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Osmoregulation in *Rhizobium meliloti*: Production of Glutamic Acid in Response to Osmotic Stress

JAMES L. BOTSFORD* AND THOMAS A. LEWIS

Department of Biology, New Mexico State University, Las Cruces, New Mexico 88003

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***Rhizobium meliloti*, like many other bacteria, accumulates high levels of glutamic acid when osmotically stressed. The effect was found to be proportional to the osmolarity of the growth medium. NaCl, KCl, sucrose, and polyethylene glycol elicited this response. The intracellular levels of glutamate and K^+ began to increase immediately when cells were shifted to high-osmolarity medium. Antibiotics that inhibit protein synthesis did not affect this increase in glutamate production. Cells growing in conventional media at any stage in the growth cycle could be suspended in medium causing osmotic stress and excess glutamate accumulated. The excess glutamate did not appear to be excreted, and the intracellular level eventually returned to normal when osmotically stressed cells were suspended in low-osmolarity medium. A *glt* mutant lacking glutamate synthase and auxotrophic for glutamate accumulated excess glutamate in response to osmotic stress. Addition of isoleucine, glutamine, proline, or arginine stimulated glutamate accumulation to wild-type levels when the mutant cells were suspended in minimal medium with NaCl to cause osmotic stress. In both wild-type and mutant cells, inhibitors of transaminase activity, including azaserine and aminooxyacetate, reduced glutamate levels. The results suggest that the excess glutamate made in response to osmotic stress is derived from degradation of amino acids and transamination of 2-ketoglutarate.**

The response of cells to high osmolarity is a fundamental biological question (42). Bacteria provide a useful model system to study the molecular biology of osmoregulation (20). In rhizobia, osmoregulation presents a complex problem. A bacterium living free in soil must contend with changes in osmotic potential as the soil dries. The bacterium must also adapt to the osmotic situation during the infection process and in a nodule as a bacteroid exchanging nutrients with the host plant. Osmoregulation, particularly in *Escherichia coli* and *Salmonella typhimurium*, has been reviewed recently (6). However, the phenomenon in *Rhizobium meliloti* has not been studied extensively. As in *E. coli*, glycine betaine serves as an osmoprotectant in *R. meliloti* (2, 30, 31, 34). Glycine betaine is obtained from oxidation of choline (35). When cells grow with osmotic stress, the enzymes involved in choline oxidation are not induced as in *E. coli* (11, 39). However, unlike *E. coli*, this bacterium can use glycine betaine as a sole source of carbon and nitrogen. The enzymes involved in its degradation are repressed when cells grow with osmotic stress (35). *R. meliloti*, like *E. coli* (3, 32, 38), accumulates the disaccharide trehalose when osmotically stressed (12, 35), and as in *E. coli*, the synthesis of membrane-derived oligosaccharides is affected by the osmolarity of the growth medium (26).

Many bacteria produce excess glutamic acid when grown with inhibitory concentrations of osmolytes (25, 40). This has been shown in a *Rhizobium* sp. that grows with mesquite, a desert legume (14); in a *Rhizobium* that grows with a leguminous tropical tree (43); in a fast-growing *Rhizobium* strain with soybeans as the host (44); and in *R. meliloti* (4). It has recently been reported (36) that *R. meliloti* accumulates not only glutamate but also a dipeptide, *N*-acetylglutaminyl glutamate, in response to osmotic stress.

It has been proposed that glutamate is made by the bacterium to act as a counterion for the K^+ that accumulates when cells grow with osmotic stress (10, 15, 33). This ion has been implicated in the regulation of genes involved in nitrogen fixation in rhizobia (13). A role for glutamate per se in nitrogen fixation has been proposed (17). Since the osmolarity in root tissue is elevated, this osmotic response could impinge on this aspect of regulation. This communication describes the production of glutamic acid in *R. meliloti* and the accompanying increase in intracellular K^+ in response to osmotic stress.

MATERIALS AND METHODS

Strains. *R. meliloti* 102f34 was obtained from Gary Ditta, University of California, San Diego. Strain 1021 was obtained from Paul Watson, University of Illinois, Champaign. Strain AK330 was obtained from Adam Kondorosi, Hungarian Academy of Science. This strain is auxotrophic for glutamic acid and lacks detectable glutamate synthase (GOGAT) (18). Glutamic dehydrogenase (GDH) levels are normal.

Medium. Cells were grown in minimal defined medium (MDM) with MOPS (morpholinepropanesulfonic acid) as a buffer (4). The pH of the medium was adjusted with NaOH. This NaOH (a few milliequivalents) was not included in calculations of the Na^+ concentration. The medium was filter sterilized. This medium contained 5 mM $(NH_4)_2SO_4$ and 1% mannitol unless noted otherwise. The medium normally included 150 μM KCl. The osmolarity of the growth media was determined with an osmometer (Westcor, Logan, Utah) as instructed by the manufacturer. The osmolarity of MDM was 112 mosM. Addition of 400 mM NaCl increased the osmolarity to 890 mosM.

