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Preferential Osmolyte Accumulation: a Mechanism of Osmotic Stress Adaptation in Diazotrophic Bacteria

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A common cellular mechanism of osmotic-stress adaptation is the intracellular accumulation of organic solutes (osmolytes). We investigated the mechanism of osmotic adaptation in the diazotrophic bacteria *Azotobacter chroococcum*, *Azospirillum brasilense*, and *Klebsiella pneumoniae*, which are adversely affected by high osmotic strength (i.e., soil salinity and/or drought). We used natural-abundance ^{13}C nuclear magnetic resonance spectroscopy to identify all the osmolytes accumulating in these strains during osmotic stress generated by 0.5 M NaCl. Evidence is presented for the accumulation of trehalose and glutamate in *Azotobacter chroococcum* ZSM4, proline and glutamate in *Azospirillum brasilense* SHS6, and trehalose and proline in *K. pneumoniae*. Glycine betaine was accumulated in all strains grown in culture media containing yeast extract as the sole nitrogen source. Alternative nitrogen sources (e.g., NH_4Cl or casamino acids) in the culture medium did not result in measurable glycine betaine accumulation. We suggest that the mechanism of osmotic adaptation in these organisms entails the accumulation of osmolytes in hyperosmotically stressed cells resulting from either enhanced uptake from the medium (of glycine betaine, proline, and glutamate) or increased net biosynthesis (of trehalose, proline, and glutamate) or both. The preferred osmolyte in *Azotobacter chroococcum* ZSM4 shifted from glutamate to trehalose as a consequence of a prolonged osmotic stress. Also, the dominant osmolyte in *Azospirillum brasilense* SHS6 shifted from glutamate to proline accumulation as the osmotic strength of the medium increased.

One area of applied research that is of global interest is the enhancement of the beneficial associations between microorganisms and plants, particularly in the rhizosphere (4). Nonsymbiotic nitrogen-fixing bacteria have been successfully used in field inoculation studies. For example, soil and/or plant inoculation with members of the genera *Azotobacter*, *Azospirillum*, and *Klebsiella* have led to significant increases in both the yield and nitrogen content of a number of forage and grain crops (1, 12, 16, 22). However, nitrogen fixation is impaired by unfavorable environmental conditions, such as osmotic stress. The adaptation of diazotrophs to osmotic stress is of particular interest because of the limited availability of arable land and fossil fuels for the production of nitrogen fertilizers (6). A practical example of the deleterious effects of salt stress is the inhibition of growth and nitrogenase activity of *Azotobacter chroococcum* (M. A. Madkour, S. M. Helmy, M. S. Hassouna, and S. I. Yacout, unpublished data), *Azospirillum brasilense* (10, 11), and *Klebsiella pneumoniae* (18, 19) observed in media of high osmotic strength supplied by NaCl, KCl, $CaCl_2$, or other electrolytes. Similar observations were made for *Rhizobium* spp. (3).

When these species are exposed to high osmolarity, they grow, albeit at a reduced rate, because of their abilities to adjust to the hyperosmotic environment. One common mechanism of adaptation to osmotic stress (osmoregulation) is the accumulation of inorganic and/or organic solutes in the cytosol to restore turgor in plants and microbes (13, 29). Several pertinent studies of the physiological and genetic responses to osmotic stress in *Escherichia coli* (5, 17), *Salmonella typhimurium* (5), and *Rhizobium meliloti* (3, 24) have been reported. In these species, potassium ions, glutamate, proline, glycine betaine, and trehalose are the prominent species of accumulated osmolytes (5). Also, the dipeptide *N*-acetylglutaminylglutamine amide was recently reported to occur in *Rhizobium meliloti* (25). Although several solutes can function as osmolytes, it appears that there is a natural ranking, or osmolyte preference, in terms of effectiveness. For example, glycine betaine accumulation seems to provide a higher degree of osmotic tolerance than either proline or glutamate (5, 13, 17). Furthermore, long-term osmoadaptation seems to be achieved by an accumulation of the nonreducing disaccharide trehalose (9, 17).

