Salt stress sensitivity of nitrogen fixation in 

*Enterobacter agglomerans* strains

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Two strains 333 to 339 of *Enterobacter agglomerans* were selected in the present study to evaluate the response of increasing concentrations of NaCl on growth, N₂-fixation, and nitrogenase activity/synthesis. *E. agglomerans* strains 333 and 339 showed optimum growth and acetylene-reducing activity with 0.5 to 1.0% NaCl in a nitrogen-free minimal medium (NFDM) with glucose, respectively, in 28 h incubation, although both strains displayed better growth and acetylene-reducing activity with 3.0% and 2.0% NaCl after 52 h and 100 h incubation periods than the 28 h culture did. Our experiments with shiftings of salt concentrations in NFDM medium indicated that a synthesis of nitrogenase enzyme was generally more sensitive to higher concentrations of NaCl than nitrogenase activity was.

**Key Words**— *Enterobacter agglomerans*; nitrogenase activity; nitrogenase synthesis; sodium chloride

Biological nitrogen fixation by diazotrophic bacteria in association with crop plant roots is well documented and has the potential to reduce nitrogen fertilizer requirements in many agricultural areas (Barber et al., 1980; Döbereiner and Day, 1976; Okon et al., 1988; Rai, 1985; Rai and Hunt, 1993; Rai et al., 1984). Various groups of nitrogen-fixing bacteria grow and fix the atmospheric N₂ to ammonia in the rhizosphere of crop plants and grasses, but their population and nitrogen-fixer ability are affected and regulated by numerous environmental factors such as temperature, pH, salt stress, water stress, excess Al and Mn, oxygen, and excess available nitrogen (Hartman et al., 1988; Kullik et al., 1989; Rai, 1983, 1987, 1991; Tripathi and Klingmüller, 1992). Salinity is the most widespread soil constraint in agriculture, and the saline-affected areas in India and elsewhere are increasing every year. Generally, excess salts in medium or soil adversely affected the survival, growth, and nitrogen fixation activity (Rai, 1987).

The ability to adapt to osmotic stress is a common process in biological systems. A hyperosmotic shock results in diffusion of water out of the cell and a reduction of positive cell turgor. To avoid dehydration, some bacteria accumulate organic substances such as L-proline or betaine. These so-called osmoprotectants reduce the inhibitory effects of high osmolarity and promote the growth under salt stress. In *Escherichia coli* and *Salmonella typhimurium*, some osmoregulated genes have been identified (Csonka, 1981; Csonka and Hanson, 1991; Landfald and Strom, 1986), and no osmoregulated genes have been described in *E. agglomerans* strains so far. Glycine betaine and its precursors choline and glycine betaine aldehyde confer high levels of osmotic tolerance in *E. coli* at an inhibitory osmotic strength. Genes governing several activities, including choline and glycine betaine aldehyde oxidation and choline uptake, have also been characterized in *E. coli* (Landfald and Strom, 1986; Styrvold et al., 1986).

*Azospirillum* spp. accumulated compatible solutes such as trehalose, glutamate, proline, and glycine betaine under salinity stress (Hartman, 1988; Hartman et al., 1991). Kallar grass (*Leptochloa fusca*) in association with *Azospirillum* spp., *Enterobacter cloacae*, *E. agglomerans*, *E. intermedium*, *Enterobacter* spp., *Klebsiella pneumoniae*, *Azotobacter* spp., *Citrobacter freundi*, and *Zoogloea* spp. has exhibited nitrogenase activity in saline soil (Malik et al., 1990). *Azospirillum* strains isolated from salt-affected soils in Brazil were more temperature- and salt-tolerant with respect to nitrogen fixation than *Azospirillum* species from other habitats were (Reinhold et al., 1988). Salt-tolerant
Azospirillum brasilense strains were isolated, and they also exhibited growth and N₂ fixation at higher concentrations of Cl⁻, SO₄²⁻, and HCO₃⁻ (Rai, 1991; Rao and Venkateswarlu, 1985). Salt- and alkali-tolerant strains of Azotobacter chroococcum isolated from calcareous saline alkali soil showed growth and nitrogen fixation at higher pH 9.6 (Ahmad et al., 1979). Nitrogen fixation of Rhizobium was more sensitive toward the NaCl (50 mM) than ammonia assimilation (Cordovilla et al., 1994). Nodulation and symbiotic nitrogen fixation of lucern were affected by different salts and their combinations, as well as the salt-tolerance limit of both symbionts (Singleton et al., 1982; Subba Rao et al., 1974). Salinity-tolerant strains of Rhizobium able to grow and fix nitrogen in symbiosis with lentil (Lens culinaris) in saline soil were evaluated (Rai, 1983). Salt-tolerant strains were found more antibiotic-resistant and also showed higher relative rates of oxidation of carbohydrates and tricarboxylic acid intermediates (Rai and Prasad, 1983; Rai et al., 1985).

