Osmoregulation in *Azospirillum brasilense*: glycine betaine transport enhances growth and nitrogen fixation under salt stress

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Addition of glycine betaine (1 mM) stimulated aerobic growth of *Azospirillum brasilense* Sp 7 in the presence of 0.3 M-NaCl. The nitrogenase activity of whole cells was particularly sensitive to salt stress, being almost totally inhibited in the presence of the same concentration of salt. Added glycine betaine strongly enhanced nitrogen fixation activity under salt stress. Under such conditions, maximal nitrogenase activity was obtained at a pO_2 value (1 kPa) that inhibits nitrogen fixation activity in the absence of salt. We demonstrated the presence of a high-affinity transport system for glycine betaine, with an apparent K_m of 10 μ M. The osmolarity of the medium regulated the activity of the transport system. The maximal transport rates were 4 and 20 nmol min^{-1} (mg protein^{-1}) in cells grown in low-salt and high-salt medium, respectively. A high intracellular concentration of glycine betaine (480 mM) was observed only at a high osmolarity (0.3 M-NaCl). Glycine betaine uptake was significantly reduced in osmotically shocked cells and a glycine betaine binding activity was detected in the crude periplasmic shock fluid. This suggests a transport mechanism involving a periplasmic glycine betaine binding protein. *A. brasilense* was unable to use the transported glycine betaine as a carbon- or nitrogen-source, in low- or high-salt medium. Intracellular glycine betaine was not catabolized.

Introduction

Azospirillum brasilense is a Gram-negative aerobic bacterium which has a tendency to grow in close association with the roots of graminaceous plants, including some important crop plants. *A. brasilense* fixes nitrogen and has been used as bacterial fertilizer (Von Bülow & Döbereiner, 1975; Okon & Kapulnik, 1986). In addition, in response to *Azospirillum* inoculant, a significant enhancement of lateral roots and root hair proliferation (Tien *et al.*, 1979) is concomitant with an increase in mineral uptake by the plant (Lin *et al.*, 1983). It was proposed that the stimulation of plant growth could be explained by phytohormone production.

In the natural environment, salinity affects microbial activity and plant growth. Substantial areas of agricultural potential in the tropics and sub-tropics are affected by salt. Many organisms that face saline or dry environments accumulate quaternary ammonium compounds such as glycine betaine. This betaine accumulates in higher plants including chenopods, composites and several cereals and grasses in response to salinity (Wyn Jones & Storey, 1981; Hanson & Hitz, 1982). Indirect evidence indicates the adaptative value of

betaine accumulation in salt-stressed and water-stressed plants (Hanson & Grumet, 1984). Glycine betaine has also been shown to be an effective osmoprotectant and to accumulate to high concentrations in both halophilic bacteria and in the enteric bacteria *Escherichia coli*, *Salmonella typhimurium* and *Klebsiella pneumoniae* in response to osmotic stress (Imhoff & Rodriguez-Valera, 1984; Le Rudulier & Bouillard, 1983; Perroud & Le Rudulier, 1985). Furthermore, glycine betaine plays a key role in osmoregulation in *Rhizobium meliloti*, the root nodule symbiont of alfalfa (Le Rudulier & Bernard, 1986). In contrast to *E. coli*, glycine betaine can function in *R. meliloti* as a carbon, nitrogen and energy source (Bernard *et al.*, 1986; Smith *et al.*, 1988).

Recently, more attention has been given to plant-associated diazotrophs showing salt tolerance or enhanced growth at high osmolarity. Salt tolerant strains of *Azospirillum halopraeferans* have been isolated from the root surface of *Leptochloa fusca* (L.) Kunth (Kallar grass) grown on salt-affected low-fertility soils (Reinhold *et al.*, 1987). However, little information is available about the osmoregulatory properties and the effect of salinity on the nitrogenase activity of *Azospirillum* strains (Hartmann, 1988; Jena *et al.*, 1988). In this study, the

consequences of salt stress on the growth and nitrogen-fixation activity of *A. brasilense* were analysed. Additionally, the beneficial effect of glycine betaine, and some characteristics of its uptake, are described.