Growth conditions. Cells were grown in Erlenmeyer flasks filled to no more than 20% capacity. Cultures were shaken at 30°C. Growth was monitored with a Klett colorimeter (green filter). Cultures were usually harvested at 150 Klett U, equivalent to 2.4×10^9 cells and 0.194 mg of protein ml^{-1}.

Cells were harvested by filtration with 47-mm-diameter filters (0.44-μm pore size; cellulose acetate or cellulose nitrate). Alternatively, cells were collected by centrifugation

* Corresponding author.

(5 min at 3,000 × *g* at room temperature). Results were comparable with both techniques.

In experiments in which cells were grown in MDM and then shifted to MDM with an inhibitory concentration of solute, cells were collected either by filtration or by centrifugation and suspended in aerated, prewarmed medium. Results were comparable with both techniques.

Extraction of cellular contents. When cells were harvested by filtration, cellular contents were extracted by placing 5 to 10 ml of boiling water on the filter with the vacuum turned off. After 1 min, the vacuum was turned on and the filtrate was collected. When cells were harvested by centrifugation, 5 ml of boiling water was added to the pelleted cells and the cell suspension was mixed with a vortex mixer. Cell debris was removed by centrifugation. The supernatant was then filtered through cellulose nitrate filters (25-mm diameter, 0.44-μm pore size).

Analytical procedures. Cellular glutamate was measured enzymatically by using bovine GDH and acetylpyridine dinucleotide by the method of Witt (41). The method provided reproducible measurements of as little as 5 nmol of glutamate. Ammonium was measured by using an ammonia electrode (Orion Corp., Cambridge, Mass.). One-milliliter samples were diluted 1:5 and treated as recommended by the manufacturer. K^+ in the aqueous extracts was measured with a 451 atomic emission spectrometer (Instrumentation Laboratory, Wilmington, Mass.) as recommended by the manufacturer. Protein was measured by the method of Lowry et al. (22) with bovine serum albumin as the standard. Normally, protein was measured directly and not inferred from the absorbance of the culture. The specific activity of GOGAT was determined by a spectrophotometric method (27) by monitoring glutamine- and 2-ketoglutarate-dependent oxidation of NADPH at 340 nm and 37°C with a recording double-beam spectrophotometer.

Estimation of protein synthesis. To determine the effect of shifting cells to medium causing osmotic stress on protein synthesis, cells were grown in MDM and suspended in MDM or MDM supplemented with 400 mM NaCl with 0.052 μCi of [^{3}H]leucine at a final concentration of 1 mM. Samples were taken as indicated by collecting 1 ml of the culture on nitrocellulose filters and washing them with 5 ml of 1 mM leucine in MDM or MDM–400 mM NaCl, followed by 5 ml of 5% perchloroacetic acid to precipitate the protein. The filters were dried, and the radioactivity was determined with a liquid scintillation counter.

RESULTS

Glutamate production when cells grow with osmotic stress. As reported previously, excess glutamic acid is found in *R. meliloti* when the bacterium is osmotically stressed (4). It was found that the amount of glutamate produced was proportional to the concentration of the osmolyte (Fig. 1). These data also show that K^+ was accumulated by the bacteria in response to osmotic stress. This has been reported in a variety of bacteria (15, 25, 40, 43). The data in Table 1 show comparable stimulation of glutamate levels when cells were grown with inhibitory concentrations of KCl, sucrose, and polyethylene glycol (Sigma Chemical Co., St. Louis, Mo.). Similar levels of glutamate were produced when cells grew with 1 mM KNO_3 replacing NH_4^+ as the nitrogen source and when cells grew with succinate replacing mannitol as the carbon and energy source (Table 1). The presence of a supplement of Casamino Acids (Difco Laboratories, Detroit, Mich.) did not affect the intracellular

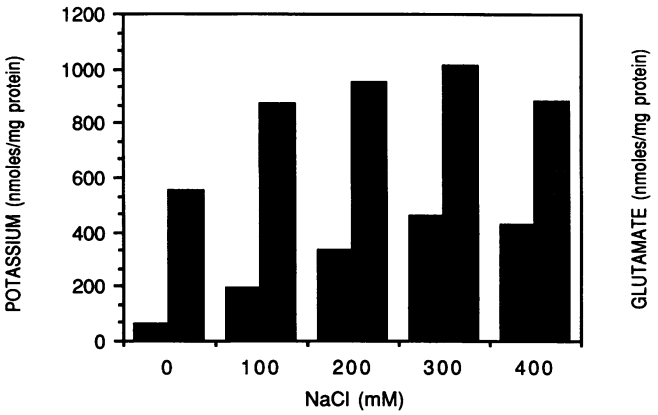

FIG. 1. Effects of increasing salt concentrations on cellular glutamate and K^+. Cells were grown in MDM with NaCl as indicated. Cells were harvested while growing exponentially, and the glutamate was measured. Solid bars represent values for glutamate, and shaded bars represent values for K^+.

accumulation of glutamate (Table 1). Casamino Acids have approximately 1 μmol of glutamate mg^{-1} when assayed enzymatically (41). The inclusion of a synthetic mixture of all of the amino acids (7) except glutamate, glutamine, and proline also had no effect on glutamate levels (data not shown).