In cyanobacteria, the inhibition of Na⁺ influx by the modulation of membrane potential or by the competitive effect on the sodium carried by specific nitrogenous compounds is a major mechanism for protection against salt stress (Apte et al., 1988; Thomas et al., 1988). Sodium chloride affected chlorophyll content, carbon fixation, and RuBisCo activity of Aphanothece stagnina (Rai, 1990).

Enterobacter species are widespread in the rhizosphere of grasses grown in tropical and temperate climates (Belly et al., 1983; Pedersen et al., 1978; Haahetela et al., 1981; Jagnow, 1988; Subba Rao, 1983; Väisänen et al., 1985), but their nitrogen-fixing efficiency under various conditions is not known. The association and nitrogen-fixing ability of E. agglomerans strains in the rhizosphere of wheat, maize, and barley are of special interest. Several wild type strains of nitrogen fixing E. agglomerans were isolated from the rhizosphere of wheat and barley (Kleeberger et al., 1983), and it was concluded that Enterobacteriaceae are typical for the innermost part of the rhizosphere of wheat. These wild-type strains were found to harbor the nitrogenase structural genes nif HDK on the large plasmids of 100 to 200 kb in size (Singh et al., 1983). The genus Enterobacter also exhibits the interesting properties that may be important to understanding more about the function and evaluation of nitrogen fixation.

The expression of nif gene(s) is complex and responds to several different environmental stimuli, including high levels of ammonia (Streicher et al., 1974; Tubb, 1979; Tubb and Postgate, 1973); some amino acids (Shanmugam and Morandi, 1976; Yoch and Pengra, 1966), oxygen (St. John et al., 1974), temperature (Hennecke and Shanmugam, 1979), and conditions that evoke a stringent response (Riesenberg and Kari, 1981; Riesenberg et al., 1982). But the possible interrelationship between salt stress and nitrogen fixation (nitrogenase activity/synthesis) in E. agglomerans strains is not known. In this paper, this study was attempted and the results are presented by using two strains of E. agglomerans and different methods of analyses. It has been shown that (a) prolonged incubation of strains with higher concentrations 0.5 and 1% of NaCl resulted in better growth and N₂ fixation; (b) Nitrogenase synthesis is more sensitive to the higher concentration of NaCl than nitrogenase activity is. These results have revealed the differences in salt stress sensitivity in E. agglomerans strains.

Materials and Methods

Bacterial strain. Two nitrogen-fixing strains of E. agglomerans 333 and 339 were selected from the culture collection, Genetics Department, University of Bayreuth, Germany, and used in the present study. These strains (E. agglomerans 333 and 339) were isolated from the rhizosphere of wheat and barley grown in sandy loam soil of the experimental farm of the university. The detailed backgrounds and characterizations of these strains have been described by Kleeberger et al. (1983).

Media, solution, and growth conditions. Luria Broth (LB) medium (containing 1 g tryptone, 0.5 g NaCl, and 0.5 g yeast extract per 100 ml water) was used throughout this study. For preparation of LB agar plates, 1.5% (w/v) Difco agar was added.

NFDM medium. Nitrogen-free minimal medium with glucose (NFDM) was made as follows. Solution I consisted of 20 g glucose, 0.7 g MgSO₄·7H₂O, 25 mg Na₂MoO₄·2H₂O, and 1,000 ml distilled water. Solution II consisted of 68 g KH₂PO₄, 241 g K₂HPO₄, and 1,000 ml distilled water (final pH 7.4). Solution III consisted of 3.6 g Fe (III) citrate and 1,000 ml distilled water. The solutions were prepared and sterilized separately. Before use, 950 ml solution I, 50 ml of solution II, and 10 ml of solution III were mixed (Kleeberger et al., 1983). Saline solution was 0.9% NaCl in demineralized water. For salt stress experiments, different final concentrations of NaCl (0.5 to 3.0%) (w/v) were included in NFDM medium and replicated five times. For preparation of the inoculum, all strains were grown in LB medium on a rotary shaker (Infors TR-125, 180 rpm) at 25°C in a culture room overnight. Growth (optical density = OD) was measured by using an LKB spectrophotometer Ultrospec II at 600 nm wavelength, after incubating the cultures in liquid up to the desired period. Cultures were diluted 1:10 in saline if the OD was higher than 0.4.