Methods

Bacterial strain and cultures. *Azospirillum brasilense* Sp 7 (ATCC 2914) was kindly supplied by Dr C. Elmerich (Institut Pasteur, Paris, France). Cultures were maintained in nutrient broth (rich medium) as described by Gauthier & Elmerich (1977). The cells were grown aerobically at 30 °C in K medium (minimal medium) according to Franche & Elmerich (1981), except that the carbon source used was sodium lactate (5 g l⁻¹) instead of malate. The nitrogen source was 20 mM-NH₄Cl. The osmolarity of the K medium was increased by adding NaCl at the concentrations indicated. The osmotic pressure of the different media was measured by freezing-point depression as described previously (Perroud & Le Rudulier, 1985). Solutions of glycine betaine were sterilized by filtration on 0.22 µm pore-size filters. When glycine betaine was used as the sole nitrogen source, it was added at 10 mM. Growth was monitored as the optical density at 420 nm after suitable dilution with the growth medium. The protein content was quantified by the Lowry method. An OD₄₂₀ of 1.0 corresponded to 0.1 mg protein ml⁻¹ in minimal medium without adding NaCl. A correcting factor was used when the cells were grown in the presence of NaCl.

Assay for nitrogen fixation. Nitrogen fixation was assayed in K medium, devoid of ammonia, described by Franche & Elmerich (1981). Aliquots (1 ml) of washed cells from preculture in K medium were inoculated into 60 ml flasks containing 8 ml of nitrogen-free medium. The flasks were sealed with stoppers and flushed with argon. Oxygen was adjusted to the appropriate concentration (pO₂ 0–2 kPa). The cultures were incubated for 4 h at 30 °C with shaking (150 r.p.m.), and 5 ml acetylene were then added. Ethylene production was measured 30 min and 1 h later with a Packard (model 427) gas chromatograph equipped with a flame ionization detector and a Porapack T column (200 × 0.3 cm) equilibrated at 60 °C.

Transport assays. When the culture density in K medium reached an OD₄₂₀ of 0.5–0.8, the cells were harvested by centrifugation, washed twice with the growth medium, and resuspended to a concentration of 0.1 mg cell protein ml⁻¹ in the same solution. Individual uptake experiments were done aerobically at 30 °C as described by Perroud & Le Rudulier (1985). Each assay contained 2.08 kBq [methyl-¹⁴C]glycine betaine and nonradioactive glycine betaine to produce the appropriate specific activity. [methyl-¹⁴C]glycine betaine was prepared from [methyl-¹⁴C]choline as described previously (Perroud & Le Rudulier, 1985). [methyl-¹⁴C]choline (2.15 MBq µmol⁻¹) was purchased from Amersham. Assays with [U-¹⁴C]succinic acid contained 1 µM radioactive substrate (4.88 kBq). [U-¹⁴C]succinic acid (5.55 MBq µmol⁻¹) was obtained from CEA (France). Each assay was done at least in triplicate, and each experiment was repeated with at least two independent cell suspensions.

Measurement of intracellular volume. The intracellular aqueous volumes were determined by the technique of Stock *et al.* (1977) using [carboxyl-¹⁴C]dextran and [³H]water purchased from New England Nuclear and CEA (France), respectively. Values were 2.2 ± 0.3 and 0.9 ± 0.3 µl (mg protein)⁻¹ for cells growing in minimal medium and minimal medium containing 0.3 M-NaCl, respectively.

Osmotic shock and binding assays. Periplasmic fractions were isolated from the cells by cold osmotic shock according to the method of Neu & Heppel (1965). Periplasmic proteins were concentrated by ultra-

filtration on Amicon YM 10 membranes. Binding activity in shock fluid was detected by an equilibrium dialysis technique (Argast & Boos, 1979; May *et al.*, 1986) in the presence of 5 µM-[methyl-¹⁴C]glycine betaine. The protein concentration was 0.5 mg ml⁻¹ and samples (20 µl) were taken in duplicate every 30 min, over 6 h. The amount of glycine betaine remaining was determined by scintillation counting. As an alternative, the binding assays were done by the filter binding procedure (Richarme & Kepes, 1983). Samples of periplasmic shock fluid (100–200 µg protein) were incubated for 30 min with the appropriate amount of [¹⁴C]glycine betaine (2.5 kBq; 2.15 MBq µmol⁻¹). Proteins were precipitated by adding 1 ml of an ice-cold saturated ammonium sulphate solution, collected by filtration onto 0.45 µm nitrocellulose filters, and washed four times with 1 ml ammonium sulphate solution. The radioactivity of the filters was determined in a scintillation counter.