Time course of increased glutamate production. It was found that cells made comparable amounts of glutamate and accumulated comparable levels of K^+ when grown in minimal medium, suspended in medium with NaCl, and incubated for 1 h rather than being grown with osmotic stress (Fig. 2). The rates of glutamate production (9.4 nmol mg of

TABLE 1. Effects of osmolytes on glutamate accumulation[a]

Addition (concn)	Glutamate concn (nmol mg of protein^{-1})
None (control)	39.2
NaCl (400 mM)	327.3
Sucrose (400 mM)	292.8
KCl (400 mM)	365.6
PEG 200 (300 mM)[b]	89.2
PEG 1000 (100 mM)[c]	113.4
KNO_3 (1 mM) replacing NH_4^+	
Control	40.6
NaCl (400 mM)	526.6
Sodium succinate (22 mM) replacing mannitol[d]	
Control	109.1
NaCl (400 mM)	509.7
Casamino Acids (0.5%) supplement[e]	
Control	71.6
NaCl (400 mM)	388.0

[a] Cells were grown to the midexponential phase with the added osmolytes. The cells were harvested, and the glutamate was extracted and assayed as described in Materials and Methods.

[b] PEG 200, Polyethylene glycol 200 (average molecular mass, 200 daltons).

[c] PEG 1000, Polyethylene glycol 1000 (average molecular mass, 1,000 daltons).

[d] The disodium salt of succinic acid was used, so these samples have an additional 44 mM NaCl.

[e] Vitamin-free Casamino Acids (Difco).

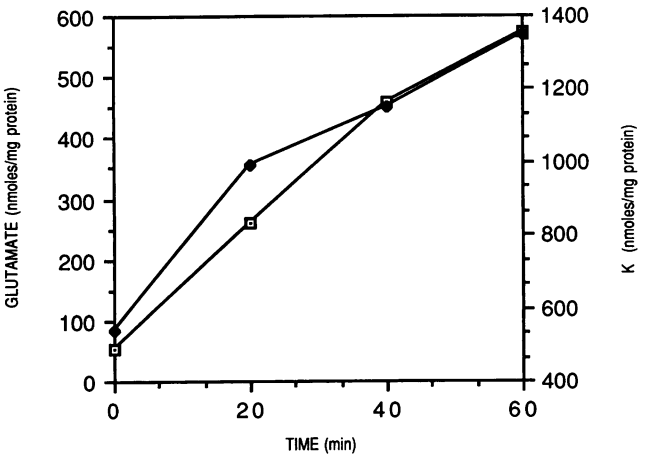

FIG. 2. Time course of glutamate production and K^+ accumulation. Cells were grown to the exponential phase in MDM, collected by filtration, and suspended in MDM–400 mM NaCl. Samples (10 ml) were taken at the indicated times and collected by filtration. The time zero sample was taken from the original culture immediately before the cells were shifted. These results represent the average of three experiments. The solid diamonds represent values for K^+, and the open squares represent values for glutamate. The rates of increase in glutamate production, 9.3 nmol min^{-1} mg of $protein^{-1}$, and K^+ accumulation, 12.4 nmol min^{-1} mg of $protein^{-1}$, were calculated from the slopes of the regression lines fitted to the data (n = 12, r^2 = 0.97 and 0.94).

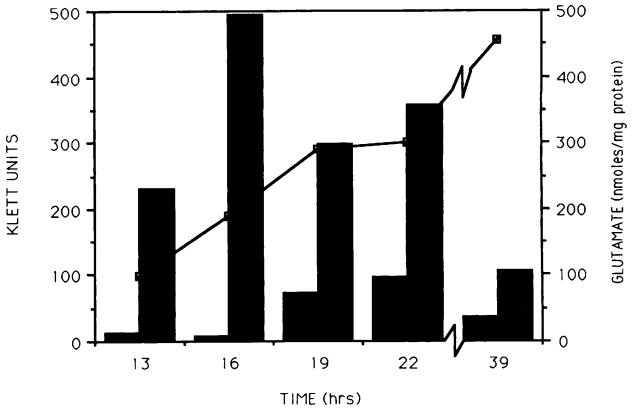

FIG. 3. Effect of growth phase on glutamate production. Cells were grown in MDM. At the times indicated, 10-ml samples of cells were removed, collected by centrifugation, and suspended in 10 ml of MDM or MDM–400 mM NaCl. The cells were incubated for 1 h, harvested by centrifugation, and assayed for protein and glutamate. The squares connected by the line represent growth of the culture in terms of Klett units. The solid bars represent glutamate levels in cells harvested and suspended in control medium. The shaded bars represent glutamate levels in cells harvested and suspended in medium with 400 mM NaCl.

$protein^{-1}$ min^{-1}) and K^+ accumulation (12.3 nmol mg of $protein^{-1}$ min^{-1}) were calculated from these data. Comparable results were obtained when cells were incubated with sucrose as the osmolyte.

