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Osmotolerance of diazotrophic rhizosphere bacteria

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Abstract

In the genus *Azospirillum* tolerance towards high concentrations of sodium chloride, sucrose or polyethylene glycol increased in the order *A. amazonense A. lipoferum A. brasilense* and *A. halopraeferens*. In *A. brasilense* and *A. halopraeferens* the compatible solutes trehaloseglutamate and an unknown compound were identified. *A. halopraeferens* only could convert choline to the potent compatible solute glycine betaine. *Acetobacter diazotrophicus* tolerated high concentrations of *sucrose* and polyethylene glycol, but was very sensitive towards sodium chloride. In contrast to the more osmotolerant *Azospirillum* spp. amino acids such as glutamate, serine and histidine were efficiently utilized as carbon and nitrogen sources and betaine, choline and proline did not relieve osmotic stress.

New halotolerant bacteria (strains BE and TC) were isolated from the rhizosphere of rice growing in alkaline, saline soil in India. They were oxidase-positive, Gram-negative, very motile bacteria, which showed pleomorphic growth. In semisolid nitrogen free mineral medium they grew and fixed nitrogen microaerobically. These isolates required sodium ions for growth and they tolerated up to 2 *M* sodium chloride in nitrogen containing mineral medium. At osmotic stress conditions the efficient compatible solute ectoine was synthesized.

Introduction

When the water potential Ψ in the cell environment increases, any microbial cell needs to regulate its turgor and intracellular composition to compensate water loss and to preserve metabolic functions. Many osmoregulatory responses involving synthesis and uptake of compatible solutes, changes in membrane composition or size regulation have evolved in the microbial world (Brown, 1990; Yancey et al., 1982). Diazotrophic rhizosphere bacteria face osmoregulatory challenges, when the plant lives in salt affected soil, the soil dries out or when they live inside the plant roots, where high organic solute concentrations may occur.

In *Azospirillum* spp. the tolerance towards sodium chloride increases in the order *A. amazonense*, *A. lipoferum*, *A. brasilense* and *A. halopraeferens* (Hartmann, 1988a,b). The utilization of amino acids, choline and glycine betaine for growth is low in the more osmotolerant species (Hartmann et al., 1988). Proline and glycine betaine can only relieve osmotic stress in *A. brasilense* and *A. halopraeferens*. Here we report on further studies on osmoregulation of *Azospirillum* spp. and *Acetobacter diazotrophicus* and about new isolates of halotolerant diazotropic bacteria from the rhizosphere of rice.

Material and methods

The *Azospirillum* strains were obtained from the Deutsche Sammlung für Mikroorganismen (DSM, Braunschweig). *Azospirillum halopraeferens* Au 4 was kindly provided by Dr. B. Reinhold (Hannover) and *Acetobacter diazo-*

trophicus PAL3 was a gift from Dr. J. Döbereiner (Rio de Janeiro). The *Azospirillum* and *Acetobacter* strains were grown in liquid mineral medium with ammonium or in semisolid nitrogen free medium as described previously (Gillis et al., 1989; Hartmann, 1988a; Reinhold et al., 1987). The strains TC and BE from the rhizosphere of rice were cultivated in *A. brasilense* mineral medium supplemented with 100 m*M* NaCl. The following minimal medium was used as osmotic stress medium to investigate the compatible solutes of TC and BE: NaCl (2 *M*), K_2HPO_4 (3 m*M*), NH_4Cl (40 m*M*), $MgSO_4$ (0,4 m*M*), $FeSO_4$ (0.04 m*M*), $CaCl_2$ (0.5 m*M*), glucose (55 m*M*), yeast extract (0.01 g L^{-1}), pH 7.5–8.0. Nitrogen fixation activity was measured in semisolid media using the acetylene reduction technique (Hartmann et al., 1988). Bacterial growth was recorded by measuring the absorbance at 560 nm.

The new isolates TC and BE were obtained in semisolid malate medium (Nfb), supplemented with 50 mg L^{-1} yeast extract according to Baldani and Döbereiner (1980). In addition 0.25% (w/v) NaCl was added. The enrichment cultures were transferred seven times to fresh Nfb medium and were finally streaked on malate and ammonium containing minimal medium agar plates. The oxidase and amino peptidase tests were performed using the reagent kit of Merck (Darmstadt).

For the determination of the compatible solutes freeze dried cells (70 mg) were processed using a modified Blye and Dyer-technique and the extracts were analyzed by the HPLC-method according to Galinski and Herzog (1990).