No reports in the literature are available on the adaptation of the nonsymbiotic diazotroph *Azotobacter chroococcum* to osmotic stress, and little is known about the osmoregulatory properties of *Azospirillum brasilense* (10, 11) or *K. pneumoniae* (18, 19). Hence, the following questions are raised. Which osmolytes are accumulated by diazotrophic bacteria? If more than one osmolyte is available, which ones do the cells prefer and why?

To answer these questions, we used natural-abundance ^{13}C nuclear magnetic resonance (NMR) spectroscopy to identify all organic compounds which accumulate intracellularly to significant levels during osmotic stress. Also, the mechanism of osmolyte accumulation (either by uptake or de novo synthesis) was investigated under conditions that might cause the order of osmolyte preference to change during the osmoadaptive process.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in this study were originally isolated from the rhizosphere of indigenous plants grown at different sites in saline and marshy soils on the northwestern borders of Egypt. Two isolates of *Azotobacter*

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chroococcum obtained from the rhizosphere of *Suaeda monoica* and *Halocnemum strobilaceum* were designated ZSM4 and ZHS2, respectively (Madkour et al., unpublished data). Two isolates of *K. pneumoniae*, KHS2 and KHS8, were obtained from the rhizosphere of *H. strobilaceum* and two isolates of *Azospirillum brasilense*, SLM5 and SHS6, were obtained from the rhizospheres of *Limoniastrum monopetalum* and *H. strobilaceum*, respectively (Madkour et al., unpublished data). Also, laboratory strains of *Azotobacter chroococcum* (ZCR), *K. pneumoniae* (KPR) and *Azospirillum brasilense* (SBR) were included.

Growth conditions. Jensen mannitol minimal (JMM) medium (15) was used for *Azotobacter chroococcum* strains. The initial pH was adjusted to 7.5, and cultures were grown on an orbital shaker (220 rpm) at 30°C. For *Azospirillum brasilense* strains, a malate minimal (MM) medium (7) was used at a pH of 6.8. Because of the microaerophilic nature of *Azospirillum* strains (26), cultures were grown without shaking at 35°C. A sucrose minimal (SM) medium (21) was used for *K. pneumoniae* at a pH of 7.2, and cultures were incubated without shaking at 37°C to provide a microaerophilic environment. The procedures which were used to obtain microaerophilic conditions yielded quantities of cells that were highly reproducible (less than 4% variation).

Casamino acids (0.1%, wt/vol) were added to growth media as a nitrogen source. In experiments designed to study the effects of different nitrogen sources in the media on osmolyte accumulation, casamino acids were replaced with 0.1% (wt/vol) of either NH_4Cl or yeast extract as indicated.

For growth rate determination, cultures were grown in 20 ml of minimal medium in 125-ml culture flasks. Growth was monitored by measuring the turbidity with a Klett-Summerson colorimeter with a no. 54 filter. All experiments were repeated at least once, with less than 5% error.

Preparation of cell extracts. Cell extracts for NMR spectral experiments were prepared as follows. A 5% (vol/vol) early-stationary-phase culture was used to inoculate 1 liter of fresh medium containing 0.5 M NaCl. Cells were harvested during late-log phase (optical density at 420 nm of 3 to 4 for *Azotobacter chroococcum*, 1.0 to 1.5 for *Azospirillum brasilense*, and 2 to 3 for *K. pneumoniae*). The protein content of the culture was determined (20), and cellular extracts were prepared as described previously (25). One hundred microliters of 1 M alanine standard was added to each sample, and the final volume was adjusted to 2.0 ml with 15% D_2O. Exactly 1.85 ml of the sample was transferred to the NMR tube for ^{13}C NMR spectroscopy. Spectra were obtained at 90.5 MHz by using a General Electric NT-360 spectrometer with a probe temperature of 25°C. The NMR parameters used were the same as those described previously (25). ^{13}C NMR chemical shifts, reported relative to tetramethylsilane, were obtained by assuming the chemical shift of internal dioxane in 15% D_2O to be 67.8 ppm. The error in amino acid quantitation in duplicate samples by NMR spectroscopy was generally less than 15%.