Fate of glycine betaine. Cells were harvested in mid-exponential phase and incubated aerobically at 30 °C in a Warburg flask with a KOH trap (0.2 ml, 6 M). [methyl-¹⁴C]glycine betaine was added to a final concentration of 0.1 µM. At intervals, the cells were collected on nitrocellulose filters (0.22 µm pore size) and extracted with 70% (v/v) ethanol as described previously (Bernard *et al.*, 1986). The radioactivity of each resulting fraction (KOH, ethanol-insoluble, ethanol-soluble) was measured by liquid scintillation. Analysis of the intracellular products was done by high-voltage electrophoresis for 60 min at 40 V cm⁻¹ with Whatman 3MM filter paper moistened in 3% (v/v) formic acid, pH 2.0 (Le Rudulier & Bouillard, 1983).

Results

Osmoprotection of *A. brasilense* Sp 7 by exogenous glycine betaine

The effects of NaCl concentration on the growth rate of *A. brasilense* are shown in Fig. 1. Addition of 0.3 M-NaCl increased the doubling time of the cells from 2 h in the

Fig. 1. Effect of exogenous glycine betaine on the growth of *A. brasilense* Sp 7 in media containing different NaCl concentrations. Cell growth was estimated as the OD₄₂₀; values are plotted as a function of incubation time. Cells were grown in the presence of 1 mM-glycine betaine (open symbols) or in the absence of glycine betaine (filled symbols). ○, Minimal medium; □, minimal medium plus 0.3 M-NaCl; △, minimal medium plus 0.5 M-NaCl.

minimal medium to 15 h. Additionally, the maximal cell density (OD_{420}) was reduced considerably – from 4 units in the absence of NaCl to only 0.5 unit in the presence of 0.3 M-NaCl. Increasing the salt concentration to 0.5 M totally inhibited growth.

Addition of glycine betaine at a concentration of 1 mM, which causes maximal stimulation of the growth rate of enteric bacteria and *R. meliloti* in media of high osmolarity (Le Rudulier & Bouillard, 1983; Bernard *et al.*, 1986), did not modify the growth rate and yield of *A. brasilense* in low-salt medium. However, in the presence of 0.3 M-NaCl, addition of glycine betaine produced an almost 3-fold stimulation of growth, lowering the doubling time to 5.5 h. Furthermore, the maximal optimal density of the culture reached 3 units after only 24 h of growth. In the presence of 0.5 M-NaCl, the stimulation was less and the cells achieved a very low final density ($OD_{420} = 0.25$).

Enhancement of nitrogen fixation activity by glycine betaine in salt-stressed *A. brasilense*

Fig. 2 shows the pO_2 profile of the specific nitrogenase activity of whole cells grown in media of low or high osmolarity (0.3 M-NaCl), with or without 1 mM-glycine betaine. With cells grown in minimal medium (Fig. 2a), anaerobiosis or a high dissolved oxygen concentration (pO_2 1.5 kPa) almost totally inhibited nitrogenase activity. *A. brasilense* is a well-characterized micro-aerobic nitrogen-fixing bacterium and the optimal pO_2 for nitrogen fixation was 0.4 kPa, as determined previously by Fu & Burris (1989). Addition of glycine betaine did not significantly affect the nitrogen fixation activity. Addition of 0.3 M-NaCl resulted in no nitrogenase activity at low (0.4 kPa) or high (2 kPa) pO_2 values, and caused 96% inhibition at the optimal pO_2 . Addition of glycine betaine stimulated nitrogen fixation activity greatly: the increase was 14-fold at a pO_2 of 0.5 kPa and 23-fold at a pO_2 of 1 kPa. As previously observed with *K. pneumoniae* (Le Rudulier & Bouillard, 1983), the nitrogen fixation activity of salt-stressed *A. brasilense* cells grown in the presence of glycine betaine reached about 55% of the activity measured in cells grown in low-salt medium.