The conversion of choline to glycine betaine was investigated using ^{14}C-choline purchased from Amersham. Logarithmically growing cells were incubated at 33 or 41°C with 35 μM ^{14}C-choline for 2 hours. Then 300 μL acetone was added to 200 μL incubation mixture. After drying in vacuum and redissolving in 20 μL water high voltage electrophoresis (800 V, 35 mA, 1 hour) was carried out using Whatman 3 mm paper and formic acid (0.75 *M*). Finally the dried paper was scanned for radioactivity. As reference substances ^{14}C-choline and ^{14}C-glycine betaine were used. ^{14}C-glycine betaine was obtained from ^{14}C-choline by enzymatic conversion with choline oxidase from Sigma.

The water potential of the different osmotic stress media were measured psychrometrically with the Wescor HR-33-T dewpoint microvoltmeter (Wescor Inc., USA). The following readings were obtained with minimal medium for *A. amazonense* and *Acetobacter diazotrophicus* (LGI with 1% sucrose): 3.1 bar; minimal medium for *A. lipoferum* and *A. brasilense*: 6.0 bar; minimal medium for *A. halopraeferens*: 8.8 bar; LGI-medium with 10% sucrose: 9.0 bar (1 bar = 10^5 Pa).

Results and discussion

Comparison of the osmotolerance of Azospirillum *spp. and* Acetobacter diazotrophicus

Using sodium chloride, polyethylene glycol (PEG) 400 and sucrose as osmotic stress agents *A. halopraeferens* performed best among *Azospirillum* spp., followed by *A. brasilense*, *A. lipoferum* and *A. amazonese* (Fig. 1). *Acetobacter diazotrophicus* PAL3 was more tolerant towards osmotic stress by sucrose and PEG400 than *Azospirillum* spp.. However, PAL3 was very sensitive towards sodium chloride. In the presence of 0.1 *M* NaCl the specific nitrogenase activity was reduced to 50% (in medium with 1% sucrose; Fig. 1) and to 20% in cultures with 10% sucrose (not shown). The nitrogen fixation activity of *Acetobacter diazotrophicus* was increased at 20% sucrose as compared to 1% (19 and 3 bars), demonstrating the adaptation of PAL3 to the high sugar concentrations in its natural habitat, the roots and stems of sugarcane.

In contrast to *A. brasilense* and *A. halopraeferens* glutamate, proline and glycine betaine could not relieve the osmotic stress induced by NaCl, PEG400 and sucrose in *Acetobacter diazotrophicus* PAL3. Glutamate, serine, alanine and histidine were efficiently used as carbon and nitrogen sources. Nitrogenase activity of PAL3 was inhibited to less than 10% in the presence of glutamate, which was also demonstrated for the osmosensitive species *A. amazonese* and *A. lipoferum* (Hartmann et al., 1988).