Growth phase and glutamate production. The effect of the growth phase on the ability of the bacteria to make glutamate in response to osmotic stress was tested by growing cells in MDM, harvesting them at different times, and suspending them in fresh medium with or without NaCl. The results of this experiment are shown in Fig. 3. These data show that regardless of the growth phase, cells shifted to conditions of osmotic stress had more intracellular glutamate than did cells shifted to low-osmolarity medium.

Glutamate does not appear to be excreted. In several experiments, culture medium was assayed for glutamate after removal of the cells. The assay for glutamate was sensitive enough to detect less than 5% of the intracellular glutamate had it been excreted. In none of the experiments could significant amounts of glutamate be detected in the supernatant fraction or in the culture filtrates.

The excess glutamate made by cells in response to osmotic stress disappears when cells are suspended in low-osmolarity medium. In one experiment, cells that had grown with osmotic stress were harvested and incubated in medium of normal osmolarity and the glutamate levels decreased. In one experiment, cells grown in MDM–400 mM NaCl were harvested and shifted to medium containing MDM. After 60 min, the cells were harvested and the intracellular glutamate was found to be 43.0 nmol mg of $protein^{-1}$. In the control culture, cells suspended in MDM–400 mM NaCl, the intracellular glutamate was 242.5 nmol mg of $protein^{-1}$ after 60 min of incubation.

Mechanism for excess glutamate production. Production of excess glutamate in response to osmotic stress was initiated as soon as cells were suspended in high-osmolarity medium (Fig. 2). Furthermore, the presence of antibiotics that inhibit protein synthesis (Table 2) did not affect glutamate production. This observation suggested that an enzyme involved in glutamate synthesis is activated in response to osmotic stress. It has been proposed that in enteric coliforms, excess glutamate accumulates because GDH is activated by increased intracellular levels of K^+ (25). GOGAT appears to be the sole route to glutamate synthesis in *R. meliloti* (18, 23, 29). Strain AK330 has normal levels of GDH (18) but no detectable GOGAT. This mutant is auxotrophic for glutamic acid and is unable to assimilate ammonium (18). It was suggested that when cells are osmotically stressed, the intracellular ammonium concentration could increase dramatically, enabling glutamate to be made by the GDH reaction. However, measurements of the intracellular ammonium levels in strain 10f34 cells that were suspended in MDM–400 mM NaCl were little different (100 to 120 nmol mg of $protein^{-1}$) from those of the control. To determine whether GDH can be activated when *R. meliloti* grows with osmotic stress and whether it can provide the cell with glutamate when they grow with excess ammonium, strain AK330 was grown on minimal medium with 80 mM NH_4^+. The cells did not grow, even with this eightfold excess of

TABLE 2. Effect of neomycin on glutamate production[a]

Strain and addition	Glutamate concn (nmol mg of $protein^{-1}$)	
	Control	With NaCl
102f34		
None	54.1	241.0
Neomycin[b]	47.6	245.2
1021		
None	68.1	357.4
Neomycin[b]	66.3	410.5

[a] Cells were grown in MDM to the exponential phase, collected by centrifugation, and suspended in MDM or MDM–400 mM NaCl. After incubation for 60 min, samples were collected.

[b] Neomycin added as indicated to a final concentration of 200 μg ml^{-1}.

TABLE 3. Effects of inhibitors of GOGAT and aminotransferase

Strain and addition (concn)	Glutamate concn (nmol mg of protein^{-1})
102f34 (wild type)[a]	
None (control)	16.2
NaCl (400 mM)	338.7
NaCl–methionine sulfone (2 mM)	350.9
NaCl–azaserine (2 mM)	105.6
NaCl–aminooxyacetic acid (5 mM)	59.0
AK330 (*glt*)[b]	
None (control)[b]	0.0
NaCl (400 mM)	172.5
NaCl–aminooxyacetic acid (5 mM)	20.9

[a] Cells were grown in MDM, harvested, and suspended in medium with the additions indicated. The cells were then incubated for 60 min, and glutamate was extracted and assayed.

[b] Cells were grown in MDM with 10 mM glutamate, harvested, and suspended in MDM with the additions indicated but without supplemental glutamate. The cells were then incubated for 60 min, and glutamate was extracted and assayed.

[c] When cells growing in MDM with 10 mM glutamate were sampled and the glutamate was measured immediately before the shift to high-osmolarity medium, 45.9 nmol of glutamate mg of protein^{-1} was measured. The control value represents the glutamate in cells shifted to MDM without glutamate and incubated for 60 min.

ammonium. When 400 mM NaCl was included, the cells remained auxotrophic for glutamate, indicating that GDH could not provide the cells with glutamate, even with high intracellular concentrations of K^+ resulting from osmotic stress. GOGAT provides enteric coliform cells with glutamate when they grow with limiting ammonium, and GDH does so when the cells grow with excess ammonium (8, 24).

GOGAT does not appear to be responsible for increased intracellular glutamate. When wild-type cells were shifted to high-osmolarity medium, addition of methionine sulfone, a potent inhibitor of GOGAT (5), did not affect glutamate levels (Table 3). *R. meliloti* AK330 has normal levels of GDH but has no detectable GOGAT activity (18; unpublished data). This mutant also accumulated excess glutamate when shifted from MDM with 1 mM glutamate to MDM without glutamate but with 400 mM NaCl to induce osmotic stress (Tables 2 and 3). In both the *glt* mutant strain and wild-type strain 102f34, inhibitors of transaminase (aminotransferase) activity, including azaserine and aminooxyacetic acid (37), inhibited accumulation of glutamate when cells were shifted to high-osmolarity medium (Table 3).