Evidence for glycine betaine transport

The time course of glycine betaine uptake in low-salt- and high-salt-grown cells is shown in Fig. 3. Cells grown in minimal medium showed a low glycine betaine transport activity. The uptake was linear for approximately 15 min with an initial rate of $3.0 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$. After 20 min, the uptake rate was almost nil and the transported quantity of glycine betaine was

Fig. 2. Effect of NaCl, pO_2 and exogenous glycine betaine (1 mM) on the specific nitrogenase activity of whole cells of *A. brasilense* Sp 7. Nitrogenase activity was measured as acetylene reduction as described in the text with cells grown in the absence of NaCl (a) or in the presence of 0.3 M-NaCl (b). $\blacksquare$, Plus glycine betaine; $\square$, no glycine betaine. The values are means of three separate experiments, with the deviation from the mean indicated.

Fig. 3. Time course of [methyl- ^{14}C]glycine betaine uptake by *A. brasilense* Sp 7. Cells grown in the absence of NaCl or in the presence of 0.3 M-NaCl were incubated at 30 °C with 30 or 200 μM -[methyl- ^{14}C]glycine betaine, respectively. Reactions were terminated by filtration at the time shown. The points shown are the mean values of duplicate assays from three different experiments. Vertical bars indicate $\pm$ SD. $\circ$, Cells grown in minimal medium; $\square$, cells grown in minimal medium plus 0.3 M-NaCl.

only 50 nmol after 30 min. Since the measured internal water volume of *A. brasilense* was $2.15 \mu\text{l (mg protein)}^{-1}$, the estimated intracellular concentration of glycine betaine was 23 mM. When the cells were grown in high-salt medium (0.3 M-NaCl), a strong stimulation of uptake was observed. The rate of glycine betaine transport was constant for approximately 10 min with a maximal velocity of $18 \text{ nmol min}^{-1} \text{ (mg protein)}^{-1}$. The initial rapid rate was followed by a slower uptake, and after 30 min the total quantity transported was 380 nmol (mg protein^{-1}), which was 7.6 times greater than the quantity transported by cells grown in minimal medium. In the presence of 0.3 M-NaCl, the cellular water volume of the cells was only $0.85 \mu\text{l (mg protein)}^{-1}$, and the estimated internal concentration of glycine betaine reached 480 mM, which represented a 21-fold increase.

Kinetics of glycine betaine transport

To determine the concentration needed to saturate the uptake system, the kinetic parameters of glycine betaine transport by *A. brasilense* were determined with low- and high-salt-grown cells. Initial rates were calculated at 2 and 10 min at substrate concentrations from 5–200 μM . Transport was a saturable function of substrate concentration. Double-reciprocal plots gave a straight line, indicating that uptake follows typical Michaelis–Menten kinetics. The maximal velocities were 4 ± 1 and $20 \pm 3 \text{ nmol min}^{-1} \text{ (mg protein)}^{-1}$ with cells grown in minimal medium and 0.3 M-NaCl, respectively. Under both conditions, the apparent K_m was $10 \pm 2 \mu\text{M}$. Uptake tests at higher or lower substrate concentrations failed to show the presence of another transport system.

Periplasmic glycine betaine binding activity

Recent studies have identified a glycine betaine binding periplasmic protein as a product of the *proU* locus which encodes the osmoregulatory transport system for glycine