Quantitative amino acid analysis. Amino acid concentrations in the cell extracts described above were also quantitated by amino acid analysis. A 20-μl sample was brought up to 400 μl by using 0.2 M sodium citrate buffer and then diluted again (1:5) with the same buffer. A 20-μl sample which contained 5 nmol of β-thionyl alanine (as an internal standard) was loaded onto a Durrum D-500 amino acid analyzer equipped with an ion-exchange column and a ninhydrin detection system. The amino acid content is expressed on a per-milligram-of-cellular-protein basis by the method of Lowry et al. (20). The proline concentrations obtained by NMR spectroscopy and amino acid analysis agreed (less than 5% error); however, the values obtained by NMR spectroscopy for the glutamate concentrations were consistently 20% higher than those obtained by amino acid analysis. Hence, a correction factor of 0.83 was used in reporting the results obtained by the spectroscopic method.

TABLE 1. Effects of osmotic stress on growth of selected diazotrophs in minimal media supplemented with casamino acids

Strain	Generation time (h)		% Inhibition of growth yield
	Control	0.5 M NaCl	
Azotobacter chroococcum[a]			
ZCR	3.4	5.5	50.4
ZSM4	1.7	2.4	27.5
ZHS2	2.9	3.3	33.4
Azospirillum brasilense[b]			
SBR	3.4	5.4	51.7
SHS6	2.8	5.8	52.3
SLM5	3.2	7.8	69.0
Klebsiella pneumoniae[c]			
KPR	3.7	5.6	60.9
KHS2	2.7	5.8	71.5
KHS8	3.0	5.8	53.5

[a] A 5% (vol/vol) early-stationary-phase inoculum of *Azotobacter chroococcum* was allowed to grow in 20 ml of JMM medium supplemented with 1 g of casamino acids per liter. Cultures were incubated aerobically at 30°C and grown to late-log phase. Growth was monitored by turbidimetry with a Klett-Summerson colorimeter.

[b] Conditions were as described in footnote *a*, except that MM medium was used. Cultures were incubated without shaking at 35°C.

[c] Conditions were as described in footnote *a*, except that SM medium was used. Cultures were incubated without shaking at 37°C.

Quantitative trehalose determination. Trehalose was quantitated by high-pressure liquid chromatography analysis by using an Alltech NH_2 (silica-based amino propyl) column (250 by 4.6 mm) with 5 μm packing and an evaporative scattering detector (Applied Chromatography Systems, Ltd.). The mobile phase was acetonitrile-water (80:20, vol/vol), and the retention time of trehalose was 9.6 min at a flow rate of 2.0 ml/min. A Hewlett Packard 3390A integrator was used for quantitation. The intracellular osmolyte content is expressed in micromoles per milligram of cell protein. The error in duplicate samples was less than 15%.

RESULTS

Effect of osmotic stress on bacterial growth. The effect of osmotic stress on the growth of three diazotrophic bacterial genera was determined (Table 1). The *Azotobacter chroococcum* strains were the most salt tolerant (averaging 37.1% inhibition of growth yield at 0.5 M NaCl); less tolerant were *Azospirillum brasilense* and *K. pneumoniae* strains (averaging 57.7 and 62.0% inhibition, respectively). The large inhibition of growth of *K. pneumoniae* KHS2 (71.5%) was observed in cells grown microaerophilically; however, a similar trend was also observed in salt-stressed *K. pneumoniae* grown anaerobically (19). The growth of *Azotobacter chroococcum* ZSM4 showed the least inhibition in response to elevated osmolarity (27.5%).