If protein synthesis were inhibited when cells are shifted to high-osmolarity medium, production of amino acids would slow. Since virtually all assimilated ammonium passes through the glutamate-glutamine couple, this would spare the glutamate pools. The effect of shifting cells to high-osmolarity medium on protein synthesis was tested by monitoring the incorporation of radioactive leucine into acid-precipitable material. Data in Fig. 4 show that protein synthesis was inhibited when cells were shifted to high-osmolarity medium and that after 30 min, protein synthesis was once again synthesized but at a much lower rate. Presumably, this represents the ability of cells to synthesize protein and not altered amino acid transport, since incorporation of the label did eventually increase in the osmotically stressed cells. All strains of rhizobia used in these experiments could use glutamate, proline, arginine, or glutamine as a sole source of nitrogen and carbon, even when the medium contained 400 mM NaCl, indicating that amino acids can be transported when cells are osmotically stressed.

Inclusion of 0.5% Casamino Acids did not affect glutamate

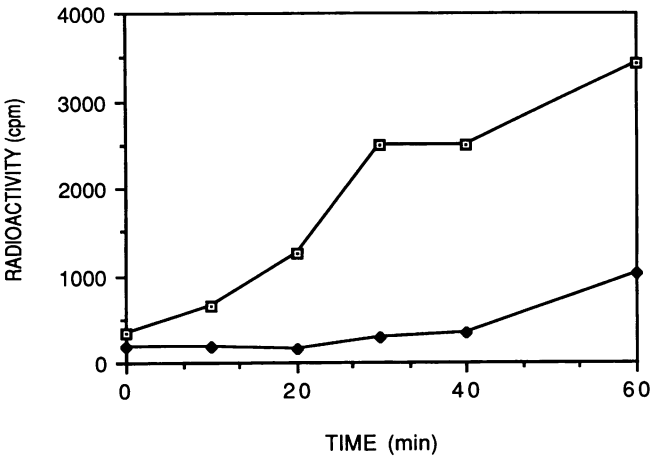

FIG. 4. Effect of osmotic stress on protein synthesis. Cells were grown in MDM–1 mM L-leucine. Cells were collected by centrifugation and suspended in MDM (open squares) or MDM–400 mM NaCl (solid diamonds) supplemented with [^{3}H]leucine at a final concentration of 1 mM as noted in Materials and Methods.

levels (Table 1). If glutamate were to accumulate because the amino acid pools increase when cells are shifted to high-osmolarity medium, this supplement should mimic the effect of osmotic stress, causing intracellular glutamate to be elevated. This was not observed.

If osmotic stress causes glutamate levels to become elevated because protein synthesis is inhibited, addition of antibiotics that inhibit protein synthesis should also cause glutamate levels to increase. This was tested (Table 2). These data show that addition of neomycin had no significant effect on glutamate production when cells were suspended in MDM. This was also tested in wild-type strain 102f34 with other antibiotics, including chloramphenicol, streptomycin, and fusidic acid, known to inhibit protein synthesis. None of these antibiotics affected intracellular glutamate levels when the cells were suspended in MDM or MDM–400 mM NaCl (data not shown).

Alternative pathways to glutamate. Strain AK330 lacks GOGAT and is auxotrophic for glutamic acid. As already noted (Tables 3 and 4), when this strain was osmotically stressed, glutamic acid accumulated. It was noted that inhibitors of aminotransferase activity inhibited accumulation of excess glutamate in both this mutant strain and the wild type (Table 3). We found that *R. meliloti* has an active aminotransferase activity using branched-chain amino acids as a source of ammonium for synthesis of glutamate with

TABLE 4. Glutamate production in strain AK330 in media with supplemental amino acids[a]

Supplement (final concn, 10 mM)	Glutamate concn (nmol mg of protein^{-1})	
	Control	With NaCl
None	0.0	103.7
Isoleucine	14.2	342.1
Glutamine	97.3	599.0
Proline	0.0	178.8
Arginine	0.8	131.0

[a] Cells were grown to the exponential phase in MDM, collected by centrifugation, and suspended in MDM on MDM–400 mM NaCl with the supplements noted. The cells were incubated for 60 min and harvested, and the glutamate was extracted and assayed.

2-ketoglutarate as the substrate (R. Gonzalez, Ph.D. dissertation, New Mexico State University, Las Cruces, 1989; submitted for publication). This transaminase activity could be involved in glutamate production in response to osmotic stress. This was tested by including isoleucine in the medium when AK330 cells were shifted to MDM–400 mM NaCl (Table 4). This amino acid stimulated glutamate production. Other amino acids readily degraded to glutamate (1), including arginine, glutamine, and proline, were also found to stimulate glutamate production (Table 4).