betaine in both *E. coli* and *S. typhimurium* (May *et al.*, 1986; Barron *et al.*, 1987; Faatz *et al.*, 1988; Higgins *et al.*, 1987). To determine whether periplasmic proteins from *A. brasilense* might function as binding proteins for glycine betaine, we used three technical approaches. First, the effect of an osmotic cold shock on glycine betaine uptake was studied (Table 1). Uptake was strongly inhibited (80% inhibition) by this treatment. Also, uptake of glycine betaine dramatically decreased from 12.4 to 0.9 $\text{nmol min}^{-1} \text{ (mg protein)}^{-1}$ after the cold shock alone. This result pointed out the necessity of a control of this type, which is very often omitted. Conversely, the cold osmotic shock did not significantly modify the uptake of succinic acid, a substrate transported via a system which is not binding-protein-dependent. Second, to investigate whether a glycine betaine binding protein was released by osmotic shock, substrate-binding experiments were done with concentrated shock fluid using the filter-binding procedure described in Methods. Periplasmic glycine betaine binding activity was very low [$45 \text{ pmol (mg protein)}^{-1}$] when the cells were grown at low osmolarity, and approximately 6-fold higher when the cells were grown in the presence of 0.3 M-NaCl. Third, in order to confirm these results, we measured the binding of [*methyl*- ^{14}C]-glycine betaine to periplasmic proteins using a dialysis technique (Argast & Boos, 1979) based on the retention phenomenon of binding proteins. Fig. 4 shows a rapid, linear exit of free glycine betaine from a dialysis bag that contained only buffer or a periplasmic fraction obtained from cells grown in minimal medium. When periplasmic proteins prepared from cells grown in the presence of 0.3 M-NaCl were used, there was an initial rapid efflux of excess glycine betaine followed by a much slower efflux which represents the dissociation of bound substrate. All these results strongly suggest that a binding protein is involved in glycine betaine transport in *A. brasilense* Sp 7.

Table 1. Effect of an osmotic cold shock on [*methyl*- ^{14}C]glycine betaine uptake and [*U*- ^{14}C]succinic acid uptake in *A. brasilense*

Uptake of $10 \mu\text{M}$ -[*methyl*- ^{14}C]glycine betaine and $1 \mu\text{M}$ -[*U*- ^{14}C]succinic acid was measured in control and shocked cells grown in the presence of 0.3 M-NaCl. Control cells were subjected to the same treatment as shocked cells, except for the osmotic shock, and were used immediately for uptake measurements. Mean results of duplicates are shown $\pm$ SD.

Time (min)	Glycine betaine uptake [nmol (mg protein) $^{-1}$]			Succinic acid uptake [pmol (mg protein) $^{-1}$]	
	Control cells (A)	Shocked cells (B)	Inhibition [(A – B/A) $\times$ 100]	Control cells (C)	Shocked cells (D)
0.5	0.41 ± 0.05	0.24 ± 0.05	42%	20 ± 3	26 ± 3
1.0	0.94 ± 0.05	0.20 ± 0.05	79%	43 ± 4	49 ± 5
2.0	1.43 ± 0.10	0.23 ± 0.05	84%	68 ± 5	73 ± 5

Fig. 4. Binding of [methyl- ^{14}C]glycine betaine ($5\ \mu\text{M}$) to periplasmic proteins of *A. brasilense* Sp 7. Periplasmic proteins were from cells grown in minimal medium (●) or in minimal medium plus $0.3\ \text{M}$ -NaCl (■). ▲, Control experiment without periplasmic proteins in the assay. Binding was assayed in $10\ \text{mM}$ -Tris/HCl, pH 7.5. The amount of radioactivity remaining in the dialysis bag was plotted against time of dialysis.

Fate of intracellular glycine betaine

In nitrogen- and carbon-free minimal medium, addition of glycine betaine ($10\ \text{mM}$) did not support growth. Incorporation of NH_4Cl ($20\ \text{mM}$) into this medium also failed to promote growth. With lactate only ($5\ \text{g l}^{-1}$) some growth was observed but the presence of glycine betaine had no additional effect. It was likely that glycine betaine cannot be metabolized by the cells.

The fate of intracellular glycine betaine in low- and high-salt grown cells was investigated. Cells were incubated with [methyl- ^{14}C]glycine betaine for 1 h, transferred into non-radioactive medium for 6 h, and then extracted with ethanol. The radioactivity found in the ethanol-insoluble fraction representing cellular material was extremely low; 91% and 95% of the label remained in the ethanol-soluble fraction obtained from low- or high-salt-grown cells, respectively. Electrophoretic analysis of this soluble fraction revealed only a single labelled compound identified as glycine betaine itself. Similar results were obtained after 24 h incubation in non-radioactive high-salt medium. These experiments indicate that intracellular glycine betaine is not catabolized by *A. brasilense*.