It was recently reported that the growth of *A. brasilense* sp7 is significantly inhibited by 0.5 M NaCl (10). However, our strains of *A. brasilense* (SBR, SHS6, and SLM5) showed relatively greater salt tolerance at the same NaCl concentration. The generation time for strain sp7 is 14.8 h, compared

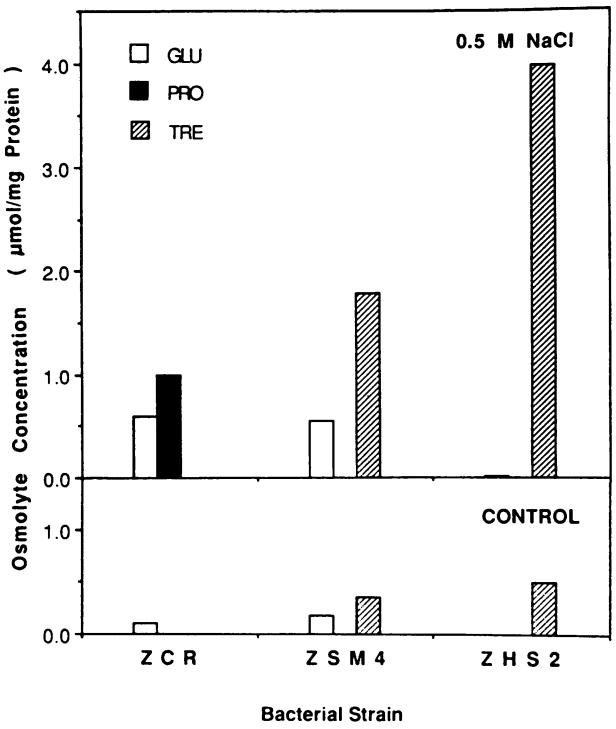

FIG. 1. Effects of osmotic stress on the accumulation of osmolytes by *Azotobacter chroococcum* ZCR, ZSM4, and ZHS2. One-liter cultures (5% inoculum) were grown for 18 h at 30°C in JMM medium supplemented with 1 g of casamino acids per liter and 0.5 M NaCl. The cells were extracted with perchloric acid, and the concentrations of osmolytes were quantitated by ^{13}C NMR spectroscopy. Osmolyte concentrations were confirmed either by amino acid analyses (for amino acids) or by high-pressure liquid chromatography (for trehalose). GLU, Glutamate; PRO, proline; TRE, trehalose.

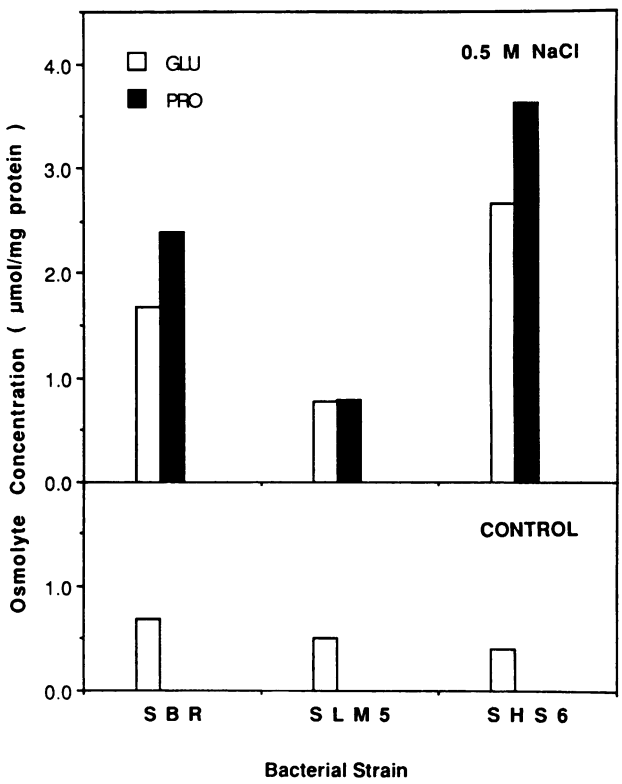

FIG. 2. Effects of osmotic stress on the accumulation of osmolytes by *Azospirillum brasilense* SBR, SLM5, and SHS6. One-liter cultures were grown for 30 h at 35°C in MM medium supplemented with 1 g of casamino acids per liter and 0.5 M NaCl. Details are given in the legend to Fig. 1. GLU, Glutamate; PRO, proline.