DISCUSSION

The results show that glutamate and K^+ accumulated when *R. meliloti* was osmotically stressed (Fig. 1). The effect was not specific for Na^+ but also occurred when cells grew with osmotic stress caused by K^+, sucrose, or polyethylene glycol (Table 1). The effect was not altered by changes in the growth medium with respect to the carbon source (mannitol or succinate) or the nitrogen source (ammonium or nitrate). When *R. meliloti* grows with nitrate, ammonium should be limiting (28). This did not reduce the ability of cells to make excess glutamate. Addition of 0.5% Casamino Acids, which should supply the cells with most of the needed amino acids, did not alter the situation (Table 1).

When cells were shifted to medium causing osmotic stress, production of excess glutamate appeared to be initiated immediately (Fig. 2). In several experiments, the first sample was taken 5 min after the cells were shifted to conditions of osmotic stress and had higher levels of glutamate than did cells shifted to conventional medium (data not shown). The increased production of glutamate was not affected by antibiotics that inhibit protein synthesis (Table 2). This indicates that de novo protein synthesis is not required.

Production of glutamate occurred regardless of the growth phase of the culture (Fig. 3). As expected, cells in exponential growth, having more resources, accumulated more glutamate when shifted than did cells in the stationary phase. However, some excess glutamate accumulated even when cells were in the stationary phase.

Cells rarely accumulated much more than 500 nmol of glutamate mg of protein^{-1}. When grown with osmotic stress, the glutamate levels gradually declined after the culture entered the stationary growth phase. When the cells were shifted to high-osmolarity medium, the glutamate levels did not continue to increase after 60 min of incubation. This suggests that the amount of glutamate in each cell is regulated.

We have isolated a clone that appears to have *glt* from *R. meliloti* Rm1021 on high-copy-number cosmid pLAFR1 (9, 21; submitted for publications). The plasmid complements AK330. The specific activity of GOGAT in strain 102f34 with this plasmid was found to be 30 times greater than in the control. However, the strain with the plasmid had amounts of intracellular glutamate comparable to those of the control. This suggests that glutamate production is actively regulated by the cells.

Virtually all of the nitrogen in bacterial cells growing in minimal defined medium with ammonium or nitrate as the nitrogen source is assimilated in reactions involving glutamate. On the basis of values for the number of moles of amino acids, purines, pyrimidines, amino sugars, and other nitrogenous compounds in *E. coli* B/r (16), it can be calculated that the bacterium must assimilate 19,730 nmol of amino moieties mg of protein^{-1}. If we assume comparable values for *R. meliloti* growing with a generation time of 3.5 h, each cell must synthesize 94 nmol of glutamate min^{-1} mg of protein^{-1}. The rate of increase in glutamate production in cells shifted to medium causing osmotic stress is 9.3 nmol min^{-1} mg of protein^{-1}. Thus, the excess glutamate made by the bacterium represents only 10% of the capacity of the cell for glutamate production. Small changes in the specific activity of one or more enzymes involved in glutamate production or utilization, as well as small changes in intracellular intermediates, could account for the excess glutamate production.

In *R. meliloti*, despite a great deal of effort working with dialyzed crude cell extracts, neither GDH nor GOGAT appears to be activated by K^+ or to be allosteric (Gonzalez, Ph.D. dissertation). Furthermore, as was noted, strain AK330 lacks detectable glutamate synthase but has normal levels of GDH. This mutant is auxotrophic for glutamate, even when growing with excess ammonium under conditions of osmotic stress. GDH does not appear to be responsible for the excess glutamate made by *R. meliloti* in response to osmotic stress. Similarly, GOGAT does not appear to be responsible either. Addition of methionine sulfone (Table 3) did not affect glutamate production when wild-type cells were shifted to high osmolarity (Table 3). *glt* mutant AK330 accumulated elevated levels of glutamate when shifted to high-osmolarity medium (Tables 2 and 3). The data indicate that some glutamic acid could be made by using the amino acids present when cells are shifted to high-osmolarity medium by using transaminase activity. Inhibitors of transaminase reduced glutamic acid made in response to osmotic stress (Table 3). The glutamate auxotroph AK330 can utilize a variety of amino acids as sources for assimilated nitrogen (18; unpublished data). Addition of a variety of amino acids to MDM with a high salt concentration to induce osmotic stress enabled the mutant to accumulate glutamate at levels comparable to those of the wild type (Table 4). The experiments with transaminase activity inhibitors (Table 3) suggest that at least some of the excess glutamate could be derived from transamination of amino acids already made by the bacterium before the shift. Glutamate production in strain AK330 shifted to MDM–400 mM NaCl was stimulated by addition of arginine, proline, and glutamine, amino acids that are readily degraded to glutamate (1). This suggests that degradation of these amino acids could also account for some of the excess glutamate.