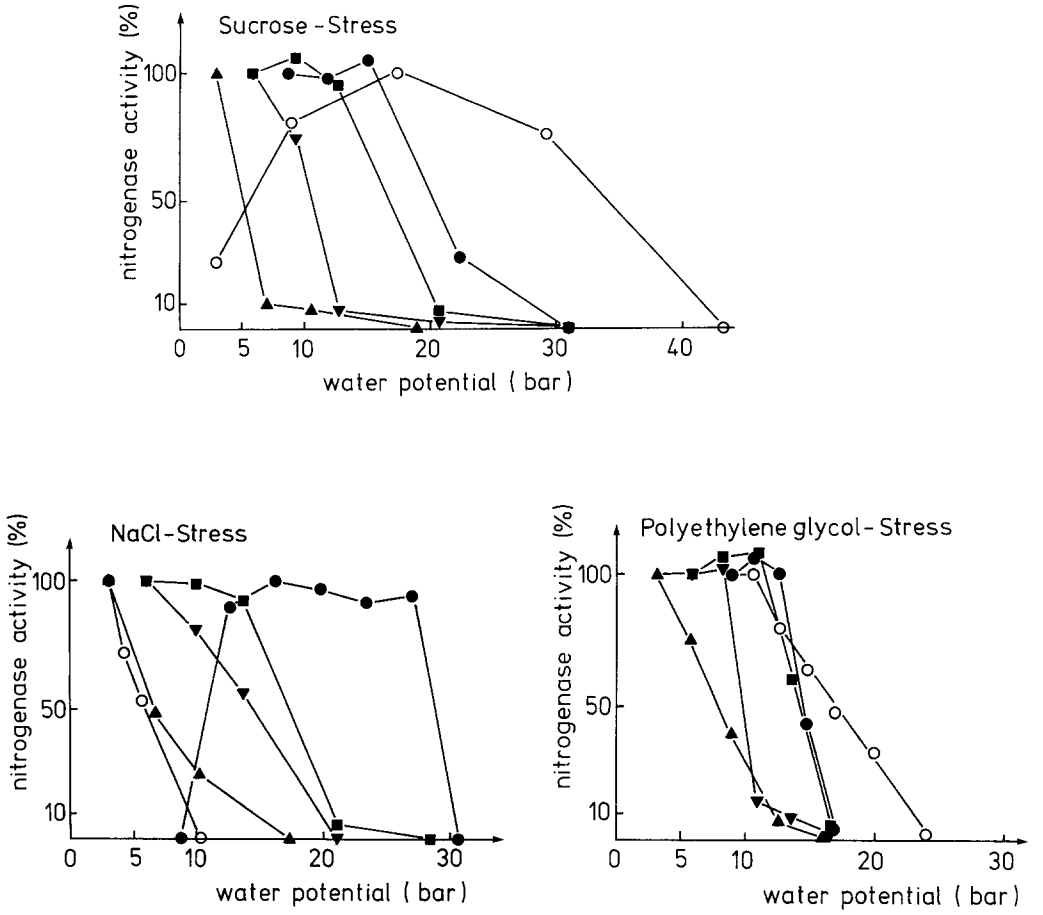

Fig. 1. Comparison of the impact of osmotic stress on nitrogenase activity of *A. amazonense* Y1 (▲), *A. lipoferum* Sp59b (▼), *A. brasilense* Sp7 (■), *A. halopraeferens* Au4 (●) and *Acetobacter diazotrophicus* PAL3 (○). The specific nitrogenase activity (nmol ethylene produced per 1 mL culture at A_{560} of 1.0) was determined in semisolid media. For each strain the highest activity was set to 100%. The following concentrations of osmotic stress solutes were used; NaCl: 0, 0.05 (PAL3), 0.1, 0.2, 0.3 (Au4), 0.4, 0.5 and 0.6 *M*; polyethylene glycol 400: 0, 2.5, 5.0, 7.5, 10.0%(w/v); sucrose: 1, 5, 10, 20, 30% (w/v).

Conversion of choline to glycine betaine in Azospirillum *spp.*

Choline and glycine betaine could relieve osmotic stress in *A. halopraeferens*, while only glycine betaine was active in *A. brasilense* (Hartmann, 1988b). The ability to convert choline to glycine betaine was tested with ^{14}C-labeled choline. The electropheretograms shown in Figure 2 clearly demonstrate, that only *A. halopraeferens* was able to convert choline to glycine betaine. Therefore *A. halopraeferens* harbours the choline-glycine betaine pathway similar to the *bet*-operon described for *E. coli* (Andresen et al., 1988). It consists of a high affinity choline uptake (Hartmann, 1988b), choline dehydrogenase and betaine aldehyde oxidase.

Isolation and osmoregulatory properties of newly isolated halotolerant N_2-fixing rhizobacteria

The strain TC was isolated from the roots of rice growing in salt affected, alkaline soil in Trichy, Tamil Nadu, India. The roots were washed several times with sterile water to get rid of loosely adhering soil. Root bits of 1 cm length were the starting material for isolation (see Material and

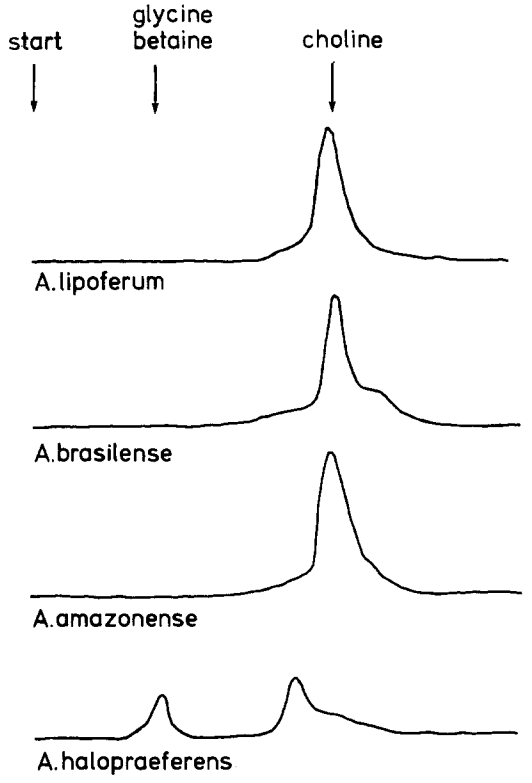

Fig. 2. High voltage paper electrophoresis of [^{14}C]-choline and its conversion products. *Azospirillum* cultures, growing at osmotic stress conditions, were incubated with 35 μM choline for 2 hours. Electrophoresis was performed as described in Material and Methods. [^{14}C]-choline and [^{14}C]-glycine betaine were used as reference substances.