to 5.4, 5.8, and 7.8 h for SBR, SHS6, and SLM5, respectively. This difference may be attributed to the nature of our strains, which were originally isolated from saline environments and therefore have developed an osmoadaptive mechanism towards stress tolerance.

Determination of osmolyte accumulation in stressed diazotrophic bacteria. Natural-abundance ^{13}C NMR spectroscopy was used to identify all the organic compounds which accumulate in osmotically stressed cells of diazotrophic bacteria. The results revealed the abilities of the stressed *Azotobacter chroococcum* ZCR, ZSM4, and ZHS2 to accumulate the amino acid glutamate or proline or the disaccharide trehalose when grown in JMM medium supplemented with casamino acids (Fig. 1). However, the types of accumulated osmolytes and their concentrations varied from strain to strain. When the three strains were harvested after 18 h of growth, the slower-growing strain, ZCR, was found to accumulate glutamate and proline while the faster-growing strains, ZSM4 and ZHS2, accumulated trehalose (1.78 and 3.98 µmol/mg of protein, respectively) as a major osmolyte. Also, glutamate accumulation was observed in ZSM4; only a trace could be detected in ZHS2. These compounds were also accumulated in nonstressed (control) cultures, but at lower concentrations (Fig. 1).

The pattern of osmolyte accumulation by *Azospirillum brasilense* SBR, SLM5, and SHS6 was quite different from that of *Azotobacter chroococcum* (Fig. 2). All of the strains accumulated glutamate and proline when grown in MM medium plus NaCl, but to different levels. The highest concentrations of osmolytes were detected in the fast-growing strain SHS6. Proline accumulation was generally higher than glutamate accumulation, except for strain SLM5, in which both proline and glutamate levels were low (0.78 µmol/mg of protein). Also, lower levels of glutamate (<0.69 µmol/mg of protein) and no proline were detected in nonstressed cultures.

K. pneumoniae KPR, KHS2, and KHS8 (Fig. 3) also accumulated glutamate and proline, but osmolyte levels were generally lower than in the *Azotobacter* and *Azospirillum* species (<0.36 µmol of glutamate/mg of protein and <1.54 µmol of proline/mg of protein). No glutamate was detected in cell extracts of stressed KHS2 (fast growing), but proline and trehalose were observed.

Mechanisms of osmolyte accumulation. To examine osmoregulation in diazotrophs more critically, three of the above strains were studied in detail: *Azospirillum brasilense* SHS6, *Azotobacter chroococcum* ZSM4, and *K. pneumoniae* KHS2. These strains were chosen because they grow more rapidly than the other strains in the absence of NaCl (Table 1). Cultures were grown in medium containing NH_4Cl, casamino acids or yeast extract as a nitrogen source. The latter two amendments contain high concentrations of glutamate and proline (23), and yeast extract also contains glycine betaine (8), which may be transported into the cell. By comparing the types of osmolytes that are accumulated under the different growth conditions, we can determine