Both K^+ and glutamate seem to be involved in nitrogen fixation (13, 17), and it is inviting to propose that rhizobia use this accumulation of glutamate and K^+ to regulate symbiotic nitrogen fixation. Addition of 100 mM NaCl increases the osmolarity of the medium to 320 mosM, and this is sufficient to cause excess glutamate and K^+ to accumulate (Fig. 1). This is in the range of the osmolarity within plant cells and presumably in the root nodule. Since glutamate plays a central role in ammonia assimilation, this could be critical to nitrogen fixation. It has been shown that K^+ is critical for derepression of nitrogenase when rhizobia grow ex planta (13). When K^+ is present as the salt of glutamic acid rather than an inorganic anion, it is more active in vitro, mediating interactions between protein and DNA, activities involved in regulation of gene expression (19). Potassium and glutamate accumulate in substantial quantities. If the volume of a bacterial cell is 1×10^{-15} liter, a glutamate concentration of 400 nmol mg of protein^{-1} is equivalent to an intracellular concentration of 30 mM. A K^+ concentration of 1,000 nmol mg of protein^{-1} is equivalent to an intracellular concentra-

tion of 75 mM. At these concentrations, either compound could have a significant effect.

The results suggest an interesting evolutionary aspect. If cells simply need to accumulate an osmoticum, glutamate is easily obtained by slight alterations of the activity of one or more enzymes involved in glutamate metabolism. The amino acid is readily utilized by the cells as a source of assimilated nitrogen and as a carbon source when the cells return to a lower-osmolarity environment. This is an ideal compound to serve as an osmoticum (38) or as counterion for K^+ (10, 25, 33).