Discussion

Azospirillum strains are widely distributed in salt-affected soils in Pakistan and Brazil (Reinhold *et al.*, 1987). Among the nitrogen-fixing bacteria associated with the roots of a salt-tolerant grass (*Leptochloa fusca* L. Kunth) used as a pioneer plant in Pakistan, a salt-tolerant *Azospirillum*, *A. halopraeferens*, has been described (Reinhold *et al.*, 1987). In *Azospirillum*, osmotolerance declines in the order *A. halopraeferens*, *A. brasilense*, *A. lipoferum* and *A. amazonense* (Hartmann, 1988). However, despite increasing interest in physiological and genetic responses of bacteria to osmotic stress, very little is known about osmoregulation in *Azospirillum*, although Hartmann (1988) demonstrated that addition of glutamate or proline ($5\ \text{mM}$) and glycine betaine ($1\ \text{mM}$) to minimal medium resulted in better growth at inhibitory NaCl concentrations in *A. brasilense* and *A. halopraeferens*, but not in *A. lipoferum* and *A. amazonense*. The results obtained in the present study demonstrate the importance of glycine betaine in both the restoration of growth and the stimulation of nitrogen fixation activity in *A. brasilense* subjected to salt stress. At high osmolarity, exogenous glycine betaine is accumulated by the cells; the internal concentration of glycine betaine after 30 min of incubation with $200\ \mu\text{M}$ substrate reached $480\ \text{mM}$. These results are similar to those obtained previously with *E. coli* and *R. meliloti* (Perroud & Le Rudulier, 1985; Bernard *et al.*, 1986). Consistent with this finding, stressed cells had a high-affinity transport system for glycine betaine. The K_t for glycine betaine, $10\ \mu\text{M}$, was not influenced by the osmolarity of the growth medium. However, the maximal velocities were 4 and $20\ \text{nmol min}^{-1} (\text{mg protein})^{-1}$ for cells grown at low salt concentrations and $0.3\ \text{M}$ -NaCl, respectively. In the presence of lower or higher substrate concentrations, no evidence was obtained for a second system of different affinity.

The regulation and activity of glycine betaine uptake has been studied in *S. typhimurium* and *E. coli*, and is similar in both. Transport is mediated by two transport systems, ProP and ProU (Cairney *et al.*, 1985a, b; May *et al.*, 1986; Barron *et al.*, 1987). *proP* encodes a constitutive, low-affinity transport system whose expression is stimulated several-fold during osmotic upshock. *proU* encodes a high-affinity system that is strongly induced at the transcriptional level by osmolarity. Both systems are also regulated at the level of transport activity (Cairney *et al.*, 1985a, b; Faatz *et al.*, 1988). The ProU system contains a periplasmic glycine betaine binding protein of $M_r 31\ 000$ (Higgins *et al.*, 1987; May *et al.*, 1986) which was purified from *E. coli* and was shown to have a K_D of $1.4\ \mu\text{M}$. Recently, the *proU* region has been shown to contain three genes, *proV*, *proW*, and *proX* in an operon

(Faatz *et al.*, 1988; Dattananda & Gowrishankar, 1989). From the determined *proU* DNA sequence (Gowrishankar, 1989), the products of these genes have been deduced. The *proV* gene encodes a hydrophilic protein (M_r 44162) which shares considerable sequence identity with ATP-binding proteins from other periplasmic transport systems (Stirling *et al.*, 1989; May *et al.*, 1989). The *proW* gene encodes a hydrophobic polypeptide (M_r 37619) which is thought to be located in the cytoplasmic membrane. The *proX* gene encodes the periplasmic glycine betaine binding protein (M_r 33729).

Since the results presented here are the first evidence for glycine betaine transport in *Azospirillum*, it is obvious that this transport is understood much less than in the enteric bacteria. However, from our results, glycine betaine transport in *A. brasilense* appears to be dependent on a periplasmic binding protein based on the following evidence. (i) Significant reduction of glycine betaine transport activity after osmotic shock, which is associated with the concomitant release of a glycine betaine binding activity into the crude shock fluid. (ii) Binding activity is not found with low-salt-grown cells, which show very low transport activity. (iii) The kinetics of glycine betaine transport show it to be a high-affinity transport system with a K_m of 10 μ M, comparable to the K_m of 1.3 μ M determined for ProU-mediated transport in *S. typhimurium* (Cairney *et al.*, 1985b). In *A. brasilense*, this system requires further exploration. It will be of interest to identify glycine betaine binding protein(s) to understand the mechanism of betaine transport. We are at present attempting to address this question.