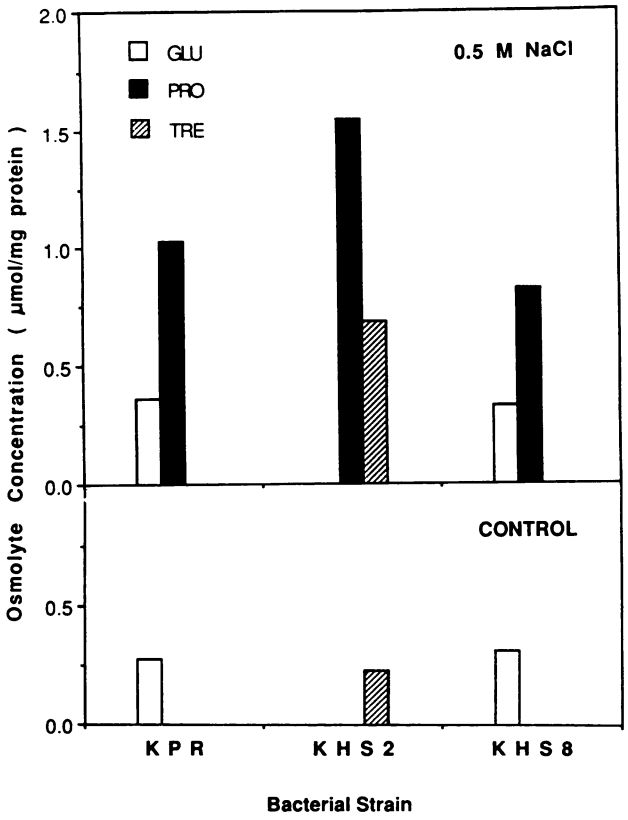

FIG. 3. Effects of osmotic stress on the accumulation of osmolytes by *K. pneumoniae* KPR, KHS2, and KHS8. One-liter cultures were grown for 18 h at 37°C in SM medium supplemented with 1 g of casamino acids per liter and 0.5 M NaCl. Details are given in the legend to Fig. 1. GLU, Glutamate; PRO, proline; TRE, trehalose.

whether this accumulation results from increased uptake or de novo synthesis or perhaps both.

Stressed *Azospirillum brasilense* SHS6 accumulated glutamate and proline in the presence of all three nitrogen sources in the medium (Table 3). However, compared to cultures grown in NH_4Cl, stressed cultures grown in casamino acids were able to accumulate even higher amounts of glutamate and (more notably) proline, presumably as a result of uptake. A reduction in the intracellular levels of glutamate and proline and the appearance of glycine betaine (2.5 μmol/mg of protein) were also observed when the medium was supplemented with yeast extract. These osmolytes were also observed in cells grown in yeast extract at low osmolarity (Table 3).

TABLE 2. Effects of the nitrogen source in the medium on the growth of diazotrophic bacteria at inhibitory osmolarity[a]

Strain	% Inhibition of growth yield of cultures grown in:		
	NH_4Cl	Casamino acids	Yeast extract
Azotobacter chroococcum ZSM4	43.8	27.5	9.6
Azospirillum brasilense SHS6	70.4	52.3	20.4
Klebsiella pneumoniae KHS2	82.1	71.5	18.3

[a] Cultures were incubated to late-log phase in the presence or absence (control) of 0.5 M NaCl. Details of media and growth conditions are given in Materials and Methods. Values given represent the percentage of control.

Glutamate is the only major osmolyte that accumulated in the osmotically stressed *Azotobacter chroococcum* ZSM4 grown in media containing casamino acids (Table 3) or NH_4Cl (data not shown). In addition to glutamate, glycine betaine (1.0 μmol/mg of protein) was observed in cells grown with yeast extract.

In stressed *K. pneumoniae* KHS2 grown with casamino acids as the nitrogen source, proline was the major osmolyte (1.5 μmol/mg of protein) and both trehalose and glutamate were observed. Just as in *A. brasilense*, the accumulation of proline was probably due to uptake from the medium since no proline was observed by us (data not shown) or by others (19) when ammonium ion was used as the nitrogen source. In cells grown in yeast extract, the most prominent osmolytes were trehalose and glycine betaine (1.9 and 0.98 μmol/mg of protein, respectively) but traces of glutamate and proline were also detected. In the absence of stress, the accumulation of both trehalose and glycine betaine was detected, but at reduced levels.

From the results given in Tables 2 and 3, it can be concluded that generally increased growth correlates with high intracellular concentrations of glycine betaine or proline. These results are discussed below.