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LITERATURE CITED

1. **Bender, D. A.** 1975. Amino acid metabolism, p. 80–111. John Wiley & Sons, Inc., New York.
2. **Bernard, T., J. A. Pocard, B. Perroud, and D. Le Rudulier.** 1986. Variations in the response of salt stressed *Rhizobium* strains to betaine. Arch. Microbiol. **143**:359–364.
3. **Boos, W., U. Ehmann, E. Bremmer, A. Middendorf, and P. Postma.** 1987. Trehalase in *Escherichia coli*. Mapping and cloning of its structural gene and identification of the enzyme as a periplasmic protein induced under high osmolarity growth conditions. J. Biol. Chem. **262**:13212–13218.
4. **Botsford, J. L.** 1984. Osmoregulation in *Rhizobium meliloti*: inhibition of growth by salts. Arch. Microbiol. **137**:124–127.
5. **Brenchley, J. E.** 1973. Effect of methionine sulfoximine and methionine sulfone on glutamate synthesis in *Klebsiella aerogenes*. J. Bacteriol. **114**:666–673.
6. **Csonka, L. N.** 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. **53**:121–147.
7. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics, p. 207. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **Dendinger, S., L. G. Patil, and J. E. Brenchley.** 1980. *Salmonella typhimurium* mutants with altered glutamate dehydrogenase and glutamate synthase activities. J. Bacteriol. **141**:190–198.
9. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77**:7347–7351.
10. **Epstein, W.** 1986. Osmoregulation by potassium transport in *Escherichia coli*. FEMS Microbiol. Rev. **39**:73–78.
11. **Eschoo, M. W.** 1988. *lac* fusion analysis of the *bet* genes of *Escherichia coli*: regulation by osmolarity, temperature, oxygen, choline, and glycine betaine. J. Bacteriol. **170**:5208–5215.
12. **Giæver, H. M., O. B. Styrvold, I. Kaasen, and A. R. Strøm.** 1988. Biochemical and genetic characterization of osmoregulatory trehalose synthesis in *Escherichia coli*. J. Bacteriol. **170**: 2841–2849.
13. **Gober, J. W., and E. R. Kashket.** 1987. K^+ regulates bacteroid associated functions of *Bradyrhizobium* sp. 32H1. Proc. Natl. Acad. Sci. USA **84**:4650–4654.
14. **Hua, S.-S. T., V. Y. Tsai, G. M. Lichens, and A. T. Noma.** 1982. Accumulation of amino acids in *Rhizobium* sp. strain WR1001 in response to sodium chloride salinity. Appl. Environ. Microbiol. **44**:135–140.
15. **Imhoff, J. F.** 1986. Osmoregulation and compatible solutes in bacteria. FEMS Microbiol. Rev. **39**:57–66.
16. **Ingraham, J. L., O. Maaløe, and F. C. Neidhardt.** 1983. Growth of the bacterial cell. Sinauer Publishers, Sunderland, Mass.
17. **Kahn, M. J., J. Kraus, and J. E. Sommerville.** 1985. A model for nutrient exchange in the *Rhizobium* legume symbiosis, p. 193–199. *In* H. J. Evans, P. J. Bottomley, and W. E. Newton, (ed.), Nitrogen fixation research programs. Martinus Nijhoff, Dordrecht, The Netherlands.
18. **Kondoroisi, A. Z., Z. Svab, G. B. Kiss, and R. A. Dixon.** 1977. Ammonia assimilation and nitrogen fixation in *Rhizobium meliloti*. Mol. Gen. Genet. **151**:221–226.
19. **Leirno, S., C. Harrison, D. S. Cayley, R. R. Burgess, and M. T. Record.** 1987. Replacement of potassium chloride by potassium glutamate enhances protein-DNA interactions in vitro. Biochemistry **26**:2095–2101.
20. **Le Rudulier, D., A. R. Strom, A. M. Dandekar, L. T. Smith, and R. C. Valentine.** 1984. Molecular biology of osmoregulation. Science **224**:1064–1068.
21. **Long, S. R., W. J. Buikema, and F. M. Ausubel.** 1982. Cloning of *Rhizobium meliloti* nodulation genes by direct complementation of Nod^- mutants. Nature (London) **298**:485–488.
22. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
23. **Ludwig, R. A.** 1980. Physiological roles for glutamine synthetases I and II in ammonium assimilation in *Rhizobium* sp. 32H1. J. Bacteriol. **141**:1209–1216.
24. **Magasanik, B.** 1982. Genetic control of nitrogen assimilation in bacteria. Annu. Rev. Genet. **16**:135–168.
25. **Measures, J. C.** 1975. Role of amino acids in osmoregulation of non-halophilic bacteria. Nature (London) **257**:398–400.
26. **Miller, K. J., E. P. Kennedy, and V. N. Reinhold.** 1985. Osmotic adaptation by gram negative bacteria: possible role for oligosaccharides. Science **231**:48–51.
27. **Miller, R. E., and E. Stadman.** 1972. Glutamate synthase of *Escherichia coli*: an iron sulfide flavoprotein. J. Biol. Chem. **247**:7407–7419.
28. **Nautyl, C. S., and V. V. Modi.** 1981. Regulation of some enzymes involved in ammonia assimilation in *Rhizobium meliloti*. Experientia **37**:121–122.
29. **Osbourne, M. S., and E. Signer.** 1980. Ammonium assimilation in *Rhizobium meliloti*. J. Bacteriol. **143**:1234–1240.
30. **Perroud, B., and D. Le Rudulier.** 1985. Glycine betaine transport in *Escherichia coli*: osmotic modulation. J. Bacteriol. **162**:393–401.
31. **Pocard, J.-A., T. Bernal, L. T. Smith, and D. Le Rudulier.** 1989. Characterization of three choline transport activities in *Rhizobium meliloti*: modulation by choline and osmotic stress. J. Bacteriol. **171**:531–537.
32. **Postma, P. W., H. G. Keizer, and P. Koolowijic.** 1986. Transport of trehalose in *Salmonella typhimurium*. J. Bacteriol. **168:** 1107–1111.
33. **Richey, B., S. Cayley, M. C. Mossing, C. Kolka, C. F. Anderson, T. C. Farr, and M. T. Record.** 1987. Variability of the intracellular environment of *Escherichia coli*. J. Biol. Chem. **262:** 7157–7164.
34. **Sauvage, D., J. Hamelin, and F. LaRher.** 1983. Glycine betaine and other structurally related compounds improve the salt tolerance of *Rhizobium meliloti*. Plant Sci. Lett. **31**:291–302.
35. **Smith, L. T., J.-A. Pocard, T. Bernal, and D. Le Rudulier.** 1988. Osmotic control of glycine betaine biosynthesis and degradation in *Escherichia coli*. J. Bacteriol. **170**:3142–3149.
36. **Smith, L. T., and G. M. Smith.** 1989. An osmoregulated dipeptide in stressed *Rhizobium meliloti*. J. Bacteriol. **171:** 4714–4717.
37. **Snapp, S. S., and C. P. Vance.** 1986. Asparagine biosynthesis in alfalfa (*Medicago sativa* L) root nodules. Plant Physiol. **82:** 390–395.
38. **Strøm, A., P. Falkenberg, and B. Landfald.** 1986. Genetics of osmoregulation in *Escherichia coli*: uptake and biosynthesis of organic osmolytes. FEMS Microbiol. Ecol. **39**:79–86.
39. **Stryvold, O. B., P. Falkenberg, B. Landfall, M. W. Eshoo, T. Bjøsen, and A. E. Strøm.** 1986. Characterization of osmoregulatory mutants of *Escherichia coli* blocked in the choline glycine-betaine pathway. J. Bacteriol. **165**:856–863.

40. **Tempest, D. W., J. L. Meers, and C. M. Brown.** 1970. Influence of environment on the content and composition of microbial free amino acid pools. J. Gen. Microbiol. **64:**171–185.
41. **Witt, I.** 1974. Determination of glutamate with glutamic dehydrogenase and 3-acetyl pyrimidine analogue of NAD (APAD). Vol. 4, p. 1713. *In* U. Berger (ed.), Methods in enzymatic analysis. Academic Press, Inc., New York.
42. **Yancey, P. H., M. E. Clark, S. C. Hand, R. D. Bowlus, and G. N. Somero.** 1982. Living with water stress: evolution of osmolyte systems. Science **217:**1214–1222.
43. **Yap, S. F., and S. T. Lim.** 1983. Response of *Rhizobium* sp. UNKL 20 to sodium chloride stress. Arch. Microbiol. **135:** 224–228.
44. **Yelton, M. M., S. S. Yang, S. A. Edie, and S. T. Lim.** 1984. Characterization of an effective salt-tolerant fast growing strain of *Rhizobium japonicum*. J. Gen. Microbiol. **129:**1537–1545.