Acetylene reduction assay. In the estimations of
N₂-dependent growth and acetylene-reducing activity, *E. agglomerans* cultures were grown in LB medium overnight under shaking conditions. They were then centrifuged and washed with saline/NFDM medium and resuspended in NFDM medium for equal OD. The cultures were inoculated in 12 ml serum bottles containing 5 ml NFDM medium and N₂ gas and were grown for 24 h, 48 h, and 96 h at 25°C under shaking conditions. Then acetylene (0.2 ml) was injected and incubated for 4 h, and ethylene and OD were determined by a gas chromatograph and spectrophotometer.

For differentiation between the effect of salt stress on nitrogenase synthesis and nitrogenase activity, the acetylene-reducing assays were designated in two sets. In the first, LB grown cells were inoculated in 5 ml NFDM medium containing different concentrations of NaCl (0.0, 0.5, 1.0, 2.0, and 3.0%) and incubated in serum bottles under shaking conditions at 25°C for 24 h. Acetylene (0.2 ml) was then injected and cultures incubated for 4 h at the same temperature and same conditions until acetylene reduction was assayed. Protein content (µg/ml cultures) was estimated (Tripathi and Klingmüller, 1992) after 24+4 h to evaluate the effects of NaCl on protein synthesis. These data represented the nitrogenase synthesis and a combination of the amount of nitrogenase present and active with each concentration of NaCl. The effect of salts on nitrogenase activity was also considered during the nitrogenase synthesis; the enzyme activity could be inhibited by salt. In the second set, LB grown cells of *E. agglomerans* strains 333 and 339 were grown into 50 ml NFDM medium into 100 ml serum bottles at 25°C under shaking conditions for 24 h. After 24 h, 5 ml cultures were shifted in small sterile serum bottles (14 ml) containing different concentrations of NaCl (0.0, 0.5, 1.0, 2.0, 3.0%). Acetylene was then injected and cultures incubated under suitable growth conditions for 4 h until acetylene reduction was assayed. These data represented the activity of nitrogenase with special concentration of NaCl. After an incubation of 24+4 h, cultures were also analyzed, especially for protein content, to learn the protein synthesis in the presence of different concentrations of NaCl for a short period. Thus observations of the first set are representative of the effects of salt stress on the nitrogenase synthesis, whereas observations in the second set are representative of the effects of salt stress on the activity of already synthesized nitrogenase. In all cases, a 0.2 ml gas phase was analyzed for the ethylene by means of a Carlo Erba strumentazione, model 180 gas chromatograph. Nitrogenase activity was calculated as follows:

$$\text{Nitrogenase activity} = \frac{\text{nmol} \text{C}_2\text{H}_4}{\text{min} \times \text{ml} \times \text{OD}}$$

**Results**

*E. agglomerans* strains 333 and 339 were grown in NFDM medium for assessing growth (OD) and acetylene-reducing activity with different concentrations of NaCl. Different responses of strains to various concentrations of NaCl were observed. However, greater growth in general was noted with strain 333 than with strain 339 at different concentrations of NaCl. For 339 strain, 0.5 to 1.0% NaCl was found to be the optimum growth concentration after prolonged incubation (94+4 h) under N₂-fixing conditions (Fig. 1), whereas strain 333 showed constant growth with 0 to 3.0% NaCl. However, strain 339 showed significantly reduced growth at higher concentrations of NaCl compared with strain 333. Thus it is concluded that the strains may be tolerant to 0.5% or higher concentrations of NaCl, but note that optimal growth is obtained at 0.5 or 1.0% NaCl.

The results of the effects of strains and of different concentrations of NaCl on acetylene-reducing activity (nitrogenase activity) after 24+4, 48+4, and 96+4 h incubation are presented in Fig. 2. The acetylene-reducing activity of both strains was inhibited significantly at higher concentrations of NaCl (3%). However, strain 333 was found to be more salt-tolerant than strain 339 was, and this resulted in greater activity at higher concentrations of NaCl. Both strains
showed maximum acetylene-reducing activity after incubation of 48 h, and prolonged incubation (96 h) resulted in reduced nitrogenase activity in all concentrations of NaCl. Only strain 333 showed reasonable growth and nitrogenase activity at 3% NaCl.

