (data not shown). This increase is probably due to the fact that the choline-activated high-affinity transport activity is not inhibited by high substrate concentrations. Therefore, the observed V_{max} is likely the result of the action of both transport activities. Because of competition between these two activities, accurate K_m determination of the low-affinity system was untrustworthy under these conditions. Hence, further analysis of the low-affinity system from cells grown in choline was not undertaken.

Finally, all of the results in Fig. 1 and Table 1 demonstrate that *R. meliloti* 102F34 possesses at least three separate transport activities which exhibit different kinetic characteristics: (i) the K_m and V_{max} values for two high-affinity transport activities and one low-affinity transport activity were distinctly different; (ii) the presence of salt in the medium significantly affected the kinetics of the high-affinity activity observed in cells grown in the absence of choline but not the kinetics of both the choline-activated high- and low-affinity activities.

Induction of high-affinity choline transport. Because the addition of choline to the medium caused dramatic changes in the kinetics of choline transport, the possibility exists that choline acts as an inducer of its transport. This possibility was investigated by blocking induction with chloramphenicol, a protein synthesis inhibitor, in cells grown in MM plus 0.3 M NaCl (Fig. 2). This medium was used to maximize the effect of choline on V_{max}, since no substrate inhibition was observed in this condition. Two-thirds of the culture was supplemented with choline, whereas the other third remained untreated. At 1.5 h after choline addition, chloramphenicol was added to one-half of the choline-treated culture. The transport rate of the choline-treated cells began to increase about 1 h after choline addition and was almost fivefold higher after 4 h (Fig. 2). This increase was not observed when chloramphenicol was added to the culture. Therefore, the choline-dependent stimulation of transport that was observed in the absence of chloramphenicol appears to be dependent on de novo protein synthesis of either a new transport protein or protein(s) regulating the activity of preexisting transport protein(s).

Modulation of choline transport activities by osmotic strength. The effects of NaCl on the constitutive and induced high-affinity transport activities were investigated further by measuring the initial rate of choline transport as a function of

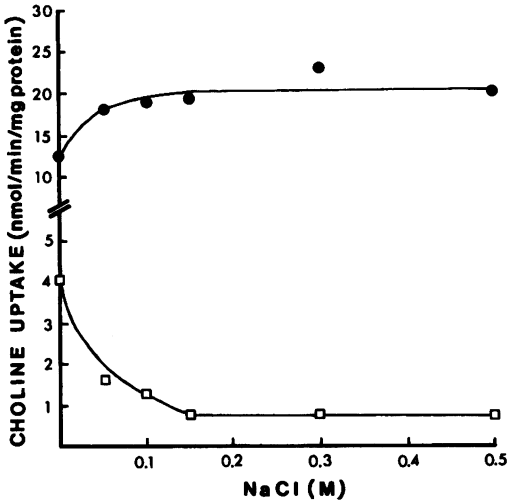

FIG. 3. Effect of NaCl in the growth medium on constitutive and induced high-affinity choline transport activity in *R. meliloti* 102F34. Cells were grown in MM plus 0 to 0.5 M NaCl in the absence (□) or in the presence (●) of 7 mM choline. Uptake was assayed with 10 μM [*methyl*-^{14}C]choline; reaction time was 5 min. The points represent means of triplicate determinations; the standard deviation was less than 10%.