Effect of medium osmolarity on osmolyte preference. Proline and glutamate were the major osmolytes found in cells of *Azospirillum brasilense* SHS6 grown in NH_4Cl at high osmolarity (Table 3). In order to determine how the external osmolarity affects the concentrations of these osmolytes in the cell, the amount of osmolyte accumulated was measured as a function of NaCl in the medium (Fig. 4). As the osmotic strength of the medium increased from 0.3 to 0.9 M, the glutamate levels decreased by 25% while proline levels increased by almost threefold. Thus, a clear shift from glutamate to proline accumulation occurred as the medium osmolarity increased. In addition, the growth rates were markedly decreased by the presence of NaCl in the medium. For example, the growth yield at late-log/early stationary phase decreased by 1.2- or 3.0-fold with 0.3 or 0.9 M NaCl, respectively, added to the growth medium. Thus, the detrimental effects of NaCl on the growth rate corresponded with the appearance of intracellular proline. This result is in agreement with the report that in a number of halophilic and nonhalophilic bacteria, proline confers a higher degree of osmotolerance than glutamate (13), a pattern consistent with our results.

Effect of the duration of high osmolarity in the medium on osmolyte preference. In *Azotobacter chroococcum* ZSM4, it was observed that trehalose accumulated only when cultures were incubated under osmotic stress for a prolonged time (18 h, early stationary phase) (Fig. 1). However, trehalose could not be detected in cultures grown for a shorter time (12 h, late-log phase) (Table 3). Hence, prolonged exposure to osmotic stress led to a 30% reduction in glutamate concentration and an increase in the accumulation of trehalose, which accounted for almost 80% of the total osmolytes observed (on a molar basis). Hence, it appears that the accumulation of glutamate initially provided a low level of osmoprotection. However, in order to withstand prolonged osmotic stress in their environment, the cells synthesized trehalose, a nonreducing disaccharide, which replaces glutamate. Thus, this environmentally linked switch in osmolyte preference seems to resemble that of the enteric bacterium *E. coli* (5, 17), in which large amounts of trehalose replaces K^+ and glutamate that accumulates after hyperosmotic shock.

TABLE 3. Osmolyte composition of stressed diazotrophic bacteria[a]

Strain	Addition to medium	Accumulated osmolyte (μmol/mg of protein)			
		Glutamate	Proline	Trehalose	Glycine betaine
Azospirillum brasilense SHS6	NH_4Cl	0.21	ND	ND	ND
	NH_4Cl + NaCl	2.3	2.3	ND	ND
	CAA	0.40	ND	ND	ND
	CAA + NaCl	2.7	3.6	ND	ND
	Yeast	1.4	1.3	ND	0.77
	Yeast + NaCl	1.7	0.71	ND	2.5
Azotobacter chroococcum ZSM4	CAA	0.17	ND	ND	ND
	CAA + NaCl	0.78	ND	ND	ND
Klebsiella pneumoniae KHS2	CAA	ND	ND	0.23	ND
	CAA + NaCl	Tr	1.5	0.69	ND
	Yeast	ND	ND	Tr	0.59
	Yeast + NaCl	Tr	Tr	1.9	0.98

[a] Cultures were grown to late-log phase in the presence or absence of 0.5 M NaCl in the medium. The media used were JMM for *Azotobacter chroococcum*, MM for *Azospirillum brasilense*, and SM for *K. pneumoniae*, and NH_4Cl, casamino acids (CAA), or yeast extract (Yeast) was added for the nitrogen source. Cell extracts were prepared as described in Materials and Methods and were used for ^{13}C NMR spectroscopy and high-pressure liquid chromatography (for quantitation of trehalose). ND, None detected.

DISCUSSION

In this report, we have shown that the intracellular accumulation of osmolytes in diazotrophic bacteria can be attributed to (i) enhanced osmolyte uptake from the medium (i.e., uptake of glycine betaine, proline, and possibly glutamate), (ii) increased net osmolyte biosynthesis (i.e., of trehalose, glutamate, and proline), or (iii) both of these mechanisms. The species of osmolyte accumulated and their preference in a given cell were subject to qualitative and quantitative changes depending on the prevailing environmental and nutritional conditions. For example, our data strongly suggest that none of the organisms tested can synthesize glycine betaine, the most potent osmoprotectant, yet all selected glycine betaine when it was available in the media. In addition, we observed that osmotically stressed cells generally favor the shift from glutamate to trehalose or proline as the culture ages or as salt levels are increased, since the latter osmolytes provide the greater osmotic stress protection needed for long-term adaptation to the new environment (29). Hence, the question that arises is why is glutamate accumulated at all if trehalose and proline are better in osmoprotection. We feel that because glutamate is a pivotal metabolite, it can be readily accumulated or disposed of, which allows it to be a reasonable response to short-term stress. On the other hand, proline and especially trehalose are specialized metabolites, the synthesis and accumulation of which likely require the induction of specific systems. It should also be noted that, because glutamate is negatively charged at a physiological pH, a cationic counterion might also be accumulated to maintain electrical neutrality. K^+ is known to be accumulated in stressed enteric bacteria (5) and may act as a counterion to glutamate in these diazotrophic bacteria as well.

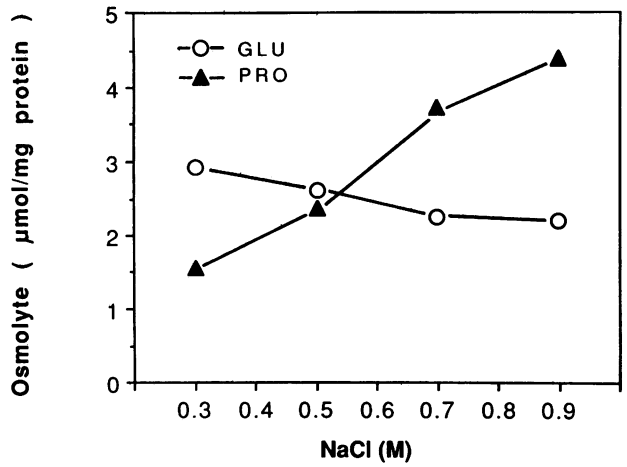

FIG. 4. Effects of osmotic strength of the medium on accumulation of osmolytes from *Azospirillum brasilense* SHS6. Cultures were grown in 1 liter of MM medium. The concentrations of osmolytes were determined by ^{13}C NMR spectroscopy and confirmed by amino acid analyses of the same samples, as described in Materials and Methods. Glutamate (GLU) and proline (PRO) were the dominant osmolytes.

The results of this study may lead to a better understanding of the osmoregulatory mechanisms of the diazotrophic bacteria that occur in nature in the rhizoplane of plants. For example, the degeneration of plant cells (especially in the root system) as well as root exudation provides a readily available supply of various amino acids, sugars, and related compounds in the rhizosphere (2). A relevant example is the observed promotion of *Azotobacter* growth by barley and wheat root exudates (28). Also, the osmolytes proline and glutamate are the major constituents of the amino acid pool, and glucose and oligosaccharides are among the essential sugars of the root exudates of these two crops (27). Another example is the accumulation of glycine betaine, which is a constitutive property of *S. monoica* (14), a salt marsh plant from the rhizosphere from which the salt-tolerant strain ZSM4 of *Azotobacter chroococcum* was isolated. Hence, it is possible that this strain, in its natural habitat, accumulates glycine betaine from the rhizosphere of *S. monoica* as part of its own osmoadaptive mechanism. The suggestion that diazotrophs in nature obtain osmoprotectants such as glycine betaine and proline from plants is presently being investigated. We believe that understanding the mechanisms of osmoadaptation in diazotrophs will contribute to the long-term goal of enhancing plant-microbe interaction for the

improvement of crops grown under arid and semiarid conditions.

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