The results of the effects of strains and different concentrations of NaCl on protein content (µg/ml culture) during the first and second sets of experiments, that is, after 24 + 4 h incubation, are presented in Fig. 3, A and B. During the first set of experiments, a prolonged incubation period (24 + 4 h) with different concentrations of NaCl resulted in an inhibition of protein synthesis of strain 339, which was significant in comparison with the higher tolerance of strain 333. A higher concentration of NaCl (3%) inhibited the protein synthesis of both strains to the maximum extent and resulted in the lowest protein content (Fig. 3A). Whereas during the second set of experiments, protein content was also measured after 24 + 4 h of growth at 0.0 level of NaCl, and data indicated that strain 333 has a more-effective protein synthesis or regulation system than strain 339 does (Fig. 3B).

Data presented in Fig. 3B on the protein content of strains 333 and 339, respectively, revealed the observations of the second set of experiments, in which cultures were grown for 24 h at the 0.0 level of NaCl, and then inoculated with different concentrations of NaCl and grown for only 4 h. A differential response of strains to various concentrations of NaCl was observed. However, higher concentrations of NaCl inhibited the protein synthesis of both strains, and strain 339 was found to be more sensitive compared with strain 333. Moreover, the data of Fig. 3, A and B clearly show that any concentration of NaCl of 0.5% or higher suppresses protein content.

Data presented in Fig. 4 on acetylene-reducing activity of strains 333 and 339, respectively, revealed the observations of the first set of experiments related to nitrogenase synthesis and the second set of experiments indicating the nitrogenase activity at different concentrations of NaCl. In the preliminary experiments 0.5% and 1.0% NaCl were found to be the optima for the acetylene-reducing activities of strains 339 and 333, respectively. Present experiments with salt concentrations shifting (see MATERIALS AND METHODS) revealed that strain 339 requires relatively lower concentrations of NaCl to express its nitrogenase synthesis than 333 does (Fig. 4), and maximum synthesis
was noted with 0.0% NaCl followed by 0.5% NaCl. Nitrogenase synthesis is inhibited with any NaCl concentrations of 0.5% or higher. However, nitrogenase activity of both strains was less affected. It is interesting to note that once the nitrogenase is synthesized, it remains active with a wide range of salt concentrations. Moreover, nitrogenase activity of strain 339 was adversely affected at higher concentrations of NaCl than 333 was.

Discussion

The present findings describe the salt stress sensitivity of synthesis and nitrogenase activity in two strains of *E. agglomerans*. The effect of different concentrations of NaCl on growth and nitrogenase activity of strains was variable, and it appears that like most *Rhizobium* and *Azospirillum* strains, *E. agglomerans* strains are also sensitive to higher concentrations of salts (Rai, 1987). Biological nitrogen fixation (BNF) is a multistep complex process, and the effectiveness of each step can be affected by various environmental factors. The applicability of BNF in cereals requires further study on nitrogen fixation effectiveness in *E. agglomerans* strains and its reaction to salt stress. Genetic determinants in strain 333 may be responsible for salt tolerance and resulted in more growth and nitrogenase activity in batch cultures (under stress). Key bacterial genes respond to environmental changes and undergo depression of a set of globally regulated genes. At least 13 different multigene systems (stimulants) are known to be induced in the response to a variety of stress stimuli such as heat, cold, and salt shock (Van Bogelen et al., 1990). The proteins encoded by these stress genes must cope with unfavorable situations to ensure survival under stress conditions. Changes in extracellular osmolality usually bring about changes in the expression for a few genes, which for the most part encode proteins involved in synthesis or transport of compatible solutes. Fundamental studies in *E. coli* and *Salmonella typhimurium* have revealed that sodium chloride increased the transcription of several genes, including an expression of outer membrane proteins (encoded by *OmpF* and *OmpC*) and regulated by *OmpR* and osmotic signal-sensing proteins (*EnvZ*) (Csonka and Hanson, 1991).

Our observations on growth and nitrogen fixation have shown that N₂-dependent growth was optimal at 0.5% and 1.0% NaCl in strains 339 and 333, but at the same concentrations nitrogenase activity in these two strains was less than optimal. Response for the good growth at 0.5% and 1.0% NaCl could be that these concentrations may be favorable for many other metabolic activities of the cells. Furthermore, it is interesting to mention that once a strain is incubated at a concentration of NaCl favorable for maximum acetylene-reducing activity, then shifted to a less favorable or an unfavorable concentration of NaCl, acetylene-reducing activity was not inhibited as strongly as it would have been if culture was incubated at less favorable or unfavorable concentrations of NaCl right from the beginning. It can be concluded that activity of nitrogenase is less sensitive to changes in concentrations of NaCl than the nitrogenase synthesis is.

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References