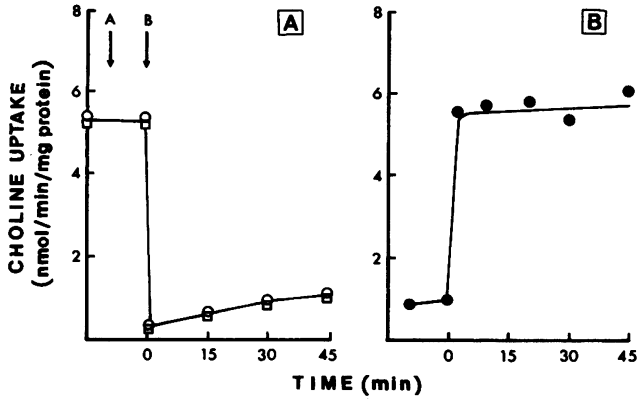

FIG. 4. Effect of osmotic upshock or downshock on choline high-affinity transport activities in *R. meliloti* 102F34. Cultures were grown overnight in MM or MM plus 0.3 M NaCl. Cultures were osmotically shocked, and samples were assayed for uptake activity. (A) Osmotic upshock of uninduced cells in the absence (○) or in the presence (□) of chloramphenicol (100 μg/ml); arrows A and B indicate the times of chloramphenicol addition and osmotic upshock, respectively. (B) Osmotic downshock of uninduced cells performed at time zero. Uptake was assayed with 10 μM [*methyl*-^{14}C]choline.

NaCl concentration in the medium (Fig. 3). The transport activity of cells grown in the absence of choline decreased steadily as the NaCl concentration increased. As little as 0.05 M NaCl caused a 60% decrease in transport activity, even though this level of salt did not affect the growth rate (data not shown). Maximal inhibition (80%) was achieved with only 0.15 M NaCl.

In contrast, the high-affinity transport rate from cultures grown in choline seemed to be slightly stimulated by NaCl. Maximum activity occurred with only 0.05 M NaCl and remained constant as the salt concentration was increased up to 0.5 M. It should be noted that the relatively low transport activity (12 nmol/min per mg of protein) observed in the absence of salt was a consequence of substrate inhibition. The apparent stimulation was due primarily to the disappearance of such substrate inhibition when the cells were grown in the presence of salt but could also be the consequence of a minor activation.

The results presented here provide additional evidence for the existence of two distinct high-affinity transport activities. They also serve to demonstrate that these activities are sensitive to relatively small changes in the concentration of NaCl in the growth medium.

To determine whether the NaCl-dependent change in high affinity choline transport rates is specific to NaCl or is an osmotic effect, other solutes were tested. All solutes added to growth media were used at isoosmotic concentrations equivalent to that of MM plus 0.3 M NaCl (14.8 bars). All solutes tested caused a clear inhibition of the uptake observed with uninduced cultures (Table 2). Hence, there was no specific effect of any given solute, and the effect shown by NaCl appeared to be osmotic in nature.

The effect of NaCl was also tested on the low-affinity transport activity; no significant modification of the transport rate could be detected as the NaCl concentration increased. However, a time course study showed that the linear period of uptake was about 10 min in low-NaCl-grown cells, whereas it could reach 1 h in cells grown in 0.3 M NaCl (data not shown).

High-affinity transport after osmotic upshock or downshock. The results described above demonstrate that the osmolarity of the medium affects the rates of choline high-affinity transport activity. To investigate the mechanism by which the medium osmolarity modulates these activities, cultures were subjected to sudden osmotic upshock or downshock (Fig. 4). First, an uninduced culture was subjected to an osmotic upshock from 0 to 0.3 M NaCl; a rapid and dramatic decrease in the transport rate was followed by a slow increase (Fig. 4A). After 40 min the specific activity was about 1 nmol/min per mg of protein, the same rate that is usually observed in cells grown in MM plus 0.3 M NaCl (Table 2). Similar results occurred when upshock was only from 0 to 0.05 M NaCl (data not shown). The addition of chloramphenicol did not decrease the rate of recovery after upshock, indicating that this small increase in activity is not the result of a newly synthesized protein(s). It was verified that the cell viability was not affected by sudden osmotic upshock or downshock.

TABLE 2. Effect of medium osmolarity and exogenous choline on choline high-affinity transport in *R. meliloti* 102F34

Growth medium[a]	Choline transport (nmol/min per mg of protein) in[b]:	
	Uninduced cultures	Induced cultures
MM	4.1 ± 0.4	10.5 ± 1.1
MM–0.3 M NaCl	0.9 ± 0.1	21.8 ± 2.0
MM–0.3 M KCl	1.5 ± 0.2	21.4 ± 2.0
MM–0.3 M LiCl	1.8 ± 0.2	23.8 ± 2.3
MM–0.3 M K_2SO_4	1.9 ± 0.2	16.1 ± 1.7

[a] Cells were grown in MM (1.1 bar) plus the indicated solutes in concentrations osmotically equivalent to that of MM plus 0.3 M NaCl (14.8 bars).

[b] For induced cultures, the choline concentration in the growth medium was 7 mM. Cells were harvested by centrifugation, rinsed once in the corresponding choline-free medium, and assayed for uptake with 10 (uninduced cultures) or 50 μM (induced cultures) choline. Each number represents the mean of triplicate determinations ± the standard deviation.

TABLE 3. Effect of unlabeled analogs on [*methyl*-^{14}C]choline transport by *R. meliloti* 102F34[a]

Inhibitor	% Inhibition[b] of activity		
	High affinity	High affinity induced	Low affinity
None	0	0	0
Ethanolamine	9	−9	33
N-Methylethanolamine	12	ND[c]	36
N,*N*-Dimethylethanolamine	18	−3	52
N-Methylglycine	4	−9	36
N-,*N*-Dimethylglycine	29	6	43
Glycine betaine	45	50	34
Chlorocholine	34	67	ND
Acetylcholine	40	79	44
Betainal	82	56	68

[a] Cultures were grown in MM (uninduced activities) or in MM plus 0.3 M NaCl and 7 mM choline (induced activity). To assay for choline transport, washed cells were incubated for 5 min in a mixture containing 10 (high-affinity activities) or 400 μM (low-affinity activity) [*methyl*-^{14}C]choline and the indicated inhibitor at a 25-fold molar excess. Data are given as the percent reduction of the uninhibited transport rates, which were 4.3 (high-affinity activity), 21.0 (high-affinity induced activity) and 37.3 (low-affinity activity) nmol/min per mg of protein.

[b] Standard deviation from triplicate cultures was less than 15%.

[c] ND, Not done.

When an uninduced culture was subjected to osmotic downshock from 0.3 M NaCl to MM medium, the high-affinity transport activity rapidly increased from 1 to 5.5 nmol/min per mg of protein, without further increase (Fig. 4B). Both upshock and downshock experiments demonstrated that high-affinity transport in uninduced cells was osmotically controlled by a rapid mechanism.

When an induced culture was subjected to osmotic shock, less pronounced changes occurred. After either upshock or downshock the transport activity decreased only about 35%. This rapid decrease was followed by a slower increase, so that within 30 min the transport rates were completely recovered. Similar patterns were obtained for the low-affinity uptake activity from cultures grown in the absence of choline (data not shown). Hence, high osmotic strength in the medium decreased only the constitutive high-affinity transport activity significantly.

Substrate specificity of the transport activities. To assess the relative substrate specificities of the three transport activities, choline transport was assayed in the presence of a 25-fold excess of various unlabeled analogs (Table 3). Several differences were observed among the three transport activities in the level of inhibition caused by some of these compounds. Glycine betaine, chlorocholine, acetylcholine, and betainal, which are closely related to choline in structure, were almost equally effective inhibitors of all three transport activities. However, the other more distantly related analogs, ethanolamine, *N*-methylethanolamine, *N*,*N*-dimethylethanolamine, *N*-methylglycine, and *N*,*N*-dimethylglycine, caused different effects on each transport system. The low-affinity transport activity was the most inhibited (about 40%), whereas the constitutive high-affinity activity showed less inhibition (from 4 to 29%). On the other hand, the induced high-affinity transport activity was either only slightly inhibited (6% inhibition with *N*,*N*-dimethylglycine) or even activated by the presence of these compounds such as *N*-methylglycine or ethanolamine.

The results of these competition experiments demonstrate the following: (i) the high-affinity transport activities are much more specific in the types of analogs that can inhibit uptake than is the low-affinity activity; (ii) the constitutive and induced high-affinity transport activities behaved differently, thus providing more evidence that they are distinct.

TABLE 4. Effect of choline analogs added to the growth medium on choline high-affinity transport activity in *R. meliloti* 102F34[a]

Addition to growth medium	Choline uptake (nmol/min per mg of protein)
None	1.3 ± 0.2
Choline	20.8 ± 2.1
Chlorocholine	16.5 ± 1.7
Glycine betaine	20.9 ± 2.0
N,*N*-Dimethylglycine	13.1 ± 1.4
N-Methylglycine	4.8 ± 0.5
N,*N*-Dimethylethanolamine	2.7 ± 0.3
N-Methylethanolamine	5.2 ± 0.5

[a] Cells were grown in MM plus 0.3 M NaCl and 7 mM of the indicated compound. Cultures were washed once in MM plus 0.3 M NaCl and assayed for choline transport at 50 μM substrate; the reaction time was 5 min. Each number represents the mean of triplicate determinations ± the standard deviation.

Since the choline high-affinity transport was significantly modified in the presence of a few choline analogs, it was of interest to determine the specificity on the induction process as well. The ability of various choline analogs to induce the high affinity transport activity was examined in cells grown in MM plus 0.3 M NaCl (Table 4). Every compound added to the growth medium caused some level of stimulation. Glycine betaine and choline stimulated transport activity equally well, whereas the analogs chlorocholine and *N*,*N*-dimethylglycine were not quite as effective. The least effective were *N*-methylethanolamine, *N*-methylglycine, and *N*,*N*-dimethylethanolamine, which stimulated the activity from four- to two-fold only. These results correlate well with the inhibition data given in Table 3.

DISCUSSION

The data presented in this report clearly demonstrate that *R. meliloti* 102F34 possesses three kinetically distinct transport activities for choline uptake: a high-affinity activity greatly inhibited by high osmolarity, a second high-affinity activity that is inducible by choline itself, and a low-affinity activity that is constitutively expressed. Several lines of evidence support this interpretation, including the determination of kinetic parameters, the multiple effects of choline, chloramphenicol, and/or salt added to the medium, and the differences in inhibition by choline analogs.

We have shown that each transport activity has its own unique set of kinetic parameters when cells are grown in the absence or the presence of NaCl added to the medium (Fig. 1, Table 1). It is very common for bacteria to have multiple transport systems for uptake of important substrates. Such multiple transport systems are usually distinguished genetically, since kinetic data alone can never prove multiple uptake systems. Although the simplest interpretation of our data is that there are multiple choline transport systems, more complex alternative explanations could be postulated. It is possible that the multiple K_m and V_{max} values that we observed were due to allosteric effects and hence due to a single transport system comparable to that described for glutamate uptake by *E. coli* K-12 cells (5). With such a system operating in a wide range of substrate concentrations, the uptake rate should increase steadily as the sub-

strate concentration increases. Indeed, in both MM and MM plus 0.3 M NaCl the uptake rate increased steadily as the choline concentration increased from 0.12 to 50 μM (Fig. 1A) and from 100 μM to 4 mM (Fig. 1B). But when substrate concentration was increased from 50 to 100 μM, the uptake rate increased strikingly about 9-fold in MM and 23-fold in MM plus 0.3 M NaCl. These large increases in uptake, which occurred in a very narrow range in choline concentration, may suggest the existence of two separate high- and low-affinity transport systems.

With low-osmolarity-grown cells, all of the choline transport systems showed a decrease in their V_{max} in the presence of high choline concentration due to substrate inhibition. Choline may inhibit transport by causing, for example, conformation changes in the transport protein(s) and/or membrane. An alternative explanation could simply be the rapid accumulation of substrate inside the cells as occurs with lactose permease. In fact, choline taken up by the cells is very efficiently converted into glycine betaine and never accumulated. Interestingly, osmotic stress impairs the ability of choline to act as an inhibitor.

Osmotic shock experiments were carried out to confirm that the mechanisms by which osmotic strength regulates choline transport activity are biochemical rather than genetic in nature. In fact, two of the three transport activities were only transiently affected by osmotic shock treatment, which could be the result of a temporary perturbation of the membrane or a decrease in the required energy pool (16). On the other hand, the constitutive high-affinity system was rapidly and strongly inactivated after upshock and reactivated after downshock. Although the physiological advantage for the inhibition of one of the three uptake activities by high osmolarity in the medium cannot presently be explained, it is not surprising. It must be emphasized that choline is a potential osmoprotectant and also a potential carbon and/or nitrogen source (1, 10). As a result, choline uptake could be regulated by other environmental factors such as carbon and/or nitrogen availability in addition to osmotic strength.

Because osmotic stress causes the substrate inhibition to disappear, choline can quickly enter the cell when it is needed for osmoprotection, but only when its concentration is high in the medium. Under these conditions the constitutive high-affinity transport rate is negligible, whereas the induced high- and low-affinity transport rates are significant. The V_{max} values for these two activities in the presence of 0.3 M NaCl were 22.7 and 50 nmol/min per mg of protein for the induced high- and low-affinity transport activities, respectively (Table 1). Indeed, these values were higher than that for the osmotically regulated glycine betaine uptake activity (V_{max}, 20 nmol/min per mg of protein; K_m, 9 μM) in *R. meliloti* grown in the presence of 0.3 M NaCl. In contrast to choline, no substrate inhibition of glycine betaine transport was ever observed (J. A. Pocard, Ph.D. thesis, University of Rennes I, France, 1987). Hence, in addition to glycine betaine transport, the induced high- and low-affinity choline transport activities also likely play a major role in osmoregulation of this bacterium.

Considering the multiple roles of choline in bacteria and yeast, it is of interest to compare our results with those of other studies of choline transport. Choline transport was also reported in *Agrobacterium tumefaciens* (20), *Pseudomonas* sp. strain BAL31 (23), and *Saccharomyces cerevisiae* (7). A high-affinity system was characterized in *S. cerevisiae* (K_m, 0.56 μM), whereas a low-affinity system (K_m, 0.5 mM) was found in *Pseudomonas* sp. Both systems were constitutively expressed and displayed typical Michaelis-Menten kinetics. Although the relationships between choline uptake and osmotic stress were not investigated in these studies, choline is not metabolized by the marine organism *Pseudomonas* sp. strain BAL31. On the other hand, the osmotic control of choline transport has been well studied in *E. coli* (24) and can be contrasted to our results with *R. meliloti*. For example, choline transport is undetectable when *E. coli* is grown in medium of low osmolarity, but two genetically separate transport systems, a high- and a low-affinity system, were fully expressed about 1 h after the culture was subjected to osmotic stress (24). However, in *R. meliloti* high osmolarity does not induce any of the choline transport systems and, as discussed above, causes an inhibition of rate for the constitutive high-affinity system. Furthermore, choline is an excellent inducer of one of the transport systems in *R. meliloti* but has no effect on transport in *E. coli* (24). We believe that the differences between choline transport in *R. meliloti* and *E. coli* are the result of the fact that choline and glycine betaine have multiple roles in *R. meliloti* but not in *E. coli*. When *R. meliloti* is grown at low osmolarity, choline and glycine betaine are degraded, salvaging carbon, nitrogen, and energy (1, 9, 22). On the other hand, when external osmolarity is increased, choline is still converted into glycine betaine, but the betaine is accumulated rather than degraded. Thus, depending on the osmotic strength of the growth medium of *R. meliloti*, these two molecules are used either as nutritional sources or as osmoprotectants. On the contrary, in *E. coli*, glycine betaine functions only as an osmoprotectant and cannot be further catabolized (18).

It has also been shown that the rate of choline uptake by *Pseudomonas* sp. was increased when choline is added to the growth medium (23), but it was not indicated whether the subsequent threefold increase in the uptake rate was due to the expression of a new transport system or to the stimulation of the constitutive activity. We believe that the changes in high-affinity transport activity that we observed when choline was added to the growth medium of *R. meliloti* are likely due to genetic induction for the following reasons. First, the effect of choline on transport was slow; it took about 8 h to reach a maximum specific activity. Second, the addition of chloramphenicol to the medium completely blocked the effect of choline. Third, the induced high-affinity system was also inducible by a large number of choline analogs (Table 4).

In conclusion, we have compiled considerable evidence for the existence of three kinetically separate choline transport activities in *R. meliloti* 102F34. Although all three are important in choline uptake at low osmolarity, only the induced high-affinity and constitutive low-affinity activities are important for osmoregulation. Further work is needed to determine whether or not these systems share any (or all) structural or regulatory elements. We are currently investigating these questions.

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