Methods). The strain BE was isolated from rhizosphere soil of a local rice variety grown at Cochin, Kerala in seawater affected paddy fields. Both isolates were oxidase-positive, amino peptidase-positive, Gram-negative and very motile bacteria. In nitrogen free semisolid medium they grew in a distinct subsurface pellicle. Both isolates showed pleomorphic growth (vibroid to long rods) and cyst formation.

The isolates grew very well in minimal medium with malate or glucose as carbon source. The maximal growth rate (66 min per generation) was obtained between 100 and 500 mM sodium chloride (Fig. 3). Growth was sodium dependent with half maximal rates at 20 mM NaCl. At salt stress conditions, lag phases occurred. At 2 M sodium chloride the lag phase was about 48 hours long and was omitted from Figure 3.

Nature of the compatible solutes

A. brasilense Sp7 and *A. halopraeferens* Au4 showed a very similar pattern of compatible solutes. The main components were trehalose, glutamate and an yet unknown component (ratio 4:1:1). The occurrence and identity of trehalose and glutamate was corroborated by ^{13}C-NMR analysis. In *Acetobacter diazotrophicus* PAL3 sucrose was the only organic solute which could

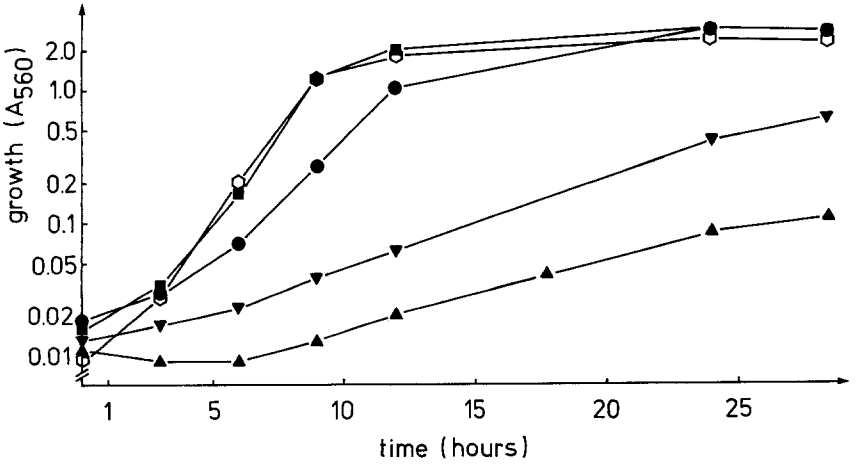

Fig. 3. Growth of the strain BE on mineral salt medium with malate as carbon and ammonium chloride (10 mM) as nitrogen source. Growth was performed in liquid shaking cultures (150 rpm, 33°C). Salt tolerance: 0.1 M NaCl (○), 0.5 M NaCl (■), 1.0 M NaCl (●), 1.5 M NaCl (▼) and 2.0 M NaCl (▲).

be observed in high concentration. Since it was used as growth substrate the quantification of the intracellular part is difficult. The osmoregulation of *Acetobacter diazotrophicus* is clearly different from *Azospirillum* or *E. coli* (Larsen et al., 1987) and might resemble the situation of *Zymomonas mobilis*. These bacteria compensate high external glucose concentrations with high internal concentrations. This is achieved by high glucose import rates (Struch et al., 1990).

The halophilic isolates TC and BE contained the compatible solutes glycine betaine, ectoine and ectoine derivate Y (ratio 2:1:1 for TC and 2:1:2 for BE) when grown on complex medium. Ectoine is probably the main osmolyte in minimal medium (with glucose and 2 *M* NaCl). The identity of ectoine needs to be proven by ^{13}C-NMR analysis. Ectoine is a cyclic amino acid discovered for the first time in halophilic, phototrophic bacteria of the genus *Ectothiorhodospira* (Galinski et al., 1985). Meanwhile ectoine and related substances were found in other genera of halophilic bacteria too (Wohlfarth et al., 1990). Its biosynthesis starts with aspartate and proceeds in five enzymatic steps as described by Peters et al. (1990). The occurrence of the biosynthesis of this very efficient compatible solute in *Azospirillum* – like diazotrophic bacteria deserves further investigation.

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