Only a few studies have examined the effects of NaCl on the acetylene-reduction activity of *Azospirillum*. In *A. amazonense* DSM 2787^T, acetylene reduction was shown to be very sensitive to the addition of salt, with 90% inhibition when only 40 mM-NaCl was added. With *A. brasilense* DSM 1690^T, and mainly with *A. halopraeferens*, increasing the NaCl concentration had much less effect: 28% and 24% of the activity observed in non-stressed cells was measured in the presence of 130 mM- and 250 mM-NaCl, respectively (Reinhold *et al.*, 1987). The nitrogenase (acetylene-reduction) activity of cultures of *A. brasilense* Sp 7 in semi-solid medium 48 h after inoculation was barely affected by 200 mM-NaCl, whereas activity was totally abolished at 400 mM (Hartmann, 1988). There is good agreement between our results obtained in liquid medium and those reported by Hartmann (1988). We have no concrete data to explain this lack of nitrogenase activity in cells grown at high osmolarity. However, it is possible that the biosynthesis of nitrogenase polypeptides is strongly affected as shown previously with *K. pneumoniae* grown at a high-salt concentration (Le Rudulier *et al.*, 1982). Other experi-

ments are needed to verify this hypothesis. More importantly, we found that glycine betaine largely restored nitrogen fixation at a totally inhibitory salt concentration. However, for maximal beneficial effect of glycine betaine on acetylene-reduction activity, the oxygen concentration had to be increased from 0.4 kPa to 1 kPa. As nitrogen fixation activity of aerobes is strictly dependent on oxygen availability, one possible explanation for high nitrogenase activity at a pO_2 higher than usual might be a modification of oxygen diffusion across the bacterial envelope in high-salt-medium. However, starting from the concept of respiratory protection most extensively studied for *Azotobacter*, it has been proposed that respiration *per se* may be protective among aerobic diazotrophs (Robson & Postgate, 1980). In *A. brasilense* Sp 7 the occurrence of a respiratory type of protection has been proposed (Kloss *et al.*, 1983). We have no data to rule out the possibility that cells grown in high-salt-medium with added glycine betaine exhibited a higher respiratory rate than cells grown in low-salt medium. One alternative explanation could be that glycine betaine uptake is, to a certain extent, dependent on pO_2 . At a pO_2 less than 0.5 kPa, the amount of glycine betaine taken up might be extremely low and not sufficient to restore nitrogen fixation activity. Increasing the pO_2 could result in a stimulation of this uptake with a concomitant beneficial effect on nitrogenase activity.

The ability to catabolize glycine betaine is widespread among micro-organisms. *Clostridium sporogenes* is able to form trimethylamine and acetate from glycine betaine in an oxidation-reduction reaction in which betaine acts as an electron acceptor for the oxidation of certain amino acids (Naumann *et al.*, 1983). The degradation scheme has been established as far as glycine in *Agrobacterium tumefaciens* (Sherr & Law, 1965) and up to the stage of glycine, pyruvate and succinyl-CoA in *Pseudomonas denitrificans* (White & Demain, 1971). In *R. meliloti*, glycine betaine is progressively demethylated through dimethylglycine and sarcosine to glycine. However, this catabolic system is blocked by increasing the osmotic strength of the medium (Bernard *et al.*, 1986). In *R. meliloti*, glycine betaine can function as a carbon, nitrogen and energy source, as well as an osmoprotectant (Smith *et al.*, 1988). In contrast, our data indicate that *A. brasilense* is unable to use glycine betaine as a source of nitrogen or carbon. This situation is similar to that observed with enteric bacteria (Perroud & Le Rudulier, 1985). However, clear differences exist in the ability of *Azospirillum* species to catabolize glycine betaine, since it has been shown that strains of *A. lipoferum* grow readily on glycine betaine as a nitrogen and carbon source, whereas *A. amazonense* and *A. halopraeferens* do not (Hartmann, 1